Pediatric immune thrombocytopenia: Catching platelets

Laarhoven, A.G.

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General Discussion 6
This thesis encompasses research performed on the topic of pediatric immune thrombocytopenia (ITP). To investigate the pathogenesis of ITP, we used samples collected as part of the TIKI study (Treatment with or without IVIg in Kids with acute ITP), in which children with newly diagnosed ITP are enrolled and subsequently randomized into those receiving intravenous immunoglobulin (IVIg) or not, to study whether IVIg will prevent development of chronic ITP. This primary outcome of the TIKI study will be evaluated after inclusion of a cohort of 200 cases. At the time of our studies, 138 children were enrolled. In this thesis we focused on the putative role of regulatory T cells (Treg) in ITP, which among other things, play an important role in protecting us from autoimmune diseases. Furthermore, we studied Fc gamma receptor (FcγR) polymorphisms in the pathogenesis of ITP in children and the role of FcγR profiles on the immediate response of the platelet count upon treatment with IVIg. Finally, the effects of platelet autoantibodies in functional platelet responses and their correlation with bleeding tendency in children with chronic ITP were investigated.

**Treg in pediatric ITP**

Immune thrombocytopenia is an autoimmune disease of heterogeneous origin, of which the pathogenesis is not completely understood. To complicate matters further, there are indications that the underlying pathology in ITP differs, not only between newly diagnosed and chronic ITP, but also between disease onset at childhood or adult age. To date, the majority of research addresses ITP pathology in adult chronic ITP. In many cases, only those who fail to respond to first line treatment are investigated, because patients and blood samples are more easily available from this group. In the past decade, research on ITP has increasingly focused on the role of effector T cells (Teff) and concomitantly, on Treg. Autoreactive T cells, responding to several epitopes of glycoprotein IIIa (GPIIIa), were identified in ITP patients. Curiously, T cells with similar specificity can apparently be found in all healthy individuals, as was shown by Filion et al. who tested 25 randomly assigned healthy blood bank donors who were negative for anti-GPIIIaIIb antibodies. However, these healthy donor cells were in an anergic state, indicating loss of tolerance in ITP patients. FOXP3-positive Tregs are pivotal cells in maintaining peripheral tolerance, which makes them a promising target in autoimmunity research. In addition, Ephrem et al. demonstrated that development of induced experimental autoimmune
encephalomyelitis (EAE) in mice could be prevented by IVIg treatment. The preventive effect was associated with increased Treg numbers with enhanced suppressive function. In addition, after depletion of Tregs with anti-CD25 monoclonal antibodies 10 days before EAE induction, IVIg therapy failed altogether,\(^5\) indicating that IVIg might function via Tregs. It has to be noted though that activated Teff may also have been depleted by this method, as they carry CD25. In addition, Nishimoto et al. observed that syngeneic nude mice injected with Treg depleted T cells spontaneously developed an ITP in 36% of the cases, whereas all remained healthy when Treg were maintained. Moreover, the mice that developed ITP also developed antiplatelet antibodies.\(^6\) Finally, although the literature is contradicting, many studies have been performed describing decreased Treg numbers and/or function in adult chronic ITP.\(^7-16\)

Taken together, we hypothesized that also in children Treg might play a crucial role in disease development, and are imperative to IVIg to exerts its beneficial effect. We assumed that Treg numbers and function would be decreased in newly diagnosed pediatric ITP patients, and would be restored or even enhanced following IVIg therapy. However, in chapter 2 we show that Treg number and suppressive function is similar to healthy controls, both during diagnosis and upon recovery. In a cross-over model in which we co-cultured PBMCs obtained at diagnosis together with Tregs from time point recovery and vice versa, we demonstrated that Teff at diagnosis are more active. Remarkably, Treg obtained at recovery failed to suppress these Teff altogether. Treg obtained at diagnosis, on the contrary, suppressed these Teff just fine. This could point at a compensatory mechanism in which Treg activity is enhanced at the onset of the disease in response to the inflammatory environment. This phenomenon has been described in mice.\(^17\) Similar findings of adequate Treg function and enhanced Teff activity have been reported in juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE) diabetes mellitus type 1 (DMT1) and other autoimmune diseases.\(^18-21\) All in all, studies proposing increased resistance to suppression of Teff rather than malfunctioning Treg in autoimmune diseases are accumulating. In our studies we even observed Treg obtained at diagnosis which seemed to have enhanced suppressive capacity and thus were well adapted to the situation at hand. This, however, was only a trend and sample size was small.

