Immunology Research Group
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Anti-human platelet antigen (HPA)-1a antibodies: For better or for worse

The development and characterization of, and exploration with, a novel human monoclonal antibody, reactive with HPA-1a, in relation to fetal and neonatal alloimmune thrombocytopenia

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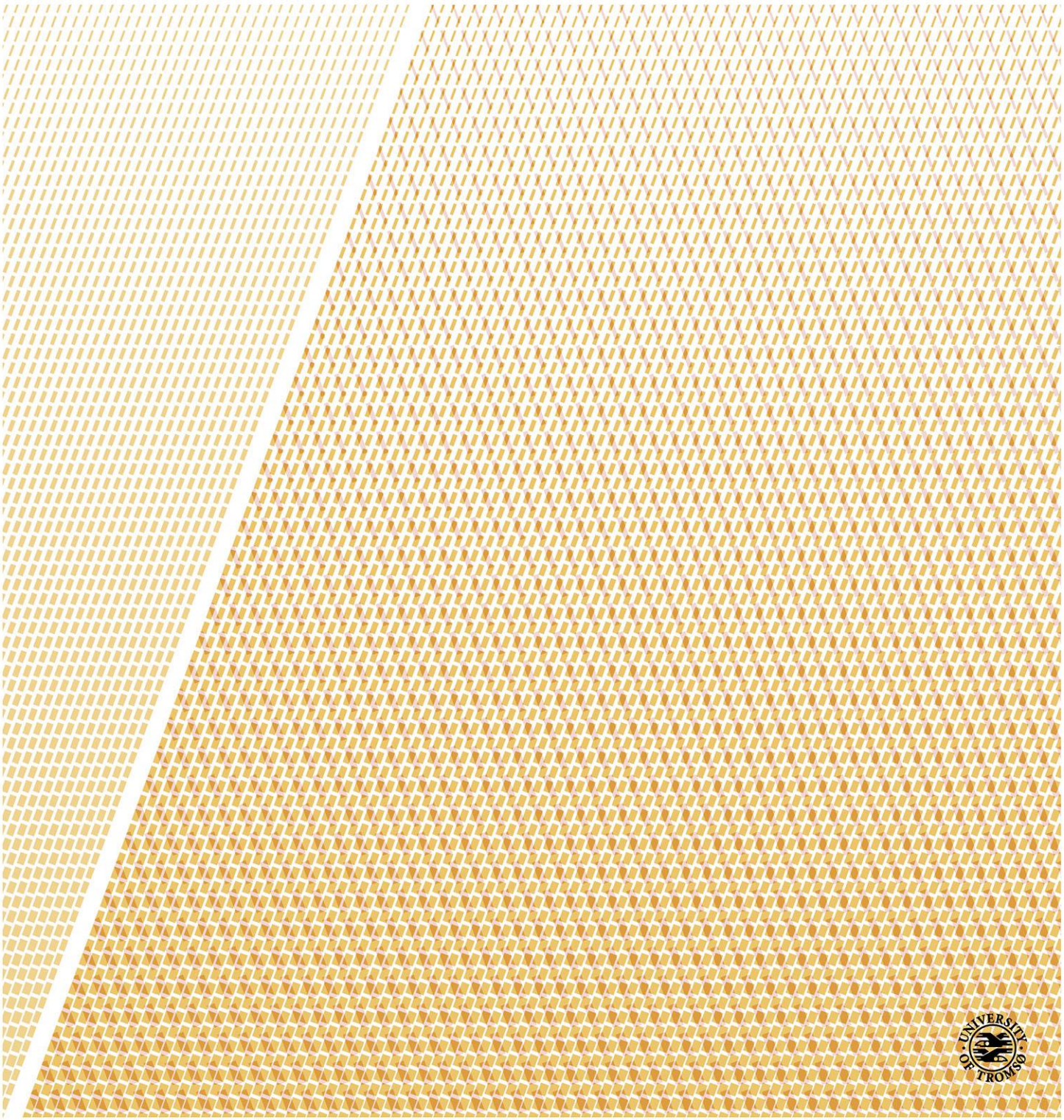
Mariana Eksteen

A dissertation for the degree of Philosophiae Doctor – October 2015



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The development and characterization of, and exploration with, a novel human monoclonal antibody, reactive with HPA-1a, in relation to fetal and neonatal alloimmune thrombocytopenia

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Tromsø, October 2015

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List of papers

I. Eksteen M, Tiller H, Averina M, Heide G, Kjaer M, Ghevaert C, Michaelsen TE. Ihle O. Husebekk A. Skogen BR. Stuge TB. Characterization of a human platelet antigen-1a-specific monoclonal antibody derived from a B cell from a woman alloimmunized in pregnancy. *The Journal of Immunology, June 15, 2015, 194:5751-5760.*

II. Eksteen M, Kjaer M, Husebekk A, Skogen BR, Stuge TB. A novel human monoclonal human platelet antigen (HPA)-1a-specific antibody can serve as a diagnostic reagent in fetal and neonatal alloimmune thrombocytopenia. *Manuscript.*

III. Eksteen M, Heide G, Tiller H, Zhou Y, Hersoug Nedberg N, Martinez IZ, Husebekk A, Skogen BR, Stuge TB, Kjaer M. Anti-human platelet antigen (HPA)-1a antibodies affect trophoblast functions crucial for placental development: A laboratory study using an *in vitro* model. *Submitted*.

Abbreviations

ADCC	antibody-dependent cell cytotoxicity
AMIS	antibody mediated immune suppression
APC	antigen-presenting cell
ASIT	antigen-specific immunotherapy
BCR	B cell receptor
BL	B-lymphoblastoid
CDC	complement-dependent cytotoxicity
CDR	complementarity determining region
CTB	cytotrophoblast
CVUE	chronic villitis of unknown etiology
dNK	decidual natural killer cells
EBV	Epstein-Barr virus
EC	endothelial cells
EGF	epidermal growth factor.
EVT	extravillous trophoblast
Fab	antibody binding fragment
FACS	fluorescence-activated cell sorting
FC	flow cytometry
Fc	fragment crystallizable
FcRn	neonatal Fc receptor
FGR	fetal growth restriction
FNAIT	fetal and neonatal alloimmune thrombocytopenia
FR	framework region
GP	glycoprotein
HDFN	hemolytic disease of the fetus and newborn
HLA	human leukocyte antigen
HNA	human neutrophil antigen
HPA	human platelet antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
ICH	intracranial hemorrhage

ITP	immune-mediated thrombocytopenic purpura
IVIG	Intravenous immunoglobulin
KIR	killer-cell immunoglobulin-like receptor
LBW	low birth weight
LDA	limiting dilution assay
LDH	lactate dehydrogenase
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAIPA	monoclonal antibody immobilization of platelet antigens
MCI	massive chronic intervillositis
MHC	major histocompatibility complex
MPR	multitransfusion platelet refractoriness
PBMC	peripheral blood mononuclear cell
PSI	plexin/semaphorin/integrin
PTP	post-transfusion purpura
R	replacement mutation
RBC	red blood cell
RhD	rhesus D
S	silent mutation
scFv	single-chain variable fragment
SPR	surface plasmon resonance
Th	helper T cell
Treg	regulatory T cell
WG	weeks gestation
VnR	vitronectin receptor

Thesis summary

Albeit a rare pregnancy complication, fetal and neonatal alloimmune thrombocytopenia (FNAIT) due to anti-human platelet antigen (HPA)-1a antibodies carries a significant risk of intracranial bleeding in the fetus and newborn. Reduced birth weight is another possible complication of FNAIT. Currently, there is no specific treatment to prevent or treat FNAIT, neither exist screening programs identifying women at risk of having a baby affected by FNAIT. Prophylactic and therapeutic strategies have been proposed, and the debate on screening programs is intensifying in several countries. The potential of polyclonal anti-HPA-1a IgG to prevent HPA-1a immunization is currently being tested in clinical trials. In this study we have developed a human monoclonal antibody (mAb) highly specific for HPA-1a, named 26.4. By *in vitro* experimentation, we have shown that this mAb can potentially be developed into a drug to specifically prevent maternal immunization to HPA-1a by potentiating the removal of fetal platelets from maternal circulation prior the immunization takes place. We have also demonstrated that this mAb outcompetes maternal anti-HPA-1a antibodies for binding to the antigen, and therefore mAb 26.4 can also be developed into a drug to treat FNAIT, by protecting fetal platelets from potentially harmful maternal anti-HPA-1a antibodies in cases when the immunization has already occurred. We have also shown that this mAb can be used as a diagnostic reagent to identify women at risk of HPA-1a immunization, as well as a standard for quantitation of anti-HPA-1a antibodies. Using an *in vitro* model, we have found that anti-HPA-1a antibodies affect trophoblast functions crucial for placental development. The latter finding sheds light on one of the possible causes of the reduced birth weight in FNAIT-affected babies.

Table of Contents

1. Introduction

In the following sections, topics relevant to this thesis will be briefly discussed. First, a short overview on the mechanisms of immune tolerance and alloimmunization in pregnancy will be given. Next, relevant information on the antibodies (Ab)s in general and human monoclonal antibodies (mAb)s will be provided, followed by an introduction to platelets and human platelet antigens (HPA)s, then fetal and neonatal alloimmune thrombocytopenia (FNAIT) due to HPA-1a and its pathophysiology. Finally, the existing strategies for prevention and therapy of FNAIT will be presented.

1.1 Maternal immune tolerance towards the fetus

The placenta forms the interface between maternal and fetal tissues, protects and nourishes the developing fetus throughout gestation. The placenta is formed early in embryonic development by the trophectoderm and extraembryonic mesoderm. The extraembryonic mesoderm forms the stromal core of the placenta, giving rise to the fibroblasts, vascular network and resident macrophage population (1). The trophectoderm differentiates into trophoblast, forming a syncytium – the epithelial lining of the placenta, and the invasive extravillous throphoblast (EVT). The syncytium produces hormones and controls nutrient, gas and waste exchange between the mother and the fetus. EVT cells invade the decidualized endometrium reaching the inner third of myometrium and control the remodeling of spiral arterioles into large diameter vessels of low resistance (Figure 1). Trophoblast invasion is highly controlled by various factors and interactions with decidual cells, and is normally completed by mid-gestation (2, 3). Insufficient remodeling of these vessels leads to inadequate placental perfusion, which in turn may lead to pregnancy complications like miscarriage, fetal growth restriction (FGR) and preeclampsia (4-8).

Trophoblast cells are in direct contact with maternal cells, and fetal cells gain access to the maternal circulation. How the maternal immune system tolerates the semi-allogeneic fetus, while effectively protecting the mother and fetus from pathogens, have intrigued scientists for decades. The maternal immune response during pregnancy, aided by endocrine pathways, is modulated to actively tolerate fetal antigens, especially in the unique environment of the decidua. Among sophisticated mechanisms of tolerance at the maternal-fetal interface (2) of particular interest is the lack of expression of the HLA class II antigens and class I HLA-A and -B by EVT cells. Instead, EVT cells express class I HLA-C and non-classical

monomorphic HLA-E and -G (9-11). This HLA expression pattern prevents direct allorecognition of fetal antigens by maternal T cells. The interaction between HLA-C on trophoblasts and the killer-cell immunoglobulin-like receptors (KIR)s on decidual natural killer (dNK) cells modulates trophoblast invasion and remodeling of the uterine vessels (12). Regulatory T (Treg) cells play an important role in mediating tolerance to the fetus and their frequency increases during pregnancy locally, in the decidua, and systemically (13-15). Recent murine studies have provided evidence for conceptus-specific immune suppression (16, 17). These studies demonstrate that conceptus-specific extrathymic Treg cells are generated during pregnancy, persist long after delivery and expand in numbers during subsequent pregnancies.

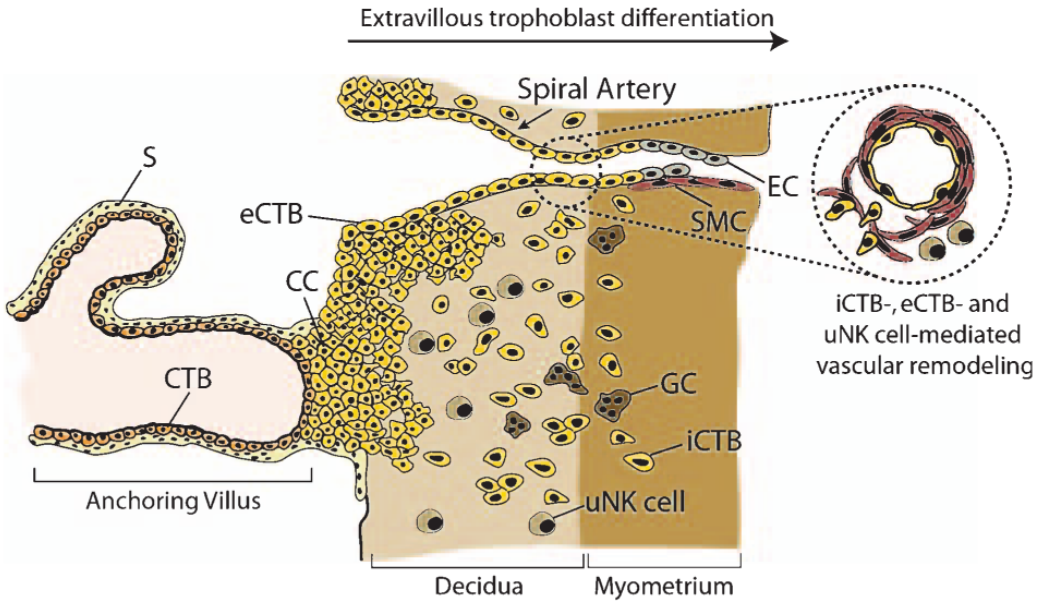

Figure 1. Differentiation pathways of the extravillous trophoblast (EVT). Reused from (18), with permission. CTB – cytotrophoblat; CC – cell column; iCTB – interstitial CTB; eCTB – endovascular CTB; GC – giant cells; SMC – smooth muscle cells; EC – endothelial cells; uNK – uterine NK cells.

A view on pregnancy as a "Th2" or anti-inflammatory state has proved to be oversimplified, since inflammatory response is crucial for implantation, placentation and parturition (19). Expectant mothers are generally not more susceptible to infections than non-pregnant women (20), however they can be more severely affected by certain infections (21, 22). Response to vaccines during pregnancy is not attenuated (23). Numbers of the B cells in human decidua are relatively low (24). Placental antigen-specific B cells are deleted in the bone marrow in mice (25). Increased estrogen levels in the third trimester reduce B lymphopoiesis (26, 27) while increasing immunoglobulin production by plasma cells (28). While sustaining humoral

immunity, the immune system during pregnancy protects the placenta from potentially harmful placenta-specific antibodies.

1.2 Alloimmunization in pregnancy

Immune tolerance during pregnancy does not completely prevent alloimmunity. The mechanisms leading to the break of maternal-fetal tolerance in some pregnancies are still not fully understood. Potential mechanisms may involve presentation of paternal antigens from the shed trophoblast microparticles and/or fetal cells leaked in to the maternal circulation on the MHC class II molecules of maternal antigen-presenting cells (APC)s in the lymph nodes and spleen (29, 30). Recognition of the paternal antigen by helper T (Th) cells in combination with 'co-stimulatory' signals from the APCs activate the Th cells (31). The dogma states that signals from the antigen bound to the B cell receptor (BCR), and signals from the antigen-specific armed Th cells, that recognize the antigen presented on the B cell MHC class II, induce the B cells to proliferate and differentiate into antibody-secreting plasma cells. This may be the case in alloimmunization as well, although responses have yet to be described in such detail. Primary response to the Ag results in predominantly class IgM Abs. Upon subsequent antigen exposure, the B cells undergo class switch, somatic hypermutation and affinity maturation, resulting in the production higher affinity antibodies of IgG class. Following an immune response, long living memory Th and B cells are also formed, and later exposure to the antigen leads to an accelerated antibody response as a result of rapid proliferation of Ag-specific clones.

Clinical manifestations of maternal alloimmune response depend on the specificity of alloantibodies. The classic alloimmune responses in incompatible pregnancies are directed to RhD, HPAs and human neutrophil antigens (HNA)s causing anemia, thrombocytopenia and neutropenia (32) respectively, in the fetus and newborn.

