Multifactorial aspects of antibody-mediated blood cell destruction
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General discussion
It is generally assumed that the antibody titer is the main determinant of antibody-mediated cell destruction and disease severity, with the higher the titer, the more cellular destruction occurring, for instance frequently reported for FNAIT.\textsuperscript{1-3} However, this correlation is not that strict considering the fact that in several cases with low antibody titers, potent platelet-destruction still occurs (and vice versa), indicating that other factors besides titer are likely to be involved.

In this thesis we started out by setting up and optimizing functional platelet-phagocytosis and respiratory burst assays with the aim of investigating potential new factors involved in antibody-mediated breakdown of platelets, which could possibly predict disease severity and/or offer new therapeutic approaches (Chapter 2). Surprisingly, we found that the IgG-specific respiratory burst reaction induced by opsonized platelets only occurred in the presence of serum, not only maternal FNAIT serum but also normal human serum (NHS). We excluded the involvement of complement, observed a variable effect with several NHS and noted there was a calcium-dependent effect. We could then identify the serum factor responsible as C-Reactive Protein (CRP), an acute phase protein known to be up regulated during infections, a known ligand for Fc-receptors on phagocytes and present at varying subclinical levels in healthy individuals. Furthermore, we found that CRP enhanced antibody-mediated platelet destruction both \textit{in vitro} (by CD16-positive monocytes and PMN) and \textit{in vivo} (using a previously established mouse model of ITP).

CRP is well established as an important acute-phase protein and is therefore utilized in daily clinical practice as a sensitive biomarker for infection and inflammation with its levels increasing from less than 0.05 mg/L to more than 500 mg/L after acute infections.\textsuperscript{4} CRP is produced by hepatocytes, predominantly under transcriptional control of IL-6. \textit{De novo} synthesis is rapidly initiated after a single stimulus, with serum concentrations rising above 5 mg/L by 6 hours and peaking around 48 hours, with a half-life of about 19 hours. When the stimulus ends, the CRP levels rapidly decrease. CRP is able to bind phosphorylcholine, which can be displayed by pathogens or damaged cell membranes.\textsuperscript{4,5} Also CRP can activate the complement system through binding of C1q and can also bind phagocytic cells via Fc-receptors,\textsuperscript{4,5} suggesting a role for CRP in the elimination of pathogens and targeted cells. In this thesis we suggest a novel role for CRP in antibody-mediated platelet destruction. We show that antibody binding to platelets triggered oxidation, resulting in exposure of phosphorylcholine residues, providing a binding platform for CRP. Low-level IgG opsonization of platelets, not sufficient to initiate phagocytosis on its own, could then be enhanced by CRP, providing a ligand for additional phagocyte FcγR, but perhaps also for FcαRI (IgA receptor), considering the fact that both types of receptor have been shown to bind CRP,\textsuperscript{6-12} further marking it for phagocyte destruction. Enhancement of antibody-mediated platelet destruction occurred at subclinical concentrations \textit{in vitro}, clearly below 10 mg/L and also measurements in FNAIT and ITP patients showed that CRP levels were increased compared to healthy controls, but
were also mainly present below 10 mg/L. Perhaps this can be explained by the fact that the stimuli in acute infections, responsible for high levels of CRP, are for instance bacterial ligands interacting with toll-like receptors. In FNAIT there are basically no bacterial ligands, preventing the production of high levels of CRP. This could of occur in ITP, but in those patients the infectious source is mostly viral, and in general bacterial infections are the ones associated with high CRP levels compared to viral infections.13-15 The observed subclinical levels of CRP in FNAIT and ITP can be explained due to the fact that CRP is always present in healthy individuals and is even known to have the tendency to be increased with age, probably due to an increasing incidence of subclinical pathologies.16