Our research is subject to some limitations, especially the small sample size, limited sample volume of only 5-10 ml of peripheral blood, and the artificial nature of our in vitro assays, which can never be fully representative of what
occurs in vivo. In addition, we investigated Treg in general, since we were unable to test antiplatelet antigen specific Treg specifically, and used peripheral blood instead of splenic cells. Zhang et al. described that platelet antigen-specific-Teff are only adequately suppressed by Treg sharing that specificity, and that it only occurs in the presence of APCs primed for this antigen. Also, Aubin et al. demonstrated in mice that the positive effect of IVIg in downregulating antigen specific T cell responses, is established by direct interaction with FcγR bearing APCs instead of T cells. These are remarkable findings, worth further exploration, especially the necessary intercession of primed APCs. Furthermore, we focused on newly diagnosed or persistent ITP in children, who recovered spontaneously, thus our findings might be specific for this cohort. We chose to address this specific group of patients because we wished to study an ITP patient cohort without possible influences of immunomodulating therapy. Moreover, we aimed to study the most common natural course of disease of ITP in children, of which ±75% does recover spontaneously. In addition, we performed a pilot experiment in which we assessed Treg suppressive capacity in 3 children with chronic ITP, showing strong suppressive function equal to the healthy control. Our findings convinced us to reject our initial hypothesis, since no clues were found pointing at decreased Treg numbers or malfunction underlying disease pathogenesis in newly diagnosed pediatric ITP. Preceding this line of thought, strengthened by the observations of Zhang and Aubin, we no longer expect IVIg to function primarily via Treg up-regulation. Therefore, we shifted our focus from Treg in ITP to FcγR bearing phagocytic cells.

FcγR polymorphisms in ITP

Other important players in ITP pathogenesis are the FcγR bearing cells, who are responsible for the destruction of opsonised platelets, particularly in the spleen. While FcγRI is a high affinity receptor, which can even bind monomeric antibodies, and as such is often occupied in vivo due to the high IgG levels in serum of 3.31-11.64 g/L in a 1 year old increasing to 7.23 - 16.85 g/L at adult age, its role in phagocytosis in vivo is not completely clear. Low-affinity FcγRs on the contrary, consisting of the activating FcγRIIIa, FcγRIIib, FcγRIIa, FcγRIIc and the inhibiting FcγRIIib, are known to bind cells or pathogens opsonised with IgG, and are capable to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis. On macrophages, DCs and in
rare cases B cells, the balance of activating and inhibiting FcγRs might determine how and if the opsonized targets are processed and presented. On B cells the inhibiting FcγRIIb can downregulate the cells’ activation and proliferation, and as such function as a negative feedback for antibody production.28,29 Besides playing a role in current ITP disease models, the relevance of FcγRs is underlined by the success rate of IVIg treatment and splenectomy.30 Recently, research has focused on SNPs and CNVs of the genes encoding the family of FcγR, namely the FCGR. Some of this genetic variation is known to alter FcγR affinity and/or function and level of expression. To date, many FcγR polymorphisms have been associated with a variety of autoimmune diseases, such as SLE,31–33 antibody positive RA,34 ITP35–41 and others.42–44 Though an association with FCGR CNVs has never been described in ITP, many studies identified SNPs with an increased prevalence in ITP. Initial studies in this field have been performed in heterogeneous and small groups of patients, resulting in conflicting data.35;36;38–41;45;46 However, most of these studies report enhanced FCGR3A*V158V allele frequency in ITP, which has an increased affinity for IgG1, IgG3 and IgG4.35;41;47 Recently, an increased incidence of the so-called FCGR2C*ORF has been described, which results in FcγRIIC expression on monocytes, B cells28 and NK cells, in contrast to the regular stop that turns FCGR2C into a pseudogene.35 To date, it is known that the FCGR2C*ORF allele has to be further specified in order to distinguish between a ‘classical’ C-ORF and a ‘non classical’ NC-ORF, as the latter does not lead to FcγRIIC expression due to an alternative splice site mutation.48 In chapter 3 we studied FCGR allele distribution in 138 children with newly diagnosed ITP. Because FcγR distribution is known to vary among ethnic background, we restricted our study to individuals from Caucasian origin. Furthermore, all FCGR2 and FCGR3 alleles are clustered in a region of 1q22, within a distance of 0.5 MB, therefore haplotypes comprising linked combinations of the various FCGR2 and FCGR3 alleles may be present. We confirmed the increased allele frequency of FCGR3A*V158 in newly diagnosed pediatric ITP (41.6% vs 32.8% in healthy controls, P=.02). However, by correcting for the high linkage disequilibrium (LD) between FCGR3A*V158 and FCGR2C*ORF (0.96),49 we showed that the increased allele frequency of FCGR3A*V158 is a concomitant finding to the enhanced prevalence of genotype FCGR2C*ORF (33.3% vs 19.6% in healthy controls, P=.005) in combination with FcγRIIC promoter haplotype 2B.2 (34.8% vs 19.6% in healthy controls, P=.002). Although the observation that enhanced prevalence of FCGR3A*V158 in pediatric ITP is a confounder to the increased
incidence of *FCGR2C*\(^*\)-ORF, it might still contribute actively to disease development. *FCGR3*\(^*\)158V results in a higher affinity for IgG and might therefore augment binding and internalization of opsonized platelets. Expression of FcγRIIC might culminate in: first, an altered balance in activating and inhibiting FcγRs, resulting in enhanced phagocytic activity; second, it might alter the inhibitory signaling of FcγRIIB expressed on B cells, being the only activating FcγR present on these cells, leading to autoantibody production\(^{26}\) and third it may play a role in the onset of pediatric ITP following a viral infection, as FcγRIIC is present on NK cells where it amplifies ADCC activity, especially under inflammatory conditions.\(^{35;48;50;51}\) No functional effects, indicating a certain dis- or advantage of the *FCGR2C* 2B.2 promoter polymorphism, have been described.\(^{33}\)