1.3 IgG effector functions

While the variable region of the antibody binding fragment (Fab) defines the Ab specificity, the Ab effector functions depend on the fragment crystallizable (Fc) part on the Ab molecule (Figure 2). Most IgG effector functions are mediated through complement and/or the Fcγ receptors (FcγR)s. Complement activation triggers the destruction of IgG-sensitized cells by complement-dependent cytotoxicity (CDC) or C3b-mediated phagocytosis. IgG bound to target cells crosslink the activating FcγRs on leucocytes mediating target cell destruction by

phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC). FcγRs have varying distribution on the effector cells (33). FcγRI expressed on macrophages, monocytes and activated granulocytes. FcγRIIa widely expressed on myeloid cells. FcγRIIIa is expressed on NK cells, macrophages and splenic monocytes. The inhibitory FcγRIIb is expressed on B cells and macrophages. All three FcγR classes are expressed early in fetal development (34). Human FcγRs are highly polymorphic and display single nucleotide polymorphisms as well as copy number variants (35) affecting their affinity for IgG and ability to engage the effector cells. FcγR polymorphisms have been associated with chronic inflammatory and autoimmune disease, susceptibility to pathogens, and responsiveness to the intravenous immunoglobulin (IVIG) therapy (35).

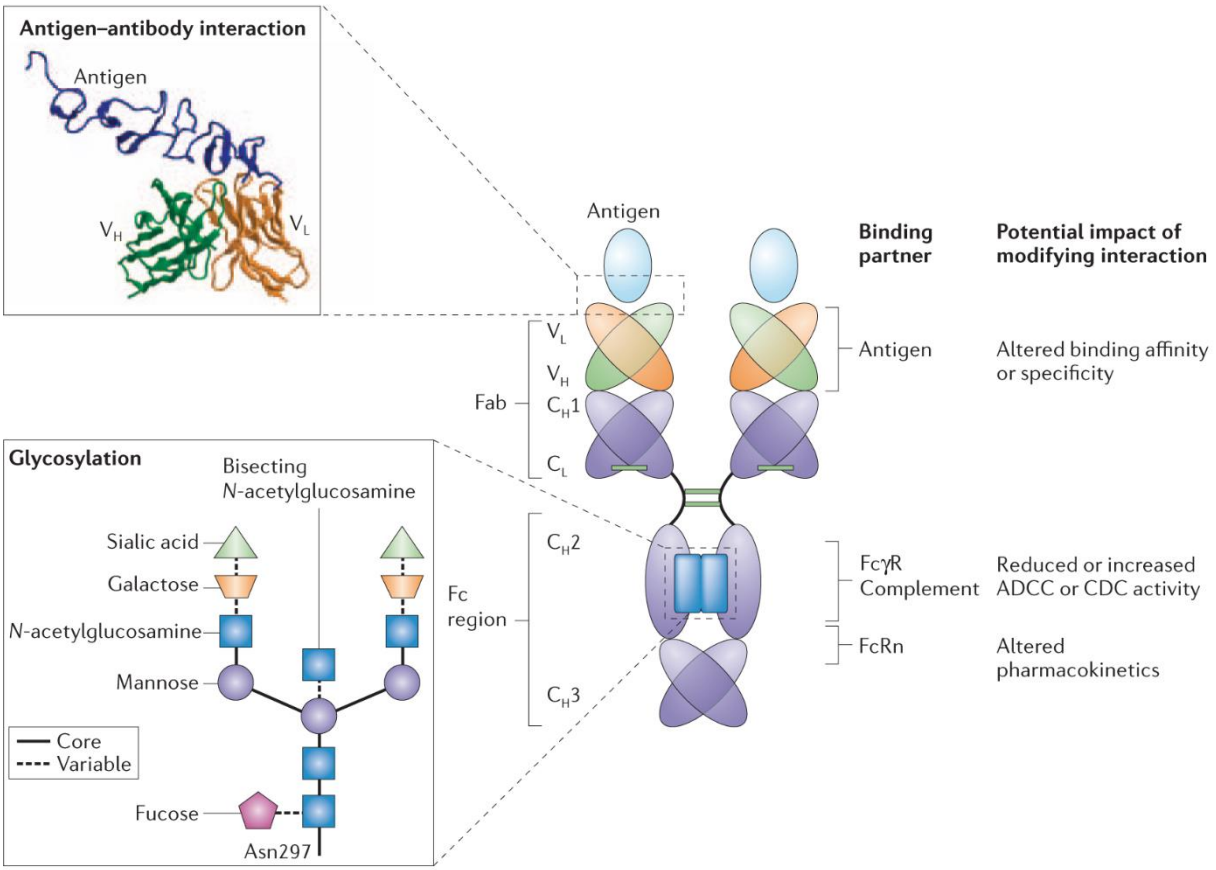

Figure 2. IgG structure and effector functions. Reused from (36), with permission.

The biological activity of the IgG, the ability to activate compliment and engage a particular FcγR, is dependent on the IgG subclass, IgG3>IgG1>>IgG2>IgG4 (37). Four IgG subclasses bind different FcγRs with varying affinity and specificity. IgG1 and IgG3, especially relevant in FNAIT (38) and hemolytic disease of the fetus or newborn (HDFN)(39), bind all of the

FcγRs. IgG2 binds FcγRIIa, and FcγRIIIa with slightly lower affinity. IgG4 binds FcγRI, and FcγRIIa with lower affinity. IgG3 is the most potent subclass in complement activation.

The composition of N-glycans on the Fc part can modulate antibody affinity to FcγRs and thus antibody effector functions. The IgG molecule contains two oligosaccharide groups attached to Asn297 of the C_H2 domain (Figure 2). In addition, N-glycans occur on 15–25% of the IgG Fab parts and can modulate antigen binding (40, 41). Each glycan has a core containing N-acetylglucosamine (GlcNAc) and mannose, with variable numbers of galactose, fucose, sialic acid and bi-sected GlcNAc attached to it. The lack of core fucose results in much stronger, up to 50-fold increased, binding to human FcγRIII (42).

1.4 Transfer of IgG to the fetus

Maternal IgM alloantibodies are not harmful to the fetus since IgM is not transported across the placenta. The neonatal Fc receptor (FcRn) expressed on syncytiotrophoblasts actively transports all four IgG subclasses, IgG1>IgG4>IgG3>IgG2, from the maternal circulation to the fetal capillaries of the placental villi (43). Maternal IgG antibodies in fetal blood are already detectable at the end of the first trimester (44). The IgG levels increase slowly during the first half of second trimester reaching approximately 10% of maternal concentration at 22 WG. Further into pregnancy, fetal IgG concentrations increase sharply, reaching levels slightly exceeding maternal IgG at term. Binding of IgG to FcRn does not involve the Fc glycans (45), therefore trans-placental transport of human IgG does not favor certain Fc glycoforms (46).

IgG3 has relatively poor placental transport as well as shorter half-life compared to that of other IgG subclasses (7 days vs 21 days) (33); IgG1 outcompetes IgG3 for binding to FcRn (47). IgG3 bears arginine at position 435 instead of histidine in other IgG subclasses. IgG3 is known to be polymorphic in humans, and the three variants containing histidine at position 435 (~ 1% in Europeans and 8% in Asians) have half-lifes and placental transport similar to other subclasses (48, 49).

1.5 Therapeutic monoclonal antibodies

Over a century ago, Paul Ehrlich hypothesized that a 'magic bullet' could selectively target disease. After the development of the hybridoma technology by Georges Köhler and César Milstein in 1975 (50), mAbs against target antigens became available and revolutionized

biomedical research and diagnostics. A hybridoma is an immortal antibody-secreting cell line derived by fusing a short-lived lymphocyte with a myeloma cell line (51). mAbs are often referred to as ‘researcher's Swiss army knife’.

Therapeutic murine mAbs entered clinical study in the early 1980s, but their success was limited by immunogenicity, the lack of effector functions and short serum half-life. Advancing technologies enabled engineering of chimeric (52) and humanized (53) antibodies, which were better tolerated and had improved effector functions. Production of human mAbs from human hybridomas, or human B-lymphocyte cell lines immortalized by Epstein-Barr virus (EBV), proved to be difficult due to the instability of cell lines and low amounts of produced antibodies (54, 55).

In the early 1990s, cloning of antibody genes (56) and expression of functional antibody fragments on the surface of filamentous phage (57, 58) enabled the bypassing of the hybridoma. Phage display was successfully used to isolate murine and human Abs from recombinant antibody libraries built from animal or human B lymphocytes, and later synthetic libraries (59). Development of transgenic mice that express human immunoglobulin genes (60-62) enabled generation of human mAbs with a broader spectrum of targets than human B cell-derived antibodies. Fully human mAbs produced by these technologies are the current state-of-art. However, both technologies have limitations. The murine immune system does not recognize some antigens as foreign and the variable part of the IgG molecule selected in mice can be immunogenic to humans. One caveat of the microbial display is that the pairing of VH and VL does not represent the selected *in vivo* antibody pairing. The other concern is that microbial display libraries do not fully represent all antibodies. Fully human antibodies with *in vivo* VH/VL pairing can be produced employing single B cell PCR, EBV immortalization of B cells or hybridoma in combination with Ab recombinant technology. Rapid growth in human mAbs in clinical research generally, and the particularly high rates of development of antineoplastic and immunomodulatory human mAbs is demonstrated in Figure 3. Currently, various Ab modifications, Ab fragments and conjugates are being developed to modify existing antibody properties or to engineer Abs with new capabilities (63).

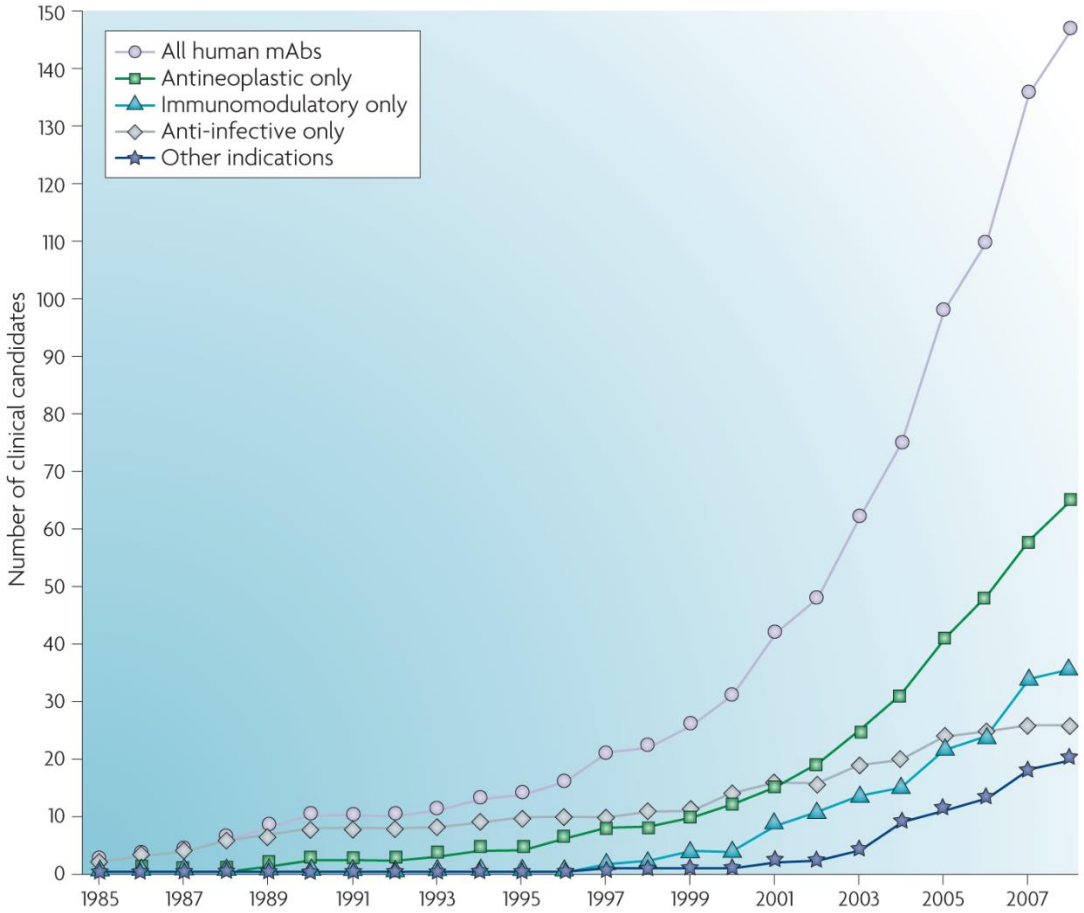

Figure 3. Number of human mAbs entering clinical study between 1985 and 2008. Reused from (64), with permission.

1.6 Platelets

Platelets are small, anucleate cells about 2 µm in diameter in resting stage, that are produced from the cytoplasm of bone marrow megakaryocytes. The lifespan of an individual platelet is only 8-10 days. Platelets are primarily destroyed by macrophages in the spleen. Normal platelet count in healthy individuals ranges 150-400 x 10^9 /L. Like erythrocytes, platelets are confined to the blood vascular system and do not enter the lymphatics or extravascular tissues. They primarily interact with leucocytes in the spleen or liver. The platelet's primary physiological role is in hemostasis. In damaged vessel endothelium, platelets recognize exposed collagen and basement membrane proteins, adhere to the site of injury and release platelet activation mediators. Activated platelets release pro-thrombotic mediators, which lead to enforcement of the coagulation cascade and formation of the hemostatic plug.

Platelets store multiple biologically active molecules in their granules and express immune receptors on the surface enabling them to modulate innate and adaptive immune responses.

Platelets play an important role in inflammation, angiogenesis, atherosclerosis, lymphatic development and tumor growth (65).

Platelets first appear in the human fetus during the first trimester of gestation and platelet count reaches the normal adult count range >150 x 10^9 /L by 17 weeks of gestation (WG) (66). Neonatal platelets have a hypofunctional phenotype compared with adult platelets (67). However, healthy full-term infants have enhanced hemostasis compared with older children and adults (68-70).

The fibrinogen receptor, GPIIbIIIa

The ability of platelets to adhere and aggregate is mediated by platelet membrane glycoproteins (GP)s, the transmembrane heterodimeric α/β receptors. GPIIbIIIa, also known as integrin heterodimer αIIbβ3 or the fibrinogen receptor, is the most abundant platelet surface membrane GP with ~80,000 molecules per platelet (71). Fibrinogen is the principle ligand of the fibrinogen receptor, but it also binds fibronectin, vitronectin and von Willebrand factor (vWF) (72). GPIIbIIIa exists in a resting conformation state and is activated to bind its ligands by inside-out signaling. GPIIbIIIa is the most immunogenic integrin on platelets, harboring multiple polymorphisms including HPA-1a/b (Figure 4).

1.7 Human platelet alloantigens

Characterization of the HPA system began in late 1950s (73) and is ongoing (for the current list see http://www.ebi.ac.uk/ipd/hpa/table1.html). To date, 34 HPAs have been identified on six functionally important platelet glycoproteins, GPIIb, GPIIIa, GPIbα, GPIbβ, GPIa and CD109 (Figure 4) (74). HPAs have been implicated in alloimmune platelet disorders such as FNAIT, posttransfusion purpura (PTP) and multitransfusion platelet refractoriness (MPR) (75). HPAs are classified numerically, following the date of discovery, and in alphabetical pairs, in order of allele frequency (‘a’ for more common and ‘b’ for less common antigens). HPA-1a and HPA-3a are expressed on fetal platelets in adult amounts as early as 16 WG (76).