When the pathogenic antibody is present, CRP is therefore always present – albeit at different levels - and can contribute to the antibody-mediated platelet destruction. CRP levels could very well be clinically relevant, also because we found CRP levels to be increased compared to healthy controls in neonatal FNAIT samples and in children with newly diagnosed ITP. Importantly, we showed in a randomized trial, that IVIg treatment of newly diagnosed ITP patients resulted in lowered CRP levels. The drop in CRP levels was accompanied with a rapid increase of the number of platelets and reduced bleeding tendency. It remains unclear if CRP levels are a cause or a consequence of disease severity or IVIG treatment, but it is challenging to confirm which is the cause. However, in a mouse model we showed that CRP injection on its own was inert, but co-injection with the anti-platelet antibody caused enhanced platelet breakdown. Therefore, CRP levels could perhaps be a useful biomarker of severities of IgG-mediated thrombocytopenias. Therapeutically, it could be beneficial to investigate oxidation inhibitors, as these could prevent the exposure of phosphorylcholine, and perhaps strategies aimed at inhibition of CRP or reduction in CRP levels may prove to be effective in FNAIT/ITP patients.17 We show this now for platelets, but as phosphorylcholine is an integral component of all cellular membranes, this mechanism of antibody-mediated intensification of cellular breakdown may be a general phenomenon and therefore important for other allo- and autoantibody-mediated diseases, and possibly be utilized for antibody-mediated therapies.

Another factor we hypothesized which could influence IgG-mediated cell destruction, and therefore of diagnostic relevance was the IgG-Fc glycosylation pattern (Chapters 4-6). First, we analyzed ten paired fetal-maternal serum samples (healthy pregnancy setting), for their IgG1, IgG2 together with IgG3, and IgG4 glycosylation (Chapter 3). Average levels of IgG-Fc galactosylation, sialylation, bisection and fucosylation were found to be similar in maternal and fetal IgGs, suggesting IgG-transport not be glycosylation selective. Therefore, the non-invasive measurements of maternal IgG-glycosylation appear to be relevant and can be diagnostically relied upon, in the disease settings in which fetal cells are targeted by maternal antibodies (FNAIT and HDFN).

Interestingly, we observed a potent and variable decrease in Fc-fucosylation levels of both anti-platelet and anti-RBC alloantibodies in pregnancy (Chapters 4-6). This is remarkable,
considering the fact that normal fucose levels of total IgG show only little variation and are high around ~94%, also in pregnancy. The same phenomenon was observed for female HID-donors (initially immunized by pregnancy), but also for male HID-donors, thereby excluding the strict requirement for a pregnancy setting in order to obtain low Fc-fucose levels (Chapter 7). However, the immune milieu defined by pregnancy could still be a contributing factor, as female HID-donors still demonstrated a lower Fc-fucosylation than male HID-donors. Although glycosylation patterns are influenced by gender, and influenced by female sex hormones, there is no effect on fucosylation. The lower Fc-fucose may perhaps be an antigen-dependent response. Possibly the location where the antigen encounters the immune-system is of importance, for instance in the blood circulation. It could also be due to a lack of a danger signal, as is the case in pregnancy and in HID-donors. The particulate nature of both the platelet and red cell antigens might also be relevant, since no decreased fucosylation has been observed upon tetanus vaccination. So far, not many antigen-specific IgG have been investigated for their glycysylation profile, and more research is required to understand which factors determine the IgG-N-glycosylation and what the physiological relevance of this regulation is. Surprisingly, IgG-fucosylation appears to be stable in time (for FNAIT even observed 7 years after delivery, but also observed many years after primary immunization of HID-donors). This indicates that IgG-fucosylation acquired during the onset of the immune response, is subject of genetic imprinting or due to long-lived plasma cells.