In addition, we observed that the *FCGR2B* promoter haplotype 2B.4, which is associated with a 1.5 fold increase in FcγRIIB expression and functional activity,\(^{33;52}\) is more prevalent in our patient cohort, a finding which is highly counterintuitive. Mice which were FcγRIIB deficient developed a SLE like symptoms.\(^{53}\) In addition, mice were prone to autoimmune diseases when their FcγRIIB promoter was subject to polymorphisms resulting in lower FcγRIIB expression levels.\(^{54;55}\) When FcγRIIB expression was restored to normal levels autoimmunity was prevented.\(^{56}\) On the contrary, Su et al. proved that in human SLE patients FcγRIIB promoter haplotype 2B.4 was overrepresented compared to healthy individuals, like in our ITP patients.\(^{57}\) We could not explain mechanistically how augmented inhibitory function would predispose to development of ITP, unless the increased expression functionally enhances the immune response, perhaps through mechanisms that are not related to B cell activation directly. Evidence for enhanced antigen presentation of intact antigens through IgG and FcγRIIB has been provided on mouse dendritic cells.\(^{58}\)

Another possibility would be that FcγRIIB expression levels are not solely dependent on the promoter haplotype. Indeed, Wu et al. described lower FcγRIIB expression on splenic macrophages of refractory adult ITP patients,\(^{59}\) which does not add up with the increased incidence of the 2B.4 promoter haplotype in ITP. Furthermore, Su et al. demonstrated that there are indeed subtleties in expression levels of FcγRIIB per cell subset. By showing a down-regulation of FcγRIIB expression on memory- and plasma B cells in SLE patients, compared to their naïve B cells, which is contrary to expression patterns in healthy controls.\(^{29;33;52}\) Together, these data show that genotype cannot be adopted in a linear fashion to expression levels on cells per se.
Next to investigating the FcγR profile in patients versus controls, we were intrigued by the possibility that FcγR isotypes might predict a patients’ response to IVIg, especially since IVIg among others, is thought to function via FcγR by either blocking FcγR or adjusting the balance of activating and inhibiting FcγR.\(^{60}\) We formulated our hypothesis based on some murine models, demonstrating that IVIg function depends on FcγRIIB, and upregulates the inhibitory FcγR.\(^{61-62}\) During our research it became known that the conclusions drawn from the murine models were probably due to different mice strains rather than FcγRIIB dependent.\(^{63}\) We hypothesized that patients with the FcγRIIB promoter haplotype 2B.4, and the common homozygous FcγRIIB*1232 genotype, would be more responsive to IVIg therapy. This would be not only because of its augmented expression levels, but also because FcγRIIB*1232 dampens activating receptors more efficiently than FcγRIIB*T232.\(^{64}\) Indeed, in the acute pediatric ITP patients the homozygous FcγRIIB*1232 genotype, in combination with promoter haplotype 2B.4, predicted a good response to IVIg in 93% of the cases, and was also associated with a quick spontaneous recovery in 44% of the patients in the observatory group. Moreover, we showed in all three individuals with a homozygous FcγRIIB*T232 genotype in our series that they failed to respond altogether. These data add up to the previous finding of Bruin et al. that pediatric patients with a heterozygous FcγRIIB*1/T232 genotype are more prone for developing chronic ITP compared to patients with a homozygous FcγRIIB*1232 genotype.\(^{65}\) Unfortunately, none of the participants in that study carried the homozygous FcγRIIB*T232 variant. Although our finding is intriguing, in particular because FcγRIIB*T232 is associated with impaired FcγRIIB function,\(^{31,39,65,66}\) it has to be considered carefully. Homozygous FcγRIIB*T232 is very rare in Caucasians.\(^{49}\) Moreover, we could not ensure whether the observed outcome was IVIg specific, since no patients were carrier of homozygous FcγRIIB*T232 in our observation group.