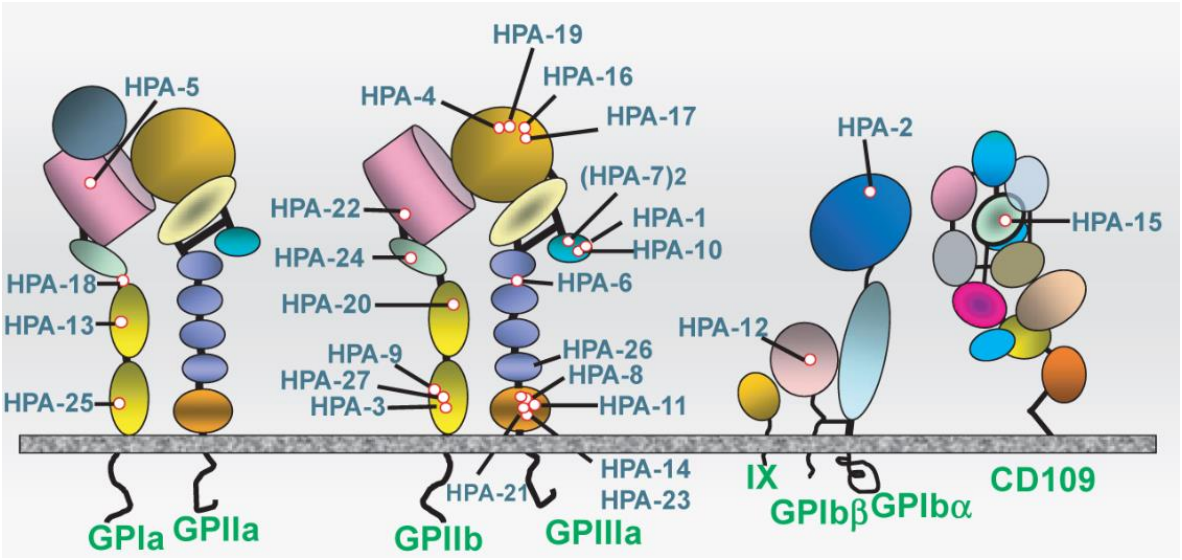

Figure 4. HPAs involved in FNAIT. Reused from (77), with permission.

Whilst called 'platelet specific', some of these antigens are present on other cell types. For example, the polymorphic integrin β3 as part of heterodimer αIIbβ3, the fibrinogen receptor, is expressed on megakaryocytes and platelets. Integrin β3 is also a part of a heterodimer αVβ3, the vitronectin receptor (VnR), and is expressed on various cell types including angiogenic endothelial cells (EC), fibroblasts, osteoclasts, trophoblasts and cancer cells.

A group of antigens expressed on blood cells and tissues, such as glycoconjugates of the blood group ABO system and the highly polymorphic HLA class I molecule, are also expressed on platelets. FNAIT can be caused by maternal IgG anti-B (and presumably anti-A) antibodies when the fetus belongs to a small group of individuals carrying large number of B (or A) antigens on platelets (78, 79). About a third of multiparous women develop IgG antibodies against fetal HLA class I antigens (80), however the evidence whether anti-HLA class I IgG antibodies can cause FNAIT is inconclusive (81-84).

1.8 HPA-1a/b polymorphism

The HPA-1 alloantigen system, previously designated $Pl^{A1/A2}$ or $Zw^{a/b}$, was first described around 1960 (85, 86). The HPA-1a epitope was later localized to GPIIIa (87). The antigen was further narrowed to 17 kDa subunit (plexin/semaphorin/integrin (PSI) domain) of GPIIIa and evidence provided that carbohydrate residues were not required for immunogenicity (88). Later, it was discovered that a single nucleotide change (C to T) results in one amino acid change (leucine to proline) at position 33 of the GPIIIa (89). A rare Val33 variant has also been described (90).

Synthetic peptides do not react with HPA-1a-specific antibodies (91), because of the 3D conformation facilitated by multiple disulfide bonds within the PSI domain (Figure 5) of native GPIIIa (92). The HPA-1a epitope is sensitive to disulfide bond reduction, or cysteine substitution, within the PSI domain (93). Epitopes of some anti-HPA-1a antibodies are not contained within the PSI domain and extend into hybrid/EGF1 domains (93). Naturally occurring substitution of the distally located residue Arg93Gln at the hybrid/PSI interface disrupts the HPA-1a epitope for some anti-HPA-1a antibodies (94).

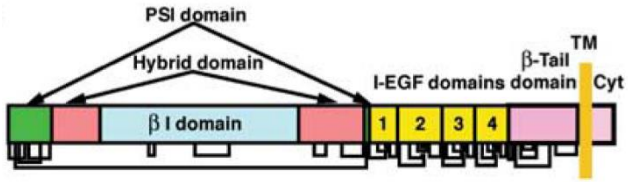

Figure 5. Schematic presentation of disulfide bonds within integrin β3. Reused from (92), with permission. PSI – plexin/semaphorin/integrin; EGF – epidermal growth factor.

Modelling of HPA-1a/b polymorphisms

To date, there is no crystallized HPA-1a antigen-antibody structure. To compare the structural effects of HPA-1a and -1b variants, 3D models of L33 and P33 integrin β3 were built using an αIIbβ3 structure available in the protein data bank and PyMOL software (Figure 6) (95). Counterintuitively, the L33P substitution did not significantly alter the local structure of the C26-C38 loop of the PSI domain, but resulted in modification of structural equilibrium of PSI, I-EGF-1 and I-EGF-2 domains. The P33 variant, compared to the L33 variant, increased structural flexibility of all three domains.

HPA-1a/b is a functionally important polymorphism

HPA-1a-negative platelets display hyper responsiveness to agonists *in vitro* (96, 97). In addition, CHO and 293 cells transfected with the HPA-1b variant of αIIbβ3, compared with the HPA-1a variant, exhibited greater adhesion to immobilized fibrinogen, greater spreading and actin reorganization, and greater fibrin clot retraction (98). It is speculated that the latter effects are due to enhanced outside-in signaling and adhesive functions of the HPA-1b variant of αIIbβ3 (98). In a large-scale meta-analysis carriage of the HPA-1b polymorphism was found to be a risk factor for ischemic strokes (99).

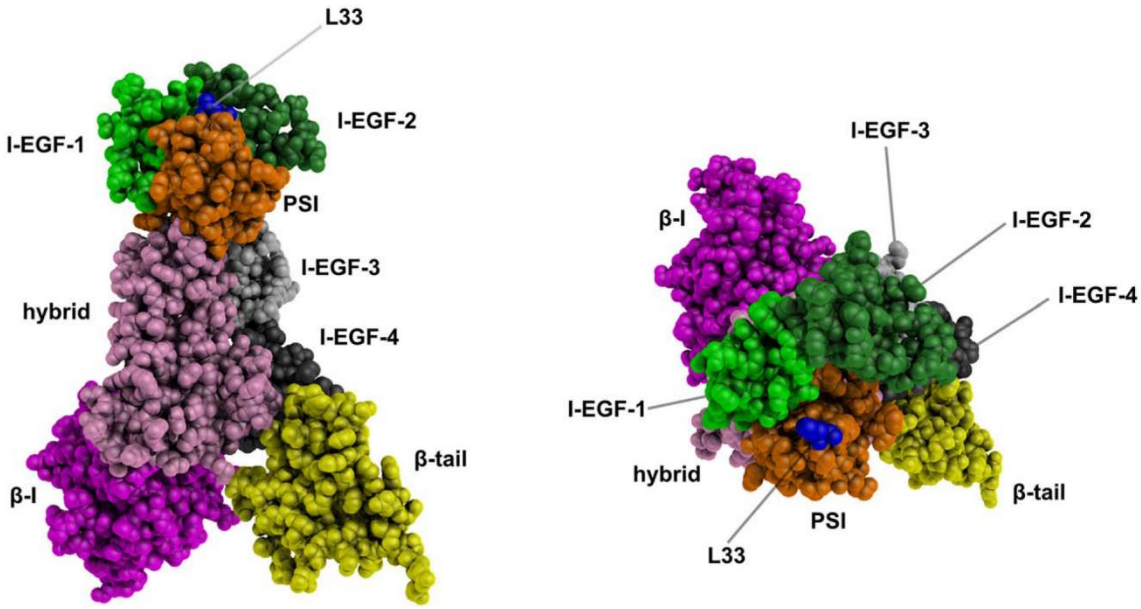

Figure 6. Ectodomain 3D structure model of the L33 integrin β3 isoform. Reused from (95), with permission. A side view of integrin β3 (left) and an apical view of the β3 knee (right) are shown. Domains are differently colored, labeled and the L33 residue is shown in blue. These static views illustrate the HPA-1 polymorphic site that is located at the top of the integrin β3 knee.

1.9 Incidence and clinical presentation of FNAIT due to HPA-1a

FNAIT is the most frequent cause of isolated severe thrombocytopenia in term neonates (100). The incidence of FNAIT is ~1 in 1000 live births (101, 102). FNAIT is caused by maternal alloantibodies against paternal antigens on fetal platelets. Maternal anti-HPA-1a antibodies account for over 80% of FNAIT cases in Caucasian populations (101, 103). The frequency of HPA-1a-negative (HPA-1bb) phenotype in Caucasians is 1.6 – 2.5%, however only 6 – 11.4% of HPA-1a-negative women become immunized in connection with an HPA-1 incompatible pregnancy (101, 103-105). Antibodies to HPA-5b are responsible in 9% of FNAIT cases, HPA-1b in 4%, and HPA-3a in 2%, followed by HPA-3b, -15a and -9b (Max^a) (106-108). FNAIT has been diagnosed in pregnancies without detectable antibodies (109-111). Also, alloimmunization does not necessarily result in FNAIT (101, 103-105). About a third of HPA-1a-positive neonates born to women with persistent anti-HPA-1a antibodies during pregnancy were born with normal platelet counts and another third had a moderate (platelet count $50\text{-}150^9$/L) thrombocytopenia (101, 103). The most common clinical presentations of FNAIT are petechiae and purpura, and less frequently, visceral and retinal bleedings. In 7-26% of severe FNAIT cases neonates suffer intracranial hemorrhage (ICH) (112, 113). ICH due to FNAIT is commonly characterized by intraventricular and intraparenchymal bleeding, which can lead to severe neurological sequelae or death (114).

The majority of ICH bleedings occur before 34 WG (115). Recurrence risk of ICH in a subsequent pregnancy is reported to be 79% (116). Recent observations have suggested reduced birth weight as another complication of HPA-1a alloimmunization (115, 117). The risk of severe FNAIT due to anti-HPA-1a antibodies is reported to be correlated to maternal ABO types (118).

1.10 Pathogenesis of FNAIT

1.10.1 Time of HPA-1a alloimmunization and source of the antigen

It is believed that fetal platelets as well as antigen-expressing trophoblast cells can cause HPA-1a alloimmunization. HPAs are expressed on fetal platelets in adult amounts as early as 16 WG (76). Fetal cells are detectable in the maternal circulation already in the first trimester (119) and fetomaternal hemorrhage is a common obstetrical occurrence. Syncytio- and invading trophoblast cells express αVβ3, carrying the HPA-1a antigen, and are in direct contact with maternal tissues (29, 120). Break of tolerance at the maternal-fetal interface and/or leakage of trophoblast cell material into maternal circulation could both potentially result in maternal immunization to HPA-1a. About a third of primigravidae women have detectable anti-HPA-1a antibodies, some of whom as early as 17 WG (103), consistent with HPA-1a expression of fetal platelets and presence of the antigen on trophoblast cells.

1.10.2 Cellular responses to HPA-1a

High affinity IgG antibody responses are likely dependent on T cell help. HPA-1a immunization is strongly associated with HLA class II allele *DRB3*01:01* (101, 103, 121, 122). HPA-1a (but not HPA-1b) peptide binds to the DRA/DRB3*01:01 molecule (123, 124) and HPA-1a-specific T cell responses can be stimulated *in vitro* in peripheral blood mononuclear cells (PBMC)s from HPA-1a alloimmunized women with HPA-1a peptide (125-127). Importantly, HPA-1a-specific T cells can be isolated from HPA-1a alloimmunized women (128, 129), and these are restricted by the DRA/DRB3*01:01 molecule. The above mentioned evidence lends strong support to the notion that anti-HPA-1a antibody responses are driven by T cells that recognize HPA-1a peptide in complex with the DRA/DRB3*01:01 molecule.

1.10.3 The mechanism of thrombocytopenia

FcRn in trans-placental transport

The critical role of fetal FcRn in trans-placental transport of anti-integrin β3 antibodies was formally proved in an integrin β3/FcRn-deficient murine model (130). Also of interest in the context of mechanisms of FNAIT, is that human IgG3 with a H435 polymorphism binds to FcRn as efficiently as IgG1, while the more common R435 IgG3 variant does not (47). It is conceivable that women positive for the H435 variant of IgG3 are more likely to give birth to children with FNAIT and HDFN, considering that this IgG3 variant predictably will have half-life and placental transport efficiency similar to IgG1, and that IgG3 has stronger effector functions compared with IgG1.

The role of FcγR and FcγR polymorphisms

It is generally accepted that anti-platelet IgG alloantibodies opsonize fetal platelets causing their recognition and destruction by FcγR-expressing phagocytic cells in the fetal liver and spleen (131-133). The important role of FcγRI and FcγRIIa in anti-HPA-1a platelet destruction has been demonstrated *in vitro* (133). In ITP patients, anti-platelet autoantibodies may contribute to platelet destruction, following activation of the complement system, by C3b-mediated phagocytosis and/or by complement-induced lysis of platelets and megakaryocytes (134, 135). Complement activation has not been studied in the context of FNAIT.

It is conceivable that carriage of certain FcγR polymorphisms is associated with severity of FNAIT. In clinical trials for prevention of RhD immunization by monoclonal anti-D IgG, individuals homozygous for FcγRIIIA-158V, which has higher binding capacity for IgG compared to the FcγRIIIA-158F variant, had the fastest clearance rate of RhD-expressing red blood cells (RBC)s (136, 137).

Antibody-mediated impairment of platelet production and aggregation

Impaired fetal platelet production, not only enhanced destruction, could contribute to the mechanism of thrombocytopenia. In support of this, it has been demonstrated that FNAIT maternal sera can suppress megakaryocyte production *in vitro* (138, 139). Sera from ITP patients containing anti-$\alpha IIb\beta_3$ antibodies also inhibited megakaryocytopoiesis *in vitro* (140, 141).

Some anti-HPA-1a sera are able to inhibit platelet aggregation (142-144). It has been hypothesized that the HPA-1a polymorphism is located near the RGD-binding site and anti-HPA-1a IgG antibodies sterically hinder access to the RGD binding site on αIIbβ3 (142, 144). To which extent, if any, inhibition of platelet aggregation by anti-HPA-1a antibodies contribute to bleeding in FNAIT is not known.

IgG subclass and glycosylation pattern

To date, only two small studies focused on the anti-HPA-1a IgG subclasses (38, 145). All sera contained IgG1, most sera contained IgG1+IgG3, while about a half of sera contained all four IgG subclasses. The levels of anti-HPA-1a IgG3 were significantly higher in the severely thrombocytopenic than in the mildly thrombocytopenic/unaffected group of sera. A higher number of maternal sera need to be tested to determine whether levels of IgG3 could be of predictive value for the severity of FNAIT.

A recent study found that maternal anti-HPA-1a-specific IgG1 from FNAIT cases had a markedly different glycosylation pattern compared to total serum IgG1: increased galactosylation and sialylation, and prominently decreased levels of core fucosylation (down to 10% while in total IgG1 is >90%) (146, 147). Decreased core fucosylation correlated with increased severity of FNAIT, possibly due to more effective phagocytosis of antibody-coated platelets (146). Similarly, a prominent decrease in Fc-fucosylation was also observed in the majority of maternal anti-D IgG1 (down to 12%) (148). The degree of fucosylation correlated significantly with low fetal-neonatal hemoglobin levels, probably due to more effective destruction of RBCs by FcγRIIIa-mediated ADCC (148).

Antibody avidity, epitope and titer

Multiple cases describe HPA-1a-negative mothers who lack detectable anti-HPA-1a antibodies gave birth to neonates affected by trombocytopenia (109-111). The antibodies were not detectable by highly sensitive and specific conventional techniques like flow cytometry (FC) and monoclonal antibody immobilization of platelet antigens (MAIPA). However, the use of surface plasmon resonance (SPR) technology enabled detection of antibodies in some of these cases and demonstrated their low avidity to the antigen (109, 111). It is possible that low avidity antibodies are washed away during extensive washing steps required in FC and MAIPA assays. Clinical significance of low avidity anti-HPA-1a antibodies has been

demonstrated in NOD/SCID murine model (110, 111). Mothers negative for the *DRB3*0101* allele are predisposed to produce anti-HPA-1a antibodies of low affinity (111).