For both anti-platelet and anti-RBC alloantibodies in pregnancy also increased levels of Fc-galactosylation were found, compared to total IgG (which is also known to be increased in pregnancy compared to a non-pregnancy setting). The increased Fc-galactosylation and lowered Fc-fucosylation levels may perhaps be regulated via higher expression of β4-galactosyltransferase and a down-regulation of FUT8 in the antibody producing plasma cells of the patients. For HIV-specific antibodies, which also were shown to have decreased fucosylation, FUT8 expression was found to be decreased in controllers and treated progressors compared to untreated progressors, while the expression of the fucosidase FUCA2, was found to be increased in controllers and untreated subjects. Recently, the transcription factor Hepatocyte Nuclear Factor (HNF)1a and its downstream target HNF4a have also been identified as transcriptional regulators of key fucosyltransferases and fucose biosynthesis genes. In addition, both the TLR9 ligand CpG oligodeoxynucleotide and IL-21 could be involved in the regulation, as they have been shown to increase Fc galactosylation. The involvement of these regulators has to be investigated for both FNAIT and HDFN.

The finding of low Fc-fucosylation in FNAIT could be very relevant for a potential FNAIT screening (Chapter 5). Screening of Fc-fucosylation might identify cases at higher risk for severe thrombocytopenia and thus for severe bleedings including intracranial hemorrhage. We did find a clinical correlation between anti-HPA-1a fucosylation and clinical disease severity, with asymptomatic cases having a high fucosylation (~85% on average). However, despite
significance, there were also cases with high fucosylation levels which resulted in intracranial hemorrhage. It is therefore important to validate these clinical data first in a larger cohort, including patients with a wide range of clinical symptoms including asymptomatic patients. Also the finding of low Fc-fucosylation in FNAIT could be relevant for a soon to be tested anti-HPA-1a immunoprophylaxis for FNAIT\textsuperscript{22}, as selection for low Fc-fucosylation would then be recommended, based on the enhanced clearance through FcγRIII and the correlation of anti-HPA-1a fucose with neonatal platelet counts that we found.

In contrast to FNAIT, for HDFN there is an accurate diagnostic test in place. It has previously been shown that the most sensitive laboratory test to predict fetal RBC destruction is the monocyte-mediated ADCC assay, for which it was shown that severe fetal anemia does not occur in RhD-alloimmunized pregnancies in which the ADCC results remained <50%, while in cases with a maximum ADCC result of >80%, 43% of the fetuses were severely anemic.\textsuperscript{23} For that reason all pregnant women in the Netherlands with anti-RBC alloantibodies are monitored using this assay and are only referred for Doppler flow measurement when the ADCC assay is above 50%. The reason why this test is such a good predictor could be because it is not only dependent on the antibody concentration but also on the interaction of the antibody with phagocytic cells. But since the monocyte-mediated ADCC is mainly effective via FcγRI, which is the high affinity receptor for IgG, and not involved in the clearance of the opsonized RBCs, its predictive potential is probably dependent on the steric presentation of the Fc-tail of the antibody. Therefore, the NK-cell mediated ADCC could add an extra dimension via IgG-fucosylation, as it exerts its function via FcγRIIIa, which seems to be highly relevant for erythrocyte-clearance,\textsuperscript{24} in combination with the fact that the observed lowered Fc-fucosylation has an increased binding affinity for FcγRIIIa and FcγRIIIb. It can be speculated that at least some of the fetuses in pregnancies with a monocyte-ADCC result of >80%, which were not severely anemic, would demonstrate a high degree of anti-D Fc-fucosylation. Therefore, the NK-cell mediated ADCC may perhaps improve the specificity of the current monocyte-mediated ADCC. Although the hemoglobin levels correlated to the degree of anti-D IgG1-fucosylation in a small pilot study we conducted (Chapter 6), the NK-cell ADCC will have to be thoroughly compared to the monocyte-mediated ADCC as regards to prediction of fetal red blood cell destruction. Furthermore, when investigating several commercial immunoprophylactic anti-D products (Chapter 7), we found a variable decrease in Fc-fucosylation in all batches, and this was lowest for immunoprophylaxis product we know for sure to be derived from mainly female HID-donors (~56%). This indicates a varying degree of biological activity in the immunoprophylactic products, as we also showed that antibodies with low Fc-fucose exhibit increased phagocytosis compared to antibodies with high Fc-fucose (Chapter 5). When investigating HID-donor immunization parameters with respect to the levels of anti-D fucosylation, we did not find any significant correlation between the level of Fc-fucosylation and the days after the first or last booster, the number of
donations, the anti-D titer or the age of donor. However, a significant positive correlation was found between the level of Fc-fucosylation and the number of administered boosters. This finding is in line with Guo et al25, who showed that repeated immunizations increased the level of Fc-fucosylation in mice. Based on the hypothesis that immunoprophylaxis exerts its effect through erythrocyte clearance via FcγRIII and the observed lowered Fc-fucosylation, it can be considered beneficial for the anti-D immunoprophylactic product to pre-select for HID-donors with low anti-D Fc-fucosylation, and to minimize the number of boosters. But it still needs to be established whether the working mechanism of anti-D immunoprophylaxis is indeed dependent on anti-D Fc-fucose.