In contrast, two children with chronic ITP who carried the homozygous FcγRIIB*T232 genotype did respond to IVIg therapy. This could point to a different mechanism in acute and chronic ITP.\(^{65}\) Another explanation would be the small group size as well as the varying time intervals after which the platelet count has been determined in this study. Homozygous FcγRIIB*T232 might be associated with an impaired short term response to IVIg. Whereas, after a few weeks, the more long term response is probably determined by other factors. Thus, no consensus on the effect of IVIg treatment in patients carrying the homozygous FcγRIIB*T232 could be reached.
Still, our studies do illustrate that SNP and CNV analysis might yield insights in both disease pathogenesis and working mechanisms of immunomodulating therapies, and as such enable personalized medicine in the future. FcγR polymorphisms on APCs are in particular of interest in ITP pathogenesis, since the subtle differences in binding and processing of opsonized platelets might determine how they are presented, and thus whether autoreactive T cells are activated and e.g. a Th1 response is favored. The APCs might be the crucial cells in determining onset of ITP or disease outcome. Nevertheless, genotyping studies are prone to a severe limitation, since they only provide a rough estimate of responsiveness to a certain stimulus. In vivo, FcγR expression levels are very flexible depending on the immune status of an individual. To that end, functional data would be very helpful. However, research in this area is challenged by great similarities among FcγR which complicate staining in flow cytometry and functional experiments, like phagocytic or ADCC assays. Ideally, APCs from patients of which the FcγR profile is known should be tested. By testing these cells in phagocytic or ADCC assays, or co-culturing these cells together with opsonised platelets and antigen specific T cells, to assess T cell activation and proliferation, might lead to a deeper understanding of the presumed pivotal role of APCs in ITP development.

**Platelet defects in chronic pediatric ITP**

In addition to pondering fundamental research questions addressing the development of, and pathogenesis underlying pediatric ITP, we wondered why the degree of thrombocytopenia does not necessarily predict bleeding. In chapter 5 we hypothesized that not only platelet numbers but also platelet function could be affected in ITP patients, resulting in the variety of bleeding phenotypes observed. Since the golden standard in platelet function tests, the light transmission aggregometry test, is not reliable with platelet counts below 50*10^9/L, we developed two new functional assays suitable for individuals with low platelet counts.67-69 The first assay, the micro platelet aggregation test, assesses platelet function directly, by measuring patients’ platelets capability to form aggregates with control platelets in response to an agonist of choice. Because autoantibodies associated with ITP are mainly directed at GPIIbIIIa, and to a lesser extent to GPIbIX, we thought that activation pathways operating via these glycoproteins were the most likely to be affected. Therefore, we chose PMA and Ristocetin as agonists respectively.70-72 The second assay, the platelet
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reactivity assay, determines to which extent platelets are activated in response to an agonists by measuring the speed and quantity of P-selectin expression, to assess platelet degranulation, and GPIIbIIIa opening, to demonstrate the conformational stage and ability to bind fibrinogen thereby indicating a pro-aggregation state. Both tests complement each other, whereas the first provides a clear cut functional outcome it does not distinguish between possible factors influencing the final platelet aggregation, other than the pathway of the agonist of choice. The second assay though gives detailed information on receptor pathways and specific manifestations of platelet activation, however the overall picture of platelet function is lacking.

We tested our hypothesis in a well-defined cohort of chronic pediatric ITP patients, who were characterized as either a mild or a severe bleeding phenotype based on the modified scale of Buchanan.