Anti-HPA-1a antibodies have a different footprint on integrin β_3. The epitope of one type of anti-HPA-1a antibodies lays within the PSI domain, the first 54 residues of the β_3 integrin. The epitope of the other type of anti-HPA-1a Abs spans to the residues distant from the PSI domain, to the hybrid and epidermal growth factor (EGF) domains. The first type of Abs is present in the majority of FNAIT sera (149). No association between the antibody epitope and the severity of FNAIT has been found (149).

The level of maternal anti-HPA-1a antibodies was reported to be strongly associated with the platelet count in the newborn (150-153) and have a predictive value for the severity of FNAIT both in the first and subsequent pregnancies (153). A decrease in levels of anti-HPA-1a antibodies during subsequent pregnancy was observed (153). A subsequent study has shown that the decrease was unlikely due to anti-idiotypic antibodies (154).

1.10.4 Anti-HPA-1a antibodies can target other fetal cells than platelets

Anti-HPA-1a antibodies can interact with HPA-1a on angiogenic ECs in the fetal brain and may impair angiogenesis (155). ICH may therefore be triggered directly by anti-HPA-1a antibodies independent of thrombocytopenia. It has been speculated that anti-HPA-1a antibodies can affect placental development (117). Integrin β3, baring HPA-1a, is expressed on EVT as part of αVβ3 integrin heterodimer (VnR) (120, 156). Earlier *in vitro* research has revealed that anti-αVβ3 antibodies can affect trophoblast cell invasion (156) and adhesion to ECs (157, 158). Integrin αVβ3 is also expressed on the apical surface of syncytiotrophoblast microvilli (29, 120). Anti-HPA-1a antibodies can bind the HPA-1a expressed on trophoblast cells (120), however the effect of this interaction has not been explored yet.

1.11 Screening for FNAIT

Currently, there are no screening programs identifying women at risk of HPA-1a immunization, and no treatment for preventing immunization exists. Advocates of the implementation of screening programs claim that it could be beneficial for clinical outcome (101, 113) and be cost effective (159). The opponents argue that there was no randomized clinical trial conducted to assess possible clinical and economical benefits of screening, and point to the lack of consensus on management of pregnancies at risk of FNAIT (100, 160).

1.12 Diagnosis of FNAIT

In the absence of a screening program, FNAIT is typically diagnosed after the birth of the first symptomatic child. The diagnosis of FNAIT is confirmed when a fetus/neonate is positive for an HPA antigen lacking in the mother with a corresponding maternal HPA-antibody. Platelet HPA-1 phenotyping is usually done by flow cytometry (161). All HPA-1a-negative samples are further subject to HPA-1 genotyping by TaqMan 5' nuclease assay (162, 163). Currently the MAIPA assay is considered the gold standard for the detection and quantitation of anti-HPA-a antibodies (164-166).

About 15% of babies born to HPA-1a-negative women are themselves HPA-1a-negative, and thus will not be affected. A noninvasive fetal HPA-1a genetic testing based on real-time PCR (167, 168) and targeted massively parallel sequencing of *ITGB3* alleles (169) using cell-free fetal DNA isolated from maternal blood could be used to identify future pregnancies at risk for FNAIT.

1.13 Management of FNAIT

Antenatal

Antenatal treatment is currently provided only in subsequent to FNAIT-affected pregnancies. Previously, fetal blood sampling (FBS) was used to verify thrombocytopenia in the fetus and for intrauterine platelet transfusions (160, 170). However, FBS in such cases is associated with 6% fetal loss rate per pregnancy (171) and centers are gradually changing their management strategies towards a completely noninvasive approach for FNAIT (172, 173). If the risk of FNAIT is considered high, in some countries intravenous immunoglobulin (IVIG), alone or in combination with prednisone, is administered antenatally to the mother (114, 160, 174, 175). The effect of IVIG on fetal platelet count is questionable, but it was shown to have a protective effect with regard to ICH independently of platelet count (115, 155, 176).

Timing and mode of delivery

There is no evidence that early birth or caesarean section increases, or reduces, morbidity/mortality in FNAIT (175). An observational study on pregnancies with FNAIT and a thrombocytopenic sibling without ICH has suggested that vaginal delivery was not associated with increased risk of ICH (177).

Postnatal

Postnatal treatment includes transfusion of HPA-compatible platelets (neonatal platelet count threshold $<35x10^9$/L in Norway) with or without IVIG (101, 178) and cranial ultrasound examination for detection of ICH.

1.14 Strategies to prevent and treat FNAIT

1.14.1 FNAIT prophylactic strategies

Two strategies have been proposed to prevent HPA-1a immunization.

Strategy 1: Anti-HPA-1a antibody-based prophylaxis

The majority of HPA-1a immunizations occur in connection with delivery. Therefore they could potentially be prevented by a strategy similar to prophylaxis of HDFN. Maternal immunization to RhD is effectively prevented by postnatal administration (together with antenatal in some countries) of polyclonal anti-D immunoglobulin (179-181). The inhibitory effect of anti-D IgG antibodies on the immunization to RhD antigen is known as antibody mediated immune suppression (AMIS) (182). AMIS is not limited to the RhD antigen and has also been observed in HLA alloimmune response (183). AMIS by maternal antibodies can greatly influence the success of vaccination of young infants (184).

Mechanisms of AMIS

Most of the knowledge on AMIS comes from studies on anti-D prophylaxis. Mechanisms of AMIS of RhD alloimmunization have been extensively studied, but the exact mode of action is still not fully understood (182, 185). Several proposed hypotheses are outlined below.

The steric hindrance or epitope masking hypothesis suggests that IgG binds the epitope preventing its recognition by BCRs and activation of B-cells with similar specificity (186). This mechanism would be Fc-independent, unlike the others. Studies with FcγR-deficient mice (187) and studies assessing the effect of the $F(ab)_2$ fragment (188) have proven that the Fc-independent mechanism is operative. Other studies documented that AMIS is Fc-dependent (136, 137). In addition, only a fraction of RhD epitopes are blocked by administered anti-D (189). Together, these findings indicate that this may not be the sole mechanism of AMIS induced by anti-D prophylaxis.

The antigen clearance hypothesis proposes that the antigen is cleared from circulation before it is recognized by the immune system. RBCs opsonized with anti-D IgG were cleared from circulation and clearance of antigen-positive cells correlated with protection from anti-D immunization (190). Anti-D-coated red cells removed from circulation by splenic macrophages (191, 192). FcγRIIIa and Fc*γ*RIIa are central in phagocytosis of anti-D-opsonized RBCs (136, 137, 193). Interestingly, RBC clearance rate with some mAbs did not always correlate with the protective anti-D effect (136, 194-196). Moreover, despite efficient antigen clearance an enhanced antibody response was observed (195).

One human study demonstrated that anti-K (Kell) IgG antibodies suppressed the anti-D response to D^+K^+ erythrocytes given to D^-K^- individuals (197), suggesting that AMIS was particle (cell) specific and not epitope specific.

The FcγRIIb-mediated B-cell inhibition hypothesis proposes that anti-D-coated red cells can crosslink inhibitory Fc*γ*RIIb and BCR triggering the blockade of B cell activation. However, multiple studies documented that Fc*γ*RIIb is not necessary to achieve the AMIS (187, 198).

Current status of anti-HPA-1a antibody-based prophylaxis

Experimentation in animal models indicate that antibody based prophylaxis for FNAIT may be possible; antibody prophylaxis prevented integrin β3 immunization and improved pregnancy outcome in a integrin β3-/- murine model (199), proving, in principle, that the administration of anti-HPA-1a antibodies can potentially prevent HPA-1a alloimmunization. In human volunteers, platelets presensitized with HPA-1a-specific mAb (B2G1) were rapidly removed from blood circulation (200). Furthermore, the potential of hyperimmune anti-HPA-1a IgG to prevent HPA-1a immunization is being tested in clinical trials (www.profnait.eu) (201).

Strategy 2: HPA-1a antigen-specific tolerization

It may be possible to induce antigen-specific T cell tolerance to HPA-1a as a strategy to prevent HPA-1a-specific antibody production. Th cells recognize short antigen-derived peptides presented by specialized APCs. The environment in which the recognition takes place determines whether a specific immune response is activated or tolerized (202). Antigen-specific immunotherapy (ASIT) to prevent allergies (203-206) and for the treatment of

autoimmune disease (207, 208) has proven successful in clinic. In murine models, the administration of peptides containing dominant Th epitopes to nasal mucosa suppressed immunization against RhD (209) and K (210) blood group antigens. Similarly, using ASIT it might be possible to prevent alloimmunization to HPA-1a. A tolerization of antigen-specific CD4+ T cells with dominant HPA-1a peptides in transgenic mice expressing MHC class II molecule HLA-DRA/DRB3*01:01 (DR52a) is an ongoing project in our group.

1.14.2 FNAIT therapeutic strategies

Several therapeutic strategies have been proposed for treatment of HPA-1a-immunized women during pregnancy. In contrast to prophylaxis, the aim of therapeutic treatment is to prevent fetal thrombocytopenia in pregnancies where anti-HPA-1a antibodies have already been formed.

Strategy 1: Protective Abs

Fetal tissues and platelets could be protected from potentially damaging anti-HPA-1a antibodies with antibodies that compete for binding to HPA-1a and lack the ability to activate immune effector functions. This is not a new concept and has been proven to function in principle with HPA-1a-specific mAbs both in a murine model (211) and in human volunteers (200). For this purpose, anti-HPA-1a mAbs have to be of high affinity to compete for the epitope and should have a non-functional Fc part (212).

Strategy 2: Targeting the FcγRs

Anti-HPA-1a-sensitized platelets are recognized by FcγR-expressing phagocytic cells, triggering platelet destruction by phagocytosis (213, 214). *In vitro* experiments demonstrated that anti-FcγR mAbs effectively, and superior to IVIG, suppress anti-HPA-1a-mediated platelet ingestion by phagocytes (133). It has been proposed that blocking of the FcγRs could potentially be used for the antenatal therapy of FNAIT.

Strategy 3: Targeting the FcRn

FcRn receptor is essential to maintain IgG homeostasis and transport of IgG across the placenta (43, 215). This receptor has been a target in development of therapeutics for autoimmune disease in recent years (215, 216). The principle of this strategy lays in the inhibition of IgG binding to FcRn, by IVIG or anti-FcRn antibodies, resulting in accelerated degradation of endogenous pathogenic IgG. Inhibition of pathogenic IgG antibody binding to FcRn, by using anti-FcRn mAb or IVIG, had a potent therapeutic effect in the improvement of

fetal and neonatal immune thrombocytopenia in a murine model (130). Whether targeting the FcRn has a significant therapeutic value for treatment of FNAIT is still to be determined.

1.15 Monoclonal antibodies specific for HPA-1a

HPA-1a-specific mAbs can serve as diagnostic tools, as well as potential prophylactic and/or therapeutic reagents in FNAIT. The existing HPA-1a-reactive mAbs have limitations. Success to generate murine mAbs specific for HPA-1a was limited due to the lack of the HPA-1 system in mice (217, 218). One murine mAb, clone SZ21, can distinguish HPA-1a and -b on intact platelets due to lower affinity binding to the HPA-1b; mAb SZ21 binds both, HPA-1a-positive and -negative platelets, at increasing mAb concentrations (218). Murine mAbs produced by immunizing mice against synthetic peptides containing the HPA-1a and -1b sequences do not react with the native GP on intact platelets due to conformational nature of the HPA-1a antigen (219). Phage display technology was used by several groups to produce human mAb fragments which bind to the HPA-1a form of GPIIIa on intact platelets (220-222). mAbs and Ab fragments distinguishing between HPA-1a and -b reported up to date are presented in Table 1.

Table 1. Antibody fragments, monoclonal or recombinant antibodies distinguishing between HPA-1a and –b.

Designated mAb or Ab fragment	Isolated product	host	Immunogen	Full molecule IgG mAb or recAb	Specificity	Commercial availability	References
3C1	mAb, hybridoma	mouse	Peptide β3 27-37, Leu33	mAb AD3	Specific for HPA-1a on the reduced form of integrin β3; does not bind intact platelets	Not found	(219)
AD3	mAb, hybridoma	mouse	Peptide β3 27-37, Pro33	mAb AD3	Specific for HPA-1b on the reduced form of integrin β3; does not bind intact platelets	Not found	(219)
LK-4	mAb, hybridoma	mouse	Human platelets	mAb, clone LK-4 (IgG1; kappa)	Specific for HPA-1a on the reduced form of integrin β3; binds both, HPA-1a and -1b on intact platelets	Hybridoma available at ATCC (#CRL-2345)	(217)
SZ21	mAb, hybridoma	mouse	Human platelets	mAb, clone SZ21 (IgG1)	Pseudospecific for HPA-1a: binds HPA-1b with lower affinity than HPA-1a	Commercially available mAb	(218)
ML-1	scFv*	HPA-1a alloimmunized woman with FNAIT child		No info	HPA-1a-specific according to ref. (221)	Not found	(221)
CamTran 007	scFv	HPA-1a alloimmunized woman with FNAIT child		recAb, clone B2G1 (IgG1)	HPA-1a-specific	Available as part of ELISA-based HPA-1a phenotyping kit from Bio-Rad (#030011)	(220)
6-14, 19, 23-24	Fab# fragments	PTP patient in acute phase		recAbs, clones 19-7, 23-15	HPA-1a-specific according to ref (149, 222)	Not found	(222), (149)

*scFv - single-chain variable fragment; #Fab- fragment antibody binding.

2. Aims of the study

The main purpose of this study was to generate human mAbs highly specific for HPA-1a and to characterize the mAbs with regard to their possible future use as a drug for prophylaxis and treatment of FNAIT, as well as a diagnostic reagent in FNAIT. The other aim was to assess the possible effect of anti-HPA-1a antibodies on invading trophoblast cells.

More specifically, the main research aims were:

- To generate HPA-1a-specific mAbs by immortalization of B cells from a woman alloimmunized in connection with pregnancy
- To characterize HPA-1a-specific mAbs concerning specificity, affinity, effect on platelet aggregation and functionality.
- To evaluate the use of a novel human HPA-1a-specific mAb as an HPA-1 phenotyping reagent and as a standard for quantitation of anti-HPA-1a antibodies in MAIPA assay
- To examine the effect of anti-HPA-1a antibody binding to αVβ3 on extravillous trophoblast cells.

3. Summary of papers

Paper I

Title: Characterization of a human platelet antigen-1a-specific monoclonal antibody derived from a B cell from a woman alloimmunized in pregnancy.

There are ongoing efforts to develop an Ab prophylaxis and therapy for FNAIT. In this study we describe the generation of a human mAb specific for HPA-1a, named 26.4. It is the only HPA-1a-specific human mAb with naturally paired H and L chains. Using flow cytometry and surface plasmon resonance technology we demonstrated mAb 26.4 high specificity and binding affinity to HPA-1a. Interestingly, mAb 26.4 bound to integrin αVβ3 (from trophoblasts) with higher affinity compared with another HPA-1a-specific human mAb, B2G1. We have demonstrated that mAb 26.4 can opsonize HPA-1a+ platelets for enhanced phagocytosis by monocytes and inhibit binding of maternal polyclonal anti-HPA-1a Abs. Mab 26.4 also weakly inhibited aggregation of HPA-1a-heterozygous platelets, however the inhibition was with no predicted clinical relevance. Thus, mAb 26.4 can potentially be developed into a drug for prophylaxis and/or therapy of FNAIT.

Paper II

Title: A novel human monoclonal human platelet antigen (HPA)-1a-specific antibody can serve as a diagnostic reagent in fetal and neonatal alloimmune thrombocytopenia.