Intriguingly, also the level of galactosylation was increased in the anti-D immunoglobulin preparations. It is known for long time that a decreased galactosylation of IgG in Rheumatoid Arthritis patients is linked to increased disease activity, and vice versa increased galactosylation is assumed to confer anti-inflammatory properties to IgG. More recently, it was shown that increased Fc-galactosylation promotes the interaction of the inhibitory FcγRIIb with the human lectin Dectin-1, which resulted in inhibition of the pro-inflammatory effector functions.26 Although we did not measure the sialic acid residues, these are probably also increased in anti-D preparations, because of the strong association between sialylation and galactosylation.27 The presence of sialic acid residues also confers anti-inflammatory properties to IgG, possibly mediated through interaction with DC-SIGN on macrophages and dendritic cells, and resulting in increased FcγRIIb expression.28 Interestingly, the binding of anti-D IgG to the inhibitory FcγRIIb has been proposed as an alternative or additional working mechanism for anti-D immunoprophylaxis.29 Our observations that plasma derived anti-D preparations demonstrate invariably high levels of galactosylation (and therefore presumably also high levels of sialylation) might contribute to the superior performance of these preparations.

In a setting of antibody-mediated platelet destruction, we identified two new factors which are of importance, namely the levels of CRP and the anti-platelet IgG-fucosylation patterns. Both of them seemed to correlate to platelet counts and clinical disease severity. As titer is also important in the antibody-mediated response, simultaneous investigation of titer, CRP levels and anti-HPA-1a-Fc fucosylation in a large group of patient samples will be warranted to investigate the diagnostic potential. The complexity can be further illustrated by other factors such as IgG subclasses with distinct affinity for various classes of the FcγRs, expression levels of FcγRs and also the genetic FcγR-polymorphisms. In a setting of ITP, viral and bacterial infections can also trigger or worsen the degree of ITP, but the exact underlying mechanisms are unknown. In this context, cross-reactivity or molecular mimicry has also been described between platelet antigens and structures on various viral and bacterial pathogens.30-33 Another factor in platelet destruction was shown to be lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, which was shown to enhance FcγR-mediated phagocytosis of IgG-
opsonized platelets *in vitro* and *in vivo*. Also there have been few reports on the myeloid inhibitory receptor signal regulatory protein (SIRP)α (also termed CD172a or SHPS-1), interacting with platelet-CD47, and thereby also able to influence the antibody-mediated phagocytic response.

In conclusion, several factors besides titer alone are involved in antibody-mediated blood cell destruction, exemplified for FNAIT in Figure 1. These multifactorial factors should all be taken into account from a diagnostic perspective, but also from a therapeutic point of view, opening up new avenues of treatment.

**Figure 1** Schematic representation of antibody-mediated platelet phagocytosis.

Red circles indicate factors which can influence the severity of antibody-mediated platelet destruction.
References