Patients were part of the CINKID study as described in chapter 4. We observed decreased platelet aggregation in response to PMA activation in patients with a severe bleeding phenotype. In addition, the reactivity assay showed most predominantly a lower expression level of P-selectin in response to ADP as well as the need of higher concentrations of ADP to open GPIIbIIIa in patients with a severe bleeding phenotype. Results augmented each other. Obviously, since autoantibodies against GPIIbIIIa are most common in ITP patients, it is tempting to suggest the involvement of autoantibodies blocking proper platelet function to explain our results. Probably a similar mechanism takes place as is known to cause acquired Glanzmann’s disease, in which autoantibodies interact with platelet function rather than opsonizing them leading to destruction by splenic macrophages.

Nevertheless, we failed to prove such a mechanism, since we were unable to perform direct autoantibody testing in our patients’ samples. Indirect MAIPA and PIFT were performed instead, however, they have such a low sensitivity and so few were considered positive that no conclusions could be drawn. It is of interest to explore what causes the observed platelet defect found in ITP patients with a severe bleeding phenotype. To that extent more sensible tests to detect autoantibodies are needed, in which a small amount of blood is sufficient. An alternative though, would be to test adult ITP patients, from whom a larger amount of blood can be drawn for testing, to enable direct autoantibody detection tests even in individuals with low platelet counts.

If indeed platelet function is crucial in developing severe bleeding tendency in patients with a form of thrombocytopenia, our assays are of great value. They
might serve as a tool to predict bleeding tendency and thereby lead to personalized medicine, in which medical treatment or platelet transfusions are reserved for those at risk of severe bleeding, and vice versa, adverse effects caused by treatment are withheld from patients who do not need therapy per se. However, several limitations have to be overcome before they can be used as such. First, platelet function has to be assessed in a variety of thrombocytopenic patients, to determine whether platelet malfunction is indeed a requisite for severe bleeding. Second, in healthy individuals platelet parameters are known to be stable over time. This might not apply for ITP patients, or patients suffering from other autoimmune diseases, in which the course of disease is often unpredictable. Many autoimmune diseases, such as SLE, M.Crohn, JIA and ITP express a pattern characterized by flares of disease activity alternated by periods of mild disease activity or even remission. This makes it less likely that a single measurement will represent a persons’ innate platelet reactivity, although it is possible that measurements performed during disease activity are representative for following flares within an individual. Therefore, longitudinal studies have to be performed. Moreover, it would be of great value to start testing in newly diagnosed patients and follow the natural course of disease. In addition, one has to realize that both assays are performed in vitro, implying that in vivo factors that might influence platelet function as well, such as endothelial function and plasma factors, are not taken into account.

In conclusion, the studies described in this thesis encompassed various approaches in pediatric ITP research. A diagnostic track was pursued by the development of laboratory assays to determine platelet function in individuals with low platelet counts. Hereby showing that some chronic pediatric ITP patients have functional platelet defects associated with severe bleeding tendency irrespective of platelet counts. Furthermore, by focusing on fundamental research to elucidate the pathogenesis of newly diagnosed pediatric ITP we showed that Treg function well and are present in normal numbers, contrary to the majority of the previous findings in chronic adult ITP patients. Due to this finding we rejected the hypothesis that the effect of IVIg is based for an important part on restoring normal Treg number and function. And likewise, the idea of Treg therapy in pediatric ITP patients. Therefore, we shifted our attention to a possible crucial step prior to T cell activation, namely FcyR. FcyR play an important role in humoral and adaptive immune responses and as such might be involved in both clearance of
Ig-opsonised platelets, as well as presenting them via APCs thereby inducing T and B cell activation. Indeed, we demonstrated that several FcγR polymorphisms, which are known to affect presence, expression level and function of the FcγR, are associated with newly diagnosed ITP, and even possible, yet contradicting, affect the outcome of IVIg function. These findings are interesting, both to obtain a deeper understanding of the mechanism of disease and to predict disease outcome or even effect of therapy. However, there is still a long way to go, in which expression levels and function of FcγR per cell subset have to be determined and where possible related to genotype. It would be even more interesting to set up experiments with patients’ FcγR-bearing cells, expose them to opsonized platelets and read out autoantigen-specific T cell responses. Yet, even then it will be in vitro experiments, which might not be representative for what occurs in vivo. Thus, although promising, personalized medicine in pediatric ITP still seems one bridge too far.
Reference List


63. Leontyev D, Katsman Y, Branch DR. Mouse background and IVIG dosage are critical in establishing the role of inhibitory Fcγ receptor for the amelioration of experimental ITP. Blood. 2012;119:5261-4