Routine HPA-1 phenotyping and anti-HPA-1a antibody quantitation could help to identify pregnancies at risk of alloimmunization and reduce FNAIT-related complications. In this study we have evaluated whether mAb 26.4 can be used as a reagent for HPA-1 phenotyping and as a standard for anti-HPA-1a antibody quantitation. We found that fluorescently conjugated mAb 26.4 could distinguished between HPA-1a and -1b platelets in a whole blood flow cytometry assay and could be used as an HPA-1a phenotyping reagent. Quantitation of anti-HPA-1a activity in serum samples using mAb 26.4 as standard was highly accurate and reproducible. Thus, mAb 26.4 can be used as a standard for anti-HPA-1a antibody quantitation.

Paper III

Title: Anti-human platelet antigen (HPA)-1a antibodies affect trophoblast functions crucial for placental development: A laboratory study using an *in vitro* model.

Recent studies have shown that in addition to bleeding complications, reduced fetal growth could be another complication of FNAIT. It has been speculated that binding of anti-HPA-1a antibodies to αVβ3 on extravillous trophoblast cells may affect function of these cells, which in turn can lead to reduced placental function and reduced fetal growth. An experimental *in vitro* model with human monoclonal anti-HPA-1a antibody (clone 26.4) and a first trimester human extravillous trophoblast-derived cell line (HTR8/SVneo) was used to study the effect of anti-HPA-1a antibodies on the function of extravillous trophoblast cells. The xCELLigence system was employed to assess the effect on adhesion and migration, and specially designed Matrigel precoated chambers to study the effect on invasive capacity of the cells. We found that anti-HPA-1a antibodies partially inhibit adhesion, migration and invasive capacity of first trimester trophoblast cells. These findings suggest that anti-HPA-1a antibodies may hinder development of placenta, and consequently, may be involved in obstetric complications such as reduced birth weight.

4. Discussion

4.1 Methodological considerations

4.1.1 Selection of HPA-1a-specific memory B cells

Human mAbs that have been naturally selected in human immune responses would be optimal for *in vivo* administration. *In vivo* BCR selection will likely drive towards minimal autoreactivity by deletion of self-reactive B cell clones. The chance of unexpected *in vivo* reactivity by, or against, this antibody is minimal. In contrast, mAbs generated by phage display have H and L chains randomly paired *in vitro*, and it has been speculated that these antibodies are more likely to be autoreactive or immunogenic (223). In addition, the antibody binding site may be altered as a result of such pairing (224, 225).

To generate human monoclonal HPA-1a-specific antibodies with a natural H and L chain pairing, HPA-1a-specific memory B cells were immortalized. Memory B cells are known to persist for a lifetime (226), retain expression of surface Ig, and thus can be antigen-selected (227). The drawback of working with memory B cells is that they are found in peripheral blood in very low numbers. Memory B cells reside in the bone marrow and only recirculate through bone marrow, peripheral blood and lymph nodes. The EBV immortalization technic described by Traggiai et al (228) was employed. The authors described that using polyclonal activator CpG during EBV transformation dramatically increased transformation efficiency of memory B cells (228). Using anti-CD22 antibodies as a B cell marker instead of anti-CD19, as described in Traggiai et al (228), yielded higher number of isolated cells and more EBV transformed cultures. Significant efforts have been made to isolate several HPA-1a-specific B cell clones (Supplemental data I).

EBV transformation efficiency was reproducible (all wells with 400 $CD22^{+}IgM^{-}IgA^{-}IgD^{-}$ cells in each resulted in transformed cultures), but the number of cultures secreting HPA-1a-specific IgG varied greatly from experiment to experiment. Specific IgG secretion in identified polyclonal B lymphoblastoid (BL) cultures declined rapidly and anti-HPA-1a antibodies were not detectable in supernatants after 2 weeks of culture. Loss of the antibody secretion by B-lymphoblastoid (BL) cells was reported previously (229). Moreover, some BL clones divided very slow and died in a few weeks. Only one clonal BL culture continuously

secreted anti-HPA-1a and grew to sufficient number of cells for fusion to a heteromyeloma cell line.

Several clonal BL cultures secreting specific antibodies have been identified, but these cultures stopped secreting specific antibodies and dividing within 2 weeks. Ig variable genes from the HPA-1a-specific clonal cultures were isolated, however, not used to generate recombinant antibodies to validate the specificity of the clones due to the unavailability of the technique in the laboratory at that time. At first, the generation of recombinant antibodies was done at The Norwegian Institute of Public Health, Oslo, Norway.

The EBV-transformed BL cells secreting specific antibodies were fused to a human-mouse heteromyeloma to stabilize and amplify the antibody production (229). BL cells are not sensitive to hypoxanthine-aminopterin-thymidine (HAT) medium, therefore BL-K6H6 hybridomas could not be selected from BL cells using HAT medium. BL cells are sensitive to ouabain. Ouabain-resistant myeloma fusion partner cell lines exist, but were not available in the lab at a time of this experiment. However, firstly, the BL cells had a very slow growth rate. Secondly, the hybridomas were isolated using two rounds of limiting dilution assay (LDA).

Isolation of single HPA-1a-specific memory B cells, exploring the memory B cell surface Ig expression, using single epitope multiple staining has also been probed (230). Integrin heterodimer αIIbβ3 was isolated from HPA-1aa and -1bb platelets, and conjugated to fluorescent dyes, FITC or PE. However, the protein was binding to cells from BL cultures negative for anti-HPA-1a IgG, suggesting that the conjugated proteins bound non-specifically to the cells. Nevertheless, double stained cells from BL cultures positive for anti-HPA-1a IgG were isolated by fluorescence-activated cell sorting (FACS) and expanded in culture. No clones producing HPA-1a-specific Abs were identified. Isolation of Ig variable genes from sorted single cells was ruled out due to the observed non-specific binding of the labelled protein.

4.1.2 Measuring mAb binding kinetics by SPR

Binding affinity (K_D) of two interacting molecules can be calculated from the ratio between their association (k_d) and dissociation (k_a) rates, $K_D = k_d/k_a$. Affinity measurements of mAb-Ag interaction by SPR (Biacore) require 1:1 molecule interactions. In this case, mAb (Ligand)

should be coupled to the sensor chip while the Ag (Analyte) injected to flow over the chip surface. Multiple attempts to couple the antibody to the sensor chip yielded low Ag-Ab binding response. It was explained by the degradation of mAb 26.4 during the coupling procedure. Coupling the Ag (GPIIbIIIa) to the chip and injecting the mAb 26.4 yielded sufficient binding response. However, in this system two Ab molecules can bind to one coupled Ag molecule leading to the avidity effect (stronger binding). Consequently, the kinetic rates to calculate the mAb binding affinity could not be measured. Relative measurements have been performed, assuming that high association and low dissociation rates indicate high affinity of the interaction, and vice versa, low association and high dissociation rates point to low affinity. In cases where the interactions had similar association rates, the antibody dissociating faster was interpreted as of lower affinity.

4.1.3 Effect of anti-HPA-1a Abs on trophoblast cells – choosing an in vitro model

Trophoblast cells

There is no perfect animal model to study human trophoblast invasion (231). Thus, trophoblast cell lines and primary trophoblast cultures are widely used for this purpose (156, 232, 233). Trophoblast invasion into the uterus and remodeling of the uterine arteries starts within the first 12 WG and continues until 20-22 WG (234). Therefore, to study the possible effect of antibodies on trophoblast function, the cells should be derived from developing placenta and thereby retain their migratory and invasive capacity. EVT cells isolated from term placenta lack migratory and invasive capacity (235). Next, the cells should have surface expression of αVβ3 integrin and be of HPA-1ab genotype, reflecting fetal HPA-1 genotype and phenotype. Also, the cells must be adherent for utilization of the xCELLigence system (236). HTR8/SVneo cells derive from 8-10 WG placenta (237), which are adherent, were tested to have surface expression of αVβ3 and HPA-1a, and genotyped HPA-1ab. It is not known whether HTR8/SVneo cells originate from female or male embryo.

HTR8/SVneo cells provide a continuously growing, relatively homogeneous population of well characterized cells. In general, trophoblast cell lines help to overcome the limitations of primary trophoblast cultures: ethical and logistical challenges to obtain the fetal material, paucity of isolated CTB cell numbers, phenotypic instability, and finally, the need for fresh preparations for each experiment (238). However, trophoblast cell lines by definition are not ‘normal’. It has been suggested that data obtained using trophoblast cell lines should always

be corroborated by data from primary culture cells (238). Therefore, in a follow-up study primary trophoblast cultures and placental villous explants will be used. Placental explants can be superior to isolated cells as they provide stromal and extracellular matrix factors, as well as variety of cells present in this complex milieu (239). Such interactions are critical for trophoblast cell viability, differentiation and function.

The method

The xCELLigence system (Roche Applied Science, Penzberg, Germany) allows continuous label-free monitoring of cell adhesion and migration in real time. It has been shown to be superior to end-point assay systems for investigating trophoblast cell function (236). While xCELLigence system was successfully used for adhesion and migration experiments, relatively high experiment to experiment variations in measurements of invasion were observed. These variations were explained by the difficulties in applying exact amounts of Matrigel on the membrane by pipetting (Figure 7). The experiment to experiment variations were reduced by using commercially available plates precoated with Matrigel for an end-point assay.

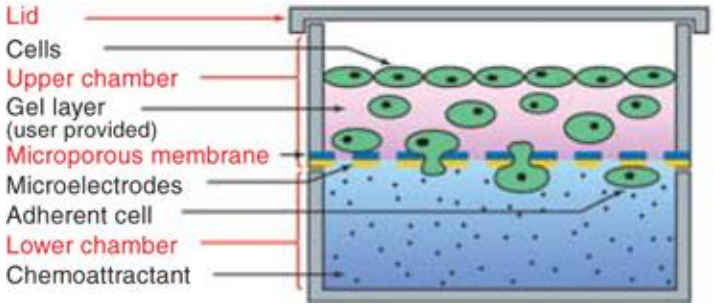

Figure 7. Graphical representation of the well of CIM-plate 16. Adopted from (240), with permission.

The antibodies

Fetal bovine serum was used as a chemoattractant in the migration and invasion experiments. Cells were applied into the upper chamber in serum-free medium and migrated towards serum-rich medium in the lower chamber. As HPA-1a IgG constitute only a fraction of the IgG isolated from maternal sera, using polyclonal antibodies instead of mAbs would have increased protein content in the upper chamber and prevented cell migration.

mAb 26.4 concentration 20 µg/ml corresponds to about 400 IU/ml anti-HPA-1a antibody activity as measured by quantitative MAIPA. Serum levels of anti-HPA-1a antibodies at 400 IU/ml are considered very high. The use of high mAb 26.4 concentrations in the functional

experiments was necessary to demonstrate in principle whether antibodies of this specificity affect function of trophoblasts or not. Furthermore, measurements of a single HPA-1a-specific mAb and specific IgG in serum can not be directly compared, because the humoral immune response to an Ag is polyclonal, resulting in antibodies of different epitope specificities and affinities.

4.2 Antibody prophylaxis for HPA-1a immunization

Traditionally it has been accepted that the majority of HPA-1a immunization cases happen during the first incompatible pregnancy. Results of a large prospective screening study conducted in Norway revealed that around 75% women were immunized to HPA-1a following delivery of an HPA-1a-positive child and only 8% were primigravidae (101). Two other prospective screening studies found that the frequency of HPA-1a immunization during the first pregnancy was not higher than 4% (102) and 24% (103). Therefore, a preventive strategy with anti-HPA-1a immunoglobulin, similar to anti-RhD immunoglobulin prophylaxis, was considered.

Such a preventive strategy has been proven successful in mice. Administration of anti-integrin β3 sera prior to injection of integrin β3+ platelets in an integrin β3-/- murine model of FNAIT prevented formation of anti-platelet antibodies and reduced bleeding complications in newborn pups (199). Currently, clinical trials are underway to test the potential of hyperimmune anti-HPA-1a IgG to prevent HPA-1a immunization (www.profnait.eu) (201). If the results of trials are favorable, the next challenge will be to obtain sufficient amounts of anti-HPA-1a serum for IgG fractionation. A potential source is donated sera from HPA-1a alloimmunized individuals. However, the numbers of women HPA-1a alloimmunized in pregnancy is relatively small, and if the prophylactic treatment works, the numbers will decrease in the future. Also, immunizing HPA-1a-negative individuals with HPA-1a-positive platelets would result in rare immunizations, because transfusion of HPA-1a-positive platelets rarely stimulate an antibody response against HPA-1a (75, 241). In addition, polyclonal anti-HPA-1a IgG would carry the risks of a human derived product. Therefore, a mAb specific for HPA-1a would be of great interest to replace polyclonal Abs as a source for prophylaxis.

Anti-D immunoglobulin prophylaxis was introduced in 1968 (181) and has been incredibly successful in preventing RhD immunization. Postnatal and antenatal anti-D administration reduced the incidence of alloimmunization from 13% (before prophylaxis) to 0.35% (242).

Many human anti-D mAbs have been developed and studied in *in vitro*, murine models, and clinical trials to replace the polyclonal anti-D product (243). However, the anti-D mAbs selected in the early 90s performed inferior to poly-D in *in vitro* functional assays, and the results of clinical trials have been rather inconclusive (243). One possible explanation for this is the complexity of the D-antigen and that a wider spectrum than one or two mAbs of anti-D specificities are required to achieve sufficient protective anti-D effect. At present, a mixture of 25 human recombinant anti-D antibodies is in advanced clinical trials for prevention of RhD alloimmunisation and treatment of ITP (244, 245). A second possible explanation has been the low functionality of the mAbs related to the Fc part of the IgG molecule. Currently, human recombinant anti-D mAbs with enhanced Fc functionality are in phase II clinical trials (242, 246).

The nature of the D antigen is different from that of HPA-1a. RhD is a highly polymorphic transmembrane protein consisting of over 400 amino acid residues in length that transverses the RBC membrane 12 times (247). The D antigen has more than 30 B-cell determinants (epitopes) (248, 249). It has been shown that anti-D epitope specificity is important for the mediation of antibody biological effect (250). In contrast to the D antigen, HPA-1a is a result of only one amino acid change, L33P, in integrin β3. Anti-HPA-1a antibodies have several epitopes (149), but all of the epitopes overlap reacting with the L33 residue. Therefore, it is conceivable that a single mAb specific for HPA-1a would be effective to prevent HPA-1a immunization.

It has become increasingly clear that the Fc-linked glycosylation pattern regulates effector functions of IgG (251). Low-fucosylated IgG Abs bind stronger to activating FcγRs, and thus confer stronger effector functions, like ADCC and phagocytosis, than do fucosylated Abs (42, 252). Anti-HPA-1a IgG1 antibodies in FNAIT are of low Fc-fucosylation (146). *In vitro*, low fucosylated IgG antibodies mediated stronger platelet phagocytosis when compared with highly fucosylated IgG (146). Similarly, decreased fucosylation of anti-D antibodies in pregnancy was also observed (253). Following the hypothesis claiming that the rapid clearance of HPA-1a+ platelets from maternal circulation prevents alloimmunization, low fucosylated anti-HPA-1a antibodies for antibody prophylaxis would be most effective. Low fucosylated anti-HPA-1a mAb B2G1 was shown to be effective in potentiating the clearance of precoated HPA-1a+ platelets in HPA-1a– human volunteers (200). Anti-D mAbs to prevent RhD alloimmunization have been extensively studied. Low fucosylated anti-D mAbs bound strongly to FcγRIIIa and FcγRIIa, and exerted superior to highly fucosylated variants the

ability to trigger ADCC *in vitro* (254), and were as potent as poly-D in clearance of RhD+ RBCs in NOD-SCID mice (254). A clinical study showed that human anti-D mAb with low-fucose content was at least as potent as the human poly-D in the clearance of RhD+ RBCs (246).

Fucose is added to the primary N-acetylglucosamine during glycoprotein processing in the Golgi apparatus and can be inhibited by prior addition of bisecting N-acetylglucosamine sugar residues. Low fucose mAbs can be produced in the rat YB2/0 cell line which has low expression of the fut8 gene (255), and in specially developed CHO cell lines that express the GnTIII enzyme (adds bisecting N-acetylglucosamine residues) (256) or knocked out for α(1-6) fucosyl transferase enzyme (257).

Described in Paper I, a phagocytosis assay was developed to test the ability of anti-HPA-1a mAb 26.4 to induce phagocytosis of sensitized platelets. This assay can be used to test biological potency of the new variants of mAb 26.4 intended for prophylaxis. Assays to test the ability of mAb 26.4 to trigger ADCC and CDC were also probed, however the measurements could not be performed due to the lack of positive control human mAbs (Supplemental data II).

The relationship between RBC clearance and prevention of RhD alloimmunization is unclear (136, 194), though poly-D preparations are potent in RBC clearance. Interestingly, while RBC clearance in humans has been associated with polymorphisms in the FcγRIIIA and FcγRIIA genes (136, 137, 193), such polymorphisms may have no effect on the prevention of alloimmunization as was shown by a clinical trial (136).

4.3 Analysis of IgV region genes of anti-HPA-1a mAbs

Antibodies undergo affinity maturation in a stepwise fashion. Every subsequent exposure to the antigen results in new mutations in Ab variable region genes and selection of B cell clones with higher affinity binding. IgV region genes of HPA-1a-specific mAbs isolated from FNAIT cases were compared in Paper I (Table 2). All three mAbs had low level of Ag-driven affinity maturation suggested by the results of Ag-driven selection analysis (258, 259), as well as low numbers of silent (S) and replacement (R) mutations in the complementarity determining regions (CDR)s and framework regions (FR)s. Interestingly, the woman from

who mAb 26.4 was derived had been exposed to HPA-1a antigen several times, having given birth to two HPA-1a-positive children with severe FNAIT. High levels of anti-HPA-1a antibodies (225 IU/ml) were detected after birth of the first child and remained high (150 IU/ml) during the second pregnancy. In light of this, the changes in the V-regions of mAb 26.4 seem relatively modest compared to changes normally associated with antigen-driven affinity maturation. Although this could be coincidental, it is possible that the low level of mutations is indirectly related to the shared structural features of the HPA-1a and HPA-1b allotypes: mutations that increase binding to the HPA-1a variant may simultaneously result in cross-reactivity to the HPA-1b variant and, in effect, autoimmunity. It is therefore possible that the low level of affinity maturation evident in mAb 26.4 is a reflection of the very fine differences between the HPA-1 allotypes, and may be a result of deletion of B cell clones with point mutations that result in cross-reactivity.

Table 2. Analysis of IgG variable gene sequences and mutations of human HPA-1a-specific mAbs.

	VH gene				**VL gene**			
Clone	**VDJ gene segments**	**CDR-H3 length**	**FR R/S**	**CDR R/S**	**VJ gene segments**	**CDR-L3 length**	**FR R/S**	**CDR R/S**
26.4	V6/D6/J6	25	4/2	4/2	KV3/KJ4	10	3/3	0/0
B2G1#	V1/D3/J6	15	6/7	2/4	-	-	-	-
ML1¤	V4/D6/J3	20	7/3	3/1	-	-	-	-
6-14*	V3	11	12/-	10/-	KV3	9	6/-	3/-
19*	V3	15	11/-	7/-	KV3	10	7/-	4/-
23-24*	V4	11	6/-	5/-	KV1	9	11/-	5/-

#The scFv fragment isolated by phage display from a woman HPA-1a immunized in connection with pregnancy (220).
¤The scFv fragment was isolated from a woman HPA-1a-immunized in connection with pregnancy who has given birth to a child with FNAIT (T.S. Kickler, personal communication, October 2013) (221).
*mAbs isolated from PTP patient (222).

For B2G1 and ML1, the H chain was isolated without an L chain; R, replacement mutations; S, silent mutations.

The results discussed above were also compared with IgV region genes of Ab fragments isolated from a PTP patient (Table 2). Three HPA-1a-specific scFv antibody fragments had significantly higher numbers of replacement mutations. In this case, the donor was an HPA-1b-homozygous woman who had received multiple transfusions over 4 year period and was in the acute thrombocytopenic phase of PTP at the time of blood collection (222). This patient

was exposed to high doses of the antigen multiple times and this could be one reason why the antibodies have relatively high levels of mutations. Although it was not determined which antibody specificities were the cause of autoimmunity, cross-reactivity to the HPA-1b allotype was not ruled as sensitive examinations for binding to HPA-1b, e.g. to HPA-1b-homozygous platelets by flow cytometry or MAIPA, was not performed. Autoimmunity is associated with a loss of tolerance to self, and this may be reflected in PTP patients as a failure in deletion of B cells that produce self-reactive anti-HPA-1a antibodies and thus allow more extensive affinity maturation than in a normal self-tolerant immune system. This speculation is preliminary, because the immune response to the antigen is polyclonal and the isolated mAbs may not represent the extent of the affinity maturation in the patient.

4.4 The epitope of mAb 26.4

The epitope of mAb 26.4 is not constrained to the PSI domain, but extends to several domains of integrin β3, as was found employing the domain-deletion peptide ELISA technique (Supplemental data III). This may have neither advantages nor disadvantages in terms of the prophylactic or therapeutic potential of the mAb 26.4. Molecular modeling of the mAb 26.4 paratope and Ab-Ag docking was complicated by the extremely long H3 loop (Supplemental data IV).

4.5 Effect of anti-HPA-1a antibodies on platelet function

In contrast to the 'inert' RBCs, platelets can be activated by anti-platelet antibodies. Firstly, platelet activation can lead to changes in hemostasis (increased blood thrombogenicity). Secondly, upon activation, platelets express surface molecules, such as P-selectin (CD62P), CD63 and annexin V. Leucocytes express P-selectin glycoprotein ligand-1 (PSGL-1). Activated platelets form monocyte-platelet aggregates and induce a pro-inflammatory phenotype in circulating monocytes (260). It is conceivable that fetal platelets (in the maternal circulation), activated by the administered anti-HPA-1a Abs, could provide the 'danger signal' for initiating the maternal immune response against HPA-1a.

Human antiplatelet antibodies can activate platelets in three different ways: by direct binding to target antigen, via Ab-mediated complement activation, and via clustering FcγRIIa on platelet membrane (261). It has been shown that anti-HPA-1a mAbs do not activate platelets by direct binding to the antigen (144). One study found that anti-HPA-1a-containing sera could induce release of pro-inflammatory cytokine RANTES from human platelets (262).

However, the effect was abolished by blockage of platelet FcγRIIa, suggesting that platelet activation resulting in RANTES release was attributed to the ability of some anti-HPA-1a antibodies to cluster FcγRIIa on platelet membrane, but not to the direct binding to αIIbβ3 integrin. Blockage of FcγRIIa did not abolish RANTES release induced by sera from PTP patients, suggesting that anti-HPA-1a antibodies from these patients activated platelets via Ab-mediated complement activation. Thus, the subclass and glycosylation of anti-HPA-1a IgG are likely critical for their ability to activate platelets. Therefore, the Fc part of the platelet-specific mAb intended for clinical use has to be designed and tested with regard to the effect on platelet activation.

4.6 mAb 26.4 as a diagnostic reagent in FNAIT

There is a need for a reagent to develop a simple, reliable and inexpensive HPA-1 phenotyping assay suitable for screening purposes. mAb 26.4 is highly specific for HPA-1a and can distinguish between HPA-1a and -1b antigens in whole blood flow cytometry. Tests utilizing the dextran acrylamide gel technique are currently in use at the primary healthcare units for blood group and RhD antigen phenotyping (263, 264). Particle gel agglutination assays for HLAs (265) and HNA-1a (266) have also been described. mAb 26.4 could potentially be used for the development of a gel card-based test for HPA-1 typing for use at the primary healthcare units.

HPA-1 phenotyping is not limited to the diagnostics of FNAIT, and is applicable in transfusion medicine and diagnostics of blood transfusion complications like PTP and MPR (267). Severe FNAIT cases have been reported when surrogate HPA-1bb women carried HPA-1aa children (268). Assisted reproductive technology procedures, like surrogacy and *in vitro* fertilization, are associated with high financial and emotional costs, and HPA-1 typing has therefore been recommended prior to the procedure.

As demonstrated in Paper II, mAb 26.4 performs similar to the polyclonal anti-HPA-1a international standard. MAIPA is a complex assay that requires multiple steps, reagents and various equipment, the conditions that vary between laboratories. It is possible that the binding of a single Ab would be more affected by local assay conditions than the pool of antibodies in the polyclonal anti-HPA-1a NIBSC reference material. Therefore, based on the present data, the mAb 26.4 can be utilized as a secondary standard: the mAb standard can be used with every run of MAIPA assay, while polyclonal anti-HPA-1a NIBSC standard would

be used for the assay establishment and calibration. Consequently, the cost of MAIPA assay and usage of the valuable polyclonal anti-HPA-1a reference material can be dramatically reduced.

mAb 26.4 concentration of 5 µg/ml corresponds to 100 IU/ml as measured by MAIPA. These measurements can not be used to quantify serum anti-HPA-1a antibodies in µg/ml, because the humoral immune response to an antigen is polyclonal, and anti-HPA-1a antibodies have different epitope specificities on the integrin β3 (although all of the epitopes overlap reacting with the L33) and differ in binding affinity to the antigen.

4.7 Anti-HPA-1a antibodies may affect placental development

The evidence is growing that the effect of anti-HPA-1a antibodies in FNAIT is beyond the effect on platelet numbers. Integrin β3, carrying HPA-1a, as part of αVβ3 is expressed on angiogenic ECs. It has recently been shown that anti-HPA-1a sera can inhibit HUVEC proliferation, formation of capillary-like networks and impaired angiogenic signaling, suggesting that anti-HPA-1a antibodies impair angiogenesis. Interaction of anti-HPA-1a antibodies with HPA-1a on angiogenic ECs in the brain impaired angiogenesis in a murine model (155). Impaired angiogenesis in the brain was suggested to be a possible cause of ICH independent of thrombocytopenia. Anti-integrin β3 sera also impaired retinal vascular development in a murine model (155). This observation may explain the visual impairment in some FNAIT cases (269). It is conceivable that placental angiogenesis, which continues throughout gestation (270), can also be affected by maternal anti-HPA-1a antibodies.

High levels of maternal anti-HPA-1a antibodies were found to be strongly associated with reduced birth weight in boys (117). Of neonates with ICH, 23% were found to be small for gestational age in an international multicenter study on ICH due to FNAIT (115). A possible link between HPA alloimmunization, chronic villitis/intervillositis and low birth weight (LBW) was also suggested in several case reports (271-273). LBW in relation to the length of gestation is known to be associated with an increased risk of morbidity and mortality in the neonatal period, and has significant negative effects on adult health (274-276). Understanding the mechanisms and identifying pregnancies at risk of having a LBW infant is therefore important. Apart from αVβ3 expression on ECs, αVβ3 is also expressed on invasive trophoblast cells (156) and on the apical surface of syncytiotrophoblast microvilli (29, 120). As reported in Paper III, in an *in vitro* model anti-HPA-1a Abs affect the adhesive, migratory and invasive capacity of

extravillous trophoblast cells – trophoblast functions crucial for placental development. Impaired placental development and reduced placenta function can lead to reduced fetal growth and LBW. An association between FNAIT due to anti-HPA-1a antibodies and increased risk of miscarriage has also been suggested, indicating that placental development may be affected in the early stages of pregnancy (277).

Chronic villitis of unknown etiology (CVUE) and massive chronic intervillositis (MCI) are placental lesions associated with infiltration of inflammatory cells in the chorionic villi and the intervillous spaces, respectively (278, 279). CVUE and MCI have been linked to alloimmunity (278-280). Both lesions have similar infiltration of immune cells: predominantly CD68+ macrophages with CD45RB+, CD3+, CD8+ and CD4+ T cells, representing maternal immune reaction to fetal antigens. A possible role of maternal Abs specific for fetal Ags has also been suggested. CVUE and MCI are associated with FGR (279, 281). CVUE was found in placentas from FNAIT-affected pregnancies not treated with IVIG (272). A case of FNAIT associated with MCI has also been described (273). A possible link between CVUE/MCI and LBW in FNAIT should be addressed in future studies.

Interaction between KIR receptors expressed on maternal dNK cells with HLA-C on invading trophoblast cells influence placentation. Both KIRs and HLA-C are polymorphic and some genotype combinations are associated with inadequate trophoblast invasion and obstetric syndromes, including pre-eclampsia, recurrent miscarriage and FGR (8, 12, 282). It is possible that ‘reproductively risky’ genotype combinations potentiate the effect of anti-HPA-1a antibodies on trophoblast, and conversely, ‘reproductively successful’ combinations limit the effect.

It has also been speculated that fetal thrombocytopenia *per se* may contribute to the reduced birth weight (117). Platelets modulate angiogenesis by selectively releasing from separate α-granules promoters (e.g. VEGF, bFGF, EGF, PDGF, MMPs) or inhibitors (e.g. endostatin, thrombospondin) of angiogenesis (283). Growth factors and chemokines released upon platelet activation enhance trophoblast invasion (284). Platelets are known to be positively involved in tumor angiogenesis and growth (285).

5. Conclusions and future perspectives

The development and characterization of a human mAb 26.4 highly specific for HPA-1a has been described in this work. This Ab has been naturally selected in a human alloimmune response, and thus, is unlikely to be autoreactive or immunogenic if administered to humans. This mAb could further be developed for use in Ab prophylaxis and therapy of FNAIT. *In vitro* experimentation have demonstrated that mAb 26.4 binds with high affinity to HPA-1a+ platelets and trophoblast-derived αVβ3. mAb 26.4 is potent in induction of phagocytosis of sensitized platelets. These qualities are thought to be important for potential Ab-based prevention of HPA-1a alloimmunization. Further, mAb 26.4 inhibited binding of polyclonal maternal anti-HPA-1a to HPA-1a+ platelets, the quality thought to be crucial for potential Ab-based therapy of FNAIT. Importantly, mAb 26.4 only weakly inhibited aggregation of HPA-1a-heterozygous platelets (fetal phenotype), an effect with no predicted clinical relevance.

As the next step in the development of a prophylactic drug, the Fc part of the mAb 26.4 can be manipulated to produce mAbs with enhanced effector functions to effectively eliminate the Ag from maternal circulation prior initiation of immune response in the mother. For possible therapeutic use, a variant of mAb 26.4 with a nonfunctional Fc part can be produced to protect fetal platelets from pathogenic maternal anti-HPA-1a Abs. mAb modifications can be introduced using recombinant Ab technology by introducing specific mutations to Fc part of the IgG molecule. Desired glycosylation profile of the mAb can be achieved by producing the mAb in specially developed cell lines. The developed variants of mAb 26.4 can further be tested *in vitro* and in murine models. It is important to test all mAb 26.4 variants for platelet activation.

It will also be interesting to further characterize mAb 26.4 concerning its epitope, as it is the only anti-HPA-1a mAb with naturally paired H and L chains. For this purpose, the mAb and mAb–Ag complex X-ray crystallization should be performed.

mAb 26.4 is highly specific for HPA-1a and, when fluorescently labeled, can distinguish between HPA-1a+ and -1a- platelets in whole blood samples. Therefore, this mAb can be used as a reagent for HPA-1 phenotyping. mAb 26.4 can be utilized for production of a simple and relatively cheap gel card-based test for HPA-1 phenotyping at primary healthcare units.

Quantitation of anti-HPA-1a antibodies in serum samples by MAIPA using mAb 26.4 as standard was highly accurate and reproducible. Thus, mAb 26.4 can be used routinely as a standard, dramatically reducing the consumption of the valuable polyclonal anti-HPA-1a NIBSC reagent. A multicenter laboratory study should be conducted to further validate the use of the mAb 26.4 as a standard.

The work presented in this thesis also assesses the possible mechanism by which maternal anti-HPA-1a antibodies can affect development of the placenta. Anti-HPA-1a antibodies hindered trophoblast adhesive, migratory and invasive capacity *in vitro*. The effect of HPA-1a antibodies on EVT invasion and remodeling of the uterine arteries should be studied further using primary trophoblast cultures and placental villous explants. To study whether anti-HPA-1a antibodies can affect trophoblast-EC interaction, a three-dimensional cell co-culture model can be employed. Histopathological studies of term placentas from FNAIT-affected pregnancies are currently planned. In the clinic, HPA-1a alloimmunized pregnancies may be closely monitored for FGR. Conversely, mothers of low birth weight neonates with unknown cause may be examined for HPA-1a alloimmunization.

Understanding the full spectrum of effects of anti-HPA-1a antibodies on the conceptus can aid the development of appropriate management of pregnancies at risk of FNAIT. The mischief of anti-HPA-1a antibodies, with the mAb 26.4 in hand, could potentially be turned into a cure for FNAIT.

Patent application covering the use of the mAb 26.4 in diagnostics, prophylaxis and therapy within FNAIT has been filed. Patent application title: FNAIT antibody. Inventors: Stuge TB, Husebekk A, Skogen BR, Tiller H, Eksteen M**,** Michaelsen TE, Ihle Ø. No.1405775.6. Filing date: 31 March 2014.

6. References

1. Burton GJ, Fowden AL. 2015. The placenta: a multifaceted, transient organ. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 370
2. Arck PC, Hecher K. 2013. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat Med* 99: 548-56
3. Knöfler M, Pollheimer J. 2012. IFPA Award in Placentology Lecture: Molecular regulation of human trophoblast invasion. *Placenta* 33, Supplement: S55-S62
4. Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC. 2006. Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. *J Pathol* 208: 535-42
5. Brosens IA, Robertson WB, Dixon HG. 1972. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu* 1: 177-91
6. Khong TY, De Wolf F, Robertson WB, Brosens I. 1986. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *Br J Obstet Gynaecol* 93: 1049-59
7. Brodsky D, Christou H. 2004. Current Concepts in Intrauterine Growth Restriction. *Journal of Intensive Care Medicine* 19: 307-19
8. Hiby SE, Apps R, Chazara O, Farrell LE, Magnus P, Trogstad L, Gjessing HK, Carrington M, Moffett A. 2014. Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. *J Immunol* 192: 5069-73
9. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248: 220-3
10. King A, Burrows TD, Hiby SE, Bowen JM, Joseph S, Verma S, Lim PB, Gardner L, Le Bouteiller P, Ziegler A, Uchanska-Ziegler B, Loke YW. 2000. Surface expression of HLA-C antigen by human extravillous trophoblast. *Placenta* 21: 376-87
11. King A, Allan DS, Bowen M, Powis SJ, Joseph S, Verma S, Hiby SE, McMichael AJ, Loke YW, Braud VM. 2000. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *Eur J Immunol* 30: 1623-31
12. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, Claas FH, Walker JJ, Redman CW, Morgan L, Tower C, Regan L, Moore GE, Carrington M, Moffett A. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest* 120: 4102-10
13. Mjösberg J, Berg G, Jenmalm MC, Ernerudh J. 2010. FOXP3+ Regulatory T Cells and T Helper 1, T Helper 2, and T Helper 17 Cells in Human Early Pregnancy Decidua. *Biology of Reproduction* 82: 698-705
14. Robertson SA, Guerin LR, Moldenhauer LM, Hayball JD. 2009. Activating T regulatory cells for tolerance in early pregnancy — the contribution of seminal fluid. *Journal of Reproductive Immunology* 83: 109-16
15. Aluvihare VR, Kallikourdis M, Betz AG. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 5: 266-71
16. Rowe JH, Ertelt JM, Xin L, Way SS. 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature* 490: 102-6
17. Samstein Robert M, Josefowicz Steven Z, Arvey A, Treuting Piper M, Rudensky Alexander Y. 2012. Extrathymic Generation of Regulatory T Cells in Placental Mammals Mitigates Maternal-Fetal Conflict. *Cell* 150: 29-38
18. Pollheimer J, Knofler M. 2012. The role of the invasive, placental trophoblast in human pregnancy. *Wien Med Wochenschr* 162: 187-90

19. Mor G, Cardenas I, Abrahams V, Guller S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences* 1221: 80-7
20. Silasi M, Cardenas I, Kwon J-Y, Racicot K, Aldo P, Mor G. 2015. Viral Infections During Pregnancy. *American Journal of Reproductive Immunology* 73: 199-213
21. Kourtis AP, Read JS, Jamieson DJ. 2014. Pregnancy and Infection. *The New England journal of medicine* 370: 2211-8
22. Kay AW, Fukuyama J, Aziz N, Dekker CL, Mackey S, Swan GE, Davis MM, Holmes S, Blish CA. 2014. Enhanced natural killer-cell and T-cell responses to influenza A virus during pregnancy. *Proceedings of the National Academy of Sciences* 111: 14506-11
23. Kay AW, Bayless NL, Fukuyama J, Aziz N, Dekker CL, Mackey S, Swan GE, Davis MM, Blish CA. 2015. Pregnancy Does Not Attenuate the Antibody or Plasmablast Response to Inactivated Influenza Vaccine. *Journal of Infectious Diseases*
24. Rieger L, Segerer S, Bernar T, Kapp M, Majic M, Morr AK, Dietl J, Kammerer U. 2009. Specific subsets of immune cells in human decidua differ between normal pregnancy and preeclampsia--a prospective observational study. *Reprod Biol Endocrinol* 7: 132
25. Ait-Azzouzene D, Caucheteux S, Tchang F, Wantyghem J, Moutier R, Langkopf A, Gendron MC, Kanellopoulos-Langevin C. 2001. Transgenic major histocompatibility complex class I antigen expressed in mouse trophoblast affects maternal immature B cells. *Biol Reprod* 65: 337-44
26. Kraus TA, Engel SM, Sperling RS, Kellerman L, Lo Y, Wallenstein S, Escribese MM, Garrido JL, Singh T, Loubeau M, Moran TM. 2012. Characterizing the pregnancy immune phenotype: results of the viral immunity and pregnancy (VIP) study. *J Clin Immunol* 32: 300-11
27. Medina KL, Smithson G, Kincade PW. 1993. Suppression of B lymphopoiesis during normal pregnancy. *J Exp Med* 178: 1507-15
28. Kanda N, Tamaki K. 1999. Estrogen enhances immunoglobulin production by human PBMCs. *J Allergy Clin Immunol* 103: 282-8
29. Kumpel BM, Sibley K, Jackson DJ, White G, Soothill PW. 2008. Ultrastructural localization of glycoprotein IIIa (GPIIIa, beta 3 integrin) on placental syncytiotrophoblast microvilli: implications for platelet alloimmunization during pregnancy. *Transfusion* 48: 2077-86
30. Anani Sarab G, Moss M, Barker RN, Urbaniak SJ. 2009. Naturally processed peptides spanning the HPA-1a polymorphism are efficiently generated and displayed from platelet glycoprotein by HLA-DRB3*0101–positive antigen-presenting cells. *Blood* 114: 1954-7
31. Stuge TB, Skogen B, Ahlen MT, Husebekk A, Urbaniak SJ, Bessos H. 2011. The cellular immunobiology associated with fetal and neonatal alloimmune thrombocytopenia. *Transfus Apher Sci* 45: 53-9
32. Tomicic M, Starcevic M, Zach V, Hundric-Haspl Z. 2007. Alloimmune Neonatal Neutropenia Due to Anti-HNA-2a Alloimmunization with Severe and Prolonged Neutropenia but Mild Clinical Course: Two Case Reports. *Archives of Medical Research* 38: 792-6
33. Kapur R, Einarsdottir HK, Vidarsson G. 2014. IgG-effector functions: "the good, the bad and the ugly". *Immunol Lett* 160: 139-44
34. Mawas F, Wiener E, Ryan G, Soothill PW, Rodeck CH. 1994. The expression of IgG Fc receptors on circulating leucocytes in the fetus and new-born. *Transfus Med* 4: 25-33

35. Bournazos S, Woof JM, Hart SP, Dransfield I. 2009. Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clinical and Experimental Immunology* 157: 244-54
36. Carter PJ. 2006. Potent antibody therapeutics by design. *Nat Rev Immunol* 6: 343-57
37. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, Daëron M. 2009. Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. *Blood* 113: 3716-25
38. Mawas F, Wiener E, Williamson LM, Rodeck CH. 1997. Immunoglobulin G subclasses of anti-human platelet antigen 1a in maternal sera: relation to the severity of neonatal alloimmune thrombocytopenia. *Eur J Haematol* 59: 287-92
39. Pollock JM, Bowman JM. 1990. Anti-Rh(D) IgG subclasses and severity of Rh hemolytic disease of the newborn. *Vox Sang* 59: 176-9
40. Vidarsson G, Dekkers G, Rispens T. 2014. IgG Subclasses and Allotypes: From Structure to Effector Functions. *Frontiers in Immunology* 5: 520
41. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. 2007. The Impact of Glycosylation on the Biological Function and Structure of Human Immunoglobulins. *Annual Review of Immunology* 25: 21-50
42. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Hennig M, Ruf A, Rufer AC, Stihle M, Umana P, Benz J. 2011. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A* 108: 12669-74
43. Roopenian DC, Akilesh S. 2007. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol* 7: 715-25
44. Simister NE. 2003. Placental transport of immunoglobulin G. *Vaccine* 21: 3365-9
45. West AP, Jr., Bjorkman PJ. 2000. Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor(,). *Biochemistry* 39: 9698-708
46. Einarsdottir HK, Selman MH, Kapur R, Scherjon S, Koeleman CA, Deelder AM, van der Schoot CE, Vidarsson G, Wuhrer M. 2013. Comparison of the Fc glycosylation of fetal and maternal immunoglobulin G. *Glycoconj J* 30: 147-57
47. Stapleton NM, Andersen JT, Stemerding AM, Bjarnarson SP, Verheul RC, Gerritsen J, Zhao Y, Kleijer M, Sandlie I, de Haas M, Jonsdottir I, van der Schoot CE, Vidarsson G. 2011. Competition for FcRn-mediated transport gives rise to short half-life of human IgG3 and offers therapeutic potential. *Nat Commun* 2: 599
48. Lefranc MP, Lefranc G. 2012. Human Gm, Km, and Am allotypes and their molecular characterization: a remarkable demonstration of polymorphism. *Methods Mol Biol* 882: 635-80
49. Einarsdottir H, Ji Y, Visser R, Mo C, Luo G, Scherjon S, van der Schoot CE, Vidarsson G. 2014. H435-containing immunoglobulin G3 allotypes are transported efficiently across the human placenta: implications for alloantibody-mediated diseases of the newborn. *Transfusion* 54: 665-71
50. Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495-7
51. Milstein C. 1999. The hybridoma revolution: an offshoot of basic research. *BioEssays* 21: 966-73
52. Boulianne GL, Hozumi N, Shulman MJ. 1984. Production of functional chimaeric mouse/human antibody. *Nature* 312: 643-6
53. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. 1986. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321: 522-5

54. Carson DA, Freimark BD. 1986. Human lymphocyte hybridomas and monoclonal antibodies. *Adv Immunol* 38: 275-311
55. Cole SP, Campling BG, Atlaw T, Kozbor D, Roder JC. 1984. Human monoclonal antibodies. *Mol Cell Biochem* 62: 109-20
56. Orlandi R, Güssow DH, Jones PT, Winter G. 1989. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proceedings of the National Academy of Sciences* 86: 3833-7
57. Hoogenboom HR. 2005. Selecting and screening recombinant antibody libraries. *Nat Biotech* 23: 1105-16
58. Clackson T, Hoogenboom HR, et al. 1991. Making Antibody Fragments Using Phage Display Libraries. *Nature* 352: 624-8
59. Sidhu SS, Fellouse FA. 2006. Synthetic therapeutic antibodies. *Nat Chem Biol* 2: 682-8
60. Lonberg N. 2005. Human antibodies from transgenic animals. *Nat Biotech* 23: 1117-25
61. Lonberg N, Taylor LD, Harding FA, Trounstine M, Higgins KM, Schramm SR, Kuo C-C, Mashayekh R, Wymore K, McCabe JG, Munoz-O'Regan D, O'Donnell SL, Lapachet ESG, Bengoechea T, Fishwild DM, Carmack CE, Kay RM, Huszar D. 1994. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 368: 856-9
62. Green LL, Hardy MC, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, Abderrahim H, Noguchi M, Smith DH, Zeng Y, David NE, Sasai H, Garza D, Brenner DG, Hales JF, McGuinness RP, Capon DJ, Klapholz S, Jakobovits A. 1994. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat Genet* 7: 13-21
63. Check E. 2007. Immunology: Pimp my antibody. *Nature* 446: 964-6
64. Nelson AL, Dhimolea E, Reichert JM. 2010. Development trends for human monoclonal antibody therapeutics. *Nat Rev Drug Discov* 9: 767-74
65. Semple JW, Italiano JE, Freedman J. 2011. Platelets and the immune continuum. *Nat Rev Immunol* 11: 264-74
66. Pahal GS, Jauniaux E, Kinnon C, Thrasher AJ, Rodeck CH. 2000. Normal development of human fetal hematopoiesis between eight and seventeen weeks' gestation. *Am J Obstet Gynecol* 183: 1029-34
67. Israels SJ, Rand ML, Michelson AD. 2003. Neonatal platelet function. *Semin Thromb Hemost* 29: 363-72
68. Andrew M, Paes B, Bowker J, Vegh P. 1990. Evaluation of an automated bleeding time device in the newborn. *Am J Hematol* 35: 275-7
69. Israels SJ, Cheang T, McMillan-Ward EM, Cheang M. 2001. Evaluation of primary hemostasis in neonates with a new in vitro platelet function analyzer. *J Pediatr* 138: 116-9
70. Boudewijns M, Raes M, Peeters V, Mewis A, Cartuyvels R, Magerman K, Rummens JL. 2003. Evaluation of platelet function on cord blood in 80 healthy term neonates using the Platelet Function Analyser (PFA-100); shorter in vitro bleeding times in neonates than adults. *Eur J Pediatr* 162: 212-3
71. Wagner C, Mascelli M, Neblock D, Weisman H, Coller B, Jordan R. 1996. *Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets*. 907-14 pp.
72. Phillips D, Charo I, Parise L, Fitzgerald L. 1988. *The platelet membrane glycoprotein IIb-IIIa complex*. 831-43 pp.

73. Van Der Weerdt CM, Veenhoven-Vonriesz LE, Nijenhuis LE, Van Loghem J. 1963. The ZW blood group system in platelets. *Vox Sang* 8: 513-30
74. Curtis BR, McFarland JG. 2014. Human platelet antigens - 2013. *Vox Sang* 106: 93-102
75. Kiefel V, König C, Kroll H, Santoso S. 2001. Platelet alloantibodies in transfused patients. *Transfusion* 41: 766-70
76. Pahal GS, Jauniaux E, Kinnon C, Thrasher AJ, Rodeck CH. 2000. Normal development of human fetal hematopoiesis between eight and seventeen weeks' gestation. *American Journal of Obstetrics and Gynecology* 183: 1029-34
77. Peterson JA, McFarland JG, Curtis BR, Aster RH. 2013. Neonatal alloimmune thrombocytopenia: pathogenesis, diagnosis and management. *Br J Haematol* 161: 3-14
78. Curtis BR, Fick A, Lochowicz AJ, McFarland JG, Ball RH, Peterson J, Aster RH. 2008. Neonatal alloimmune thrombocytopenia associated with maternal-fetal incompatibility for blood group B. *Transfusion* 48: 358-64
79. Curtis BR, Edwards JT, Hessner MJ, Klein JP, Aster RH. 2000. Blood group A and B antigens are strongly expressed on platelets of some individuals. *Blood* 96: 1574-81
80. King KE, Kao KJ, Bray PF, Casella JF, Blakemore K, Callan NA, Kennedy SD, Kickler TS. 1996. The role of HLA antibodies in neonatal thrombocytopenia: a prospective study. *Tissue Antigens* 47: 206-11
81. Saito S, Ota M, Komatsu Y, Ota S, Aoki S, Koike K, Tokunaga I, Tsuno T, Tsuruta G, Kubo T, Fukushima H. 2003. Serologic analysis of three cases of neonatal alloimmune thrombocytopenia associated with HLA antibodies. *Transfusion* 43: 908-17
82. Moncharmont P, Dubois V, Obegi C, Vignal M, Merieux Y, Gebuhrer L, Rigal D. 2004. HLA antibodies and neonatal alloimmune thrombocytopenia. *Acta Haematol* 111: 215-20
83. Thude H, Schorner U, Helfricht C, Loth M, Maak B, Barz D. 2006. Neonatal alloimmune thrombocytopenia caused by human leucocyte antigen-B27 antibody. *Transfus Med* 16: 143-9
84. Taaning E. 2000. HLA antibodies and fetomaternal alloimmune thrombocytopenia: Myth or meaningful? *Transfusion Medicine Reviews* 14: 275-80
85. Van Loghem Jj J, Dorfmeijer H, Van Hart M, Schreuder F. 1959. Serological and genetical studies on a platelet antigen (Zw). *Vox Sang* 4: 161-9
86. Shulman NR, Aster RH, Pearson HA, Hiller MC. 1962. Immunoreactions involving platelets. VI. Reactions of maternal isoantibodies responsible for neonatal purpura. Differentiation of a second platelet antigen system. *Journal of Clinical Investigation* 41: 1059-69
87. Kunicki TJ, Aster RH. 1979. Isolation and immunologic characterization of the human platelet alloantigen, P1A1. *Mol Immunol* 16: 353-60
88. Newman PJ, Martin LS, Knipp MA, Kahn RA. 1985. Studies on the nature of the human platelet alloantigen, PlA1: localization to a 17,000-dalton polypeptide. *Mol Immunol* 22: 719-29
89. Newman PJ, Derbes RS, Aster RH. 1989. The human platelet alloantigens, PlA1 and PlA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J Clin Invest* 83: 1778-81
90. Santoso S, Kroll H, Andrei-Selmer CL, Socher I, Rankin A, Kretzschmar E, Watkins NA, Ouwehand WH. 2006. A naturally occurring Leu33Val mutation in β3-integrin impairs the HPA-1a epitope: the third allele of HPA-1. *Transfusion* 46: 790-9

91. Flug F, Espinola R, Liu LX, SinQuee C, DaRosso R, Nardi M, Karpatkin S. 1991. A 13-mer peptide straddling the leucine33/proline33 polymorphism in glycoprotein IIIa does not define the PLA1 epitope. *Blood* 77: 1964-9
92. Xiao T, Takagi J, Coller BS, Wang J-H, Springer TA. 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* 432: 59-67
93. Valentin N, Visentin GP, Newman PJ. 1995. Involvement of the cysteine-rich domain of glycoprotein IIIa in the expression of the human platelet alloantigen, PlA1: evidence for heterogeneity in the humoral response. *Blood* 85: 3028-33
94. Watkins NA, Schaffner-Reckinger E, Allen DL, Howkins GJ, Brons NHC, Smith GA, Metcalfe P, Murphy MF, Kieffer N, Ouwehand WH. 2002. HPA-1a phenotype–genotype discrepancy reveals a naturally occurring Arg93Gln substitution in the platelet β3 integrin that disrupts the HPA-1a epitope. *Blood* 99: 1833-9
95. Jallu V, Poulain P, Fuchs PFJ, Kaplan C, de Brevern AG. 2012. Modeling and Molecular Dynamics of HPA-1a and -1b Polymorphisms: Effects on the Structure of the β3 Subunit of the αIIbβ3 Integrin. *PLoS ONE* 7: e47304
96. Feng D, Lindpaintner K, Larson MG, Rao VS, O'Donnell CJ, Lipinska I, Schmitz C, Sutherland PA, Silbershatz H, D'Agostino RB, Muller JE, Myers RH, Levy D, Tofler GH. 1999. Increased platelet aggregability associated with platelet GPIIIa PlA2 polymorphism: the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol* 19: 1142-7
97. Michelson AD, Furman MI, Goldschmidt-Clermont P, Mascelli MA, Hendrix C, Coleman L, Hamlington J, Barnard MR, Kickler T, Christie DJ, Kundu S, Bray PF. 2000. Platelet GP IIIa Pl(A) polymorphisms display different sensitivities to agonists. *Circulation* 101: 1013-8
98. Vijayan KV, Goldschmidt-Clermont PJ, Roos C, Bray PF. 2000. The Pl(A2) polymorphism of integrin beta(3) enhances outside-in signaling and adhesive functions. *J Clin Invest* 105: 793-802
99. Floyd CN, Ellis BH, Ferro A. 2014. The PlA1/A2 Polymorphism of Glycoprotein IIIa as a Risk Factor for Stroke: A Systematic Review and Meta-Analysis. *PLoS ONE* 9: e100239
100. Murphy MF, Bussel JB. 2007. Advances in the management of alloimmune thrombocytopenia. *British Journal of Haematology* 136: 366-78
101. Kjeldsen-Kragh J, Killie MK, Tomter G, Golebiowska E, Randen I, Hauge R, Aune B, Øian P, Dahl LB, Pirhonen J, Lindeman R, Husby H, Haugen G, Grønn M, Skogen B, Husebekk A. 2007. A screening and intervention program aimed to reduce mortality and serious morbidity associated with severe neonatal alloimmune thrombocytopenia. *Blood* 110: 833-9
102. Turner ML, Bessos H, Fagge T, Harkness M, Rentoul F, Seymour J, Wilson D, Gray I, Ahya R, Cairns J, Urbaniak S. 2005. Prospective epidemiologic study of the outcome and cost-effectiveness of antenatal screening to detect neonatal alloimmune thrombocytopenia due to anti-HPA-1a. *Transfusion* 45: 1945-56
103. Williamson LM, Hackett G, Rennie J, Palmer CR, Maciver C, Hadfield R, Hughes D, Jobson S, Ouwehand WH. 1998. The Natural History of Fetomaternal Alloimmunization to the Platelet-Specific Antigen HPA-1a (PlA1, Zwa) as Determined by Antenatal Screening. *Blood* 92: 2280-7
104. Blanchette VS, Chen L, de Friedberg ZS, Hogan VA, Trudel E, Decary F. 1990. Alloimmunization to the PlA1 platelet antigen: results of a prospective study. *Br J Haematol* 74: 209-15

105. Davoren A, McParland P, Crowley J, Barnes A, Kelly G, Murphy WG. 2003. Antenatal screening for human platelet antigen-1a: results of a prospective study at a large maternity hospital in Ireland. *BJOG: An International Journal of Obstetrics & Gynaecology* 110: 492-6
106. Davoren A, Curtis BR, Aster RH, McFarland JG. 2004. Human platelet antigen-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. *Transfusion* 44: 1220-5
107. Kaplan C, Porcelijn L, Vanlieferinghen P, Julien E, Bianchi F, Martageix C, Bertrand G, Jallu V. 2005. Anti-HPA-9bw (Maxa) fetomaternal alloimmunization, a clinically severe neonatal thrombocytopenia: difficulties in diagnosis and therapy and report on eight families. *Transfusion* 45: 1799-803
108. Peterson JA, Balthazor SM, Curtis BR, McFarland JG, Aster RH. 2005. Maternal alloimmunization against the rare platelet-specific antigen HPA-9b (Maxa) is an important cause of neonatal alloimmune thrombocytopenia. *Transfusion* 45: 1487-95
109. Socher I, Andrei-Selmer C, Bein G, Kroll H, Santoso S. 2009. Low-avidity HPA-1a alloantibodies in severe neonatal alloimmune thrombocytopenia are detectable with surface plasmon resonance technology. *Transfusion* 49: 943-52
110. Bakchoul T, Kubiak S, Krautwurst A, Roderfeld M, Siebert HC, Bein G, Sachs UJ, Santoso S. 2011. Low-avidity anti-HPA-1a alloantibodies are capable of antigen-positive platelet destruction in the NOD/SCID mouse model of alloimmune thrombocytopenia. *Transfusion* 51: 2455-61
111. Peterson JA, Kanack A, Nayak D, Bougie DW, McFarland JG, Curtis BR, Aster RH. 2013. Prevalence and clinical significance of low-avidity HPA-1a antibodies in women exposed to HPA-1a during pregnancy. *Transfusion* 53: 1309-18
112. Spencer JA, Burrows RF. 2001. Fetomaternal alloimmune thrombocytopenia: a literature review and statistical analysis. *Australian and New Zealand Journal of Obstetrics and Gynaecology* 41: 45-55
113. Kamphuis MM, Paridaans N, Porcelijn L, De Haas M, Van Der Schoot CE, Brand A, Bonsel GJ, Oepkes D. 2010. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *Bjog* 117: 1335-43
114. Kamphuis MM, Oepkes D. 2011. Fetal and neonatal alloimmune thrombocytopenia: prenatal interventions. *Prenatal Diagnosis* 31: 712-9
115. Tiller H, Kamphuis MM, Flodmark O, Papadogiannakis N, David AL, Sainio S, Koskinen S, Javela K, Wikman AT, Kekomaki R, Kanhai HH, Oepkes D, Husebekk A, Westgren M. 2013. Fetal intracranial haemorrhages caused by fetal and neonatal alloimmune thrombocytopenia: an observational cohort study of 43 cases from an international multicentre registry. *BMJ Open* 3
116. Radder CM, Brand A, Kanhai HHH. 2003. Will it ever be possible to balance the risk of intracranial haemorrhage in fetal or neonatal alloimmune thrombocytopenia against the risk of treatment strategies to prevent it? *Vox Sanguinis* 84: 318-25
117. Tiller H, Killie MK, Husebekk A, Skogen B, Ni H, Kjeldsen-Kragh J, ØIan P. 2012. Platelet antibodies and fetal growth: maternal antibodies against fetal platelet antigen 1a are strongly associated with reduced birthweight in boys. *Acta Obstetricia et Gynecologica Scandinavica* 91: 79-86
118. Ahlen MT, Husebekk A, Killie MK, Kjeldsen-Kragh J, Olsson ML, Skogen B. 2012. The Development of Severe Neonatal Alloimmune Thrombocytopenia due to Anti-HPA-1a Antibodies Is Correlated to Maternal ABO Genotypes. *Clinical and Developmental Immunology* 2012: 5

119. Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H. 1993. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet* 91: 427-32
120. Vanderpuye OA, Labarrere CA, McIntyre JA. 1991. A vitronectin-receptor-related molecule in human placental brush border membranes. *Biochem J* 280 (Pt 1): 9-17
121. L'Abbe D, Tremblay L, Filion M, Busque L, Goldman M, Decary F, Chartrand P. 1992. Alloimmunization to platelet antigen HPA-1a (PIA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Hum Immunol* 34: 107-14
122. Valentin N, Vergracht A, Bignon JD, Cheneau ML, Blanchard D, Kaplan C, Reznikoff-Etievant MF, Muller JY. 1990. HLA-DRw52a is involved in alloimmunization against PL-A1 antigen. *Hum Immunol* 27: 73-9
123. Wu S, Maslanka K, Gorski J. 1997. An integrin polymorphism that defines reactivity with alloantibodies generates an anchor for MHC class II peptide binding: a model for unidirectional alloimmune responses. *J Immunol* 158: 3221-6
124. Parry CS, Gorski J, Stern LJ. 2007. Crystallographic structure of the human leukocyte antigen DRA, DRB3*0101: models of a directional alloimmune response and autoimmunity. *J Mol Biol* 371: 435-46
125. Maslanka K, Yassai M, Gorski J. 1996. Molecular identification of T cells that respond in a primary bulk culture to a peptide derived from a platelet glycoprotein implicated in neonatal alloimmune thrombocytopenia. *J Clin Invest* 98: 1802-8
126. Sukati H, Bessos H, Barker RN, Urbaniak SJ. 2005. Characterization of the alloreactive helper T-cell response to the platelet membrane glycoprotein IIIa (integrin-β3) in human platelet antigen-1a alloimmunized human platelet antigen-1b1b women. *Transfusion* 45: 1165-77
127. Jackson DJ, Murphy MF, Soothill PW, Lucas GF, Elson CJ, Kumpel BM. 2005. Reactivity of T cells from women with antibodies to the human platelet antigen (HPA)-1a to peptides encompassing the HPA-1 polymorphism. *Clinical & Experimental Immunology* 142: 92-102
128. Ahlen MT, Husebekk A, Killie MK, Skogen B, Stuge TB. 2009. T-cell responses associated with neonatal alloimmune thrombocytopenia: isolation of HPA-1a–specific, HLA-DRB3*0101–restricted CD4+ T cells. *Blood* 113: 3838-44
129. Rayment R, Kooij TW, Zhang W, Siebold C, Murphy MF, Allen D, Willcox N, Roberts DJ. 2009. Evidence for the specificity for platelet HPA-1a alloepitope and the presenting HLA-DR52a of diverse antigen-specific helper T cell clones from alloimmunized mothers. *J Immunol* 183: 677-86
130. Chen P, Li C, Lang S, Zhu G, Reheman A, Spring CM, Freedman J, Ni H. 2010. Animal model of fetal and neonatal immune thrombocytopenia: role of neonatal Fc receptor in the pathogenesis and therapy. *Blood* 116: 3660-8
131. Clarkson SB, Bussel JB, Kimberly RP, Valinsky JE, Nachman RL, Unkeless JC. 1986. Treatment of refractory immune thrombocytopenic purpura with an anti-Fc gamma-receptor antibody. *N Engl J Med* 314: 1236-9
132. McKenzie SE, Taylor SM, Malladi P, Yuhan H, Cassel DL, Chien P, Schwartz E, Schreiber AD, Surrey S, Reilly MP. 1999. The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model. *J Immunol* 162: 4311-8
133. Wiener E, Abeyakoon O, Benchetrit G, Lyall M, Keler T, Rodeck CH. 2003. Anti-HPA-1a-mediated platelet phagocytosis by monocytes in vitro and its inhibition by Fc gamma receptor (FcgammaR) reactive reagents. *Eur J Haematol* 70: 67-74

134. Peerschke EIB, Andemariam B, Yin W, Bussel JB. 2010. Complement activation on platelets correlates with a decrease in circulating immature platelets in patients with immune thrombocytopenic purpura. *British Journal of Haematology* 148: 638-45
135. Najaoui A, Bakchoul T, Stoy J, Bein G, Rummel MJ, Santoso S, Sachs UJ. 2012. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP). *European Journal of Haematology* 88: 167-74
136. Miescher S, Spycher MO, Amstutz H, De Haas M, Kleijer M, Kalus UJ, Radtke H, Hubsch A, Andresen I, Martin RM, Bichler J. 2004. A single recombinant anti-RhD IgG prevents RhD immunization: association of RhD-positive red blood cell clearance rate with polymorphisms in the FcgammaRIIA and FcgammaIIIA genes. *Blood* 103: 4028-35
137. Kumpel BM, De Haas M, Koene HR, Van De Winkel JG, Goodrick MJ. 2003. Clearance of red cells by monoclonal IgG3 anti-D in vivo is affected by the VF polymorphism of Fcgamma RIIIa (CD16). *Clin Exp Immunol* 132: 81-6
138. Warwick RM, Vaughan J, Murray N, Lubenko A, Roberts I. 1994. In vitro culture of colony forming unit-megakaryocyte (CFU-MK) in fetal alloimmune thrombocytopenia. *Br J Haematol* 88: 874-7
139. Liu ZJ, Bussel JB, Lakkaraja M, Ferrer-Marin F, Ghevaert C, Feldman HA, McFarland JG, Chavda C, Sola-Visner M. 2015. Suppression of in vitro megakaryopoiesis by maternal sera containing anti-HPA-1a antibodies. *Blood*
140. Chang M, Nakagawa PA, Williams SA, Schwartz MR, Imfeld KL, Buzby JS, Nugent DJ. 2003. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood* 102: 887-95
141. McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. 2004. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. *Blood* 103: 1364-9
142. van Leeuwen EF, Leeksma OC, van Mourik JA, Engelfriet CP, von dem Borne AE. 1984. Effect of the binding of anti-Zwa antibodies on platelet function. *Vox Sang* 47: 280-9
143. Beadling WV, Herman JH, Stuart MJ, Keashen-Schnell M, Miller JL. 1995. Fetal bleeding in neonatal alloimmune thrombocytopenia mediated by anti-PlAl is not associated with inhibition of fibrinogen binding to platelet GPIIb/IIIa. *Am J Clin Pathol* 103: 636-41
144. Joutsi-Korhonen L, Preston S, Smethurst PA, Ijsseldijk M, Schaffner-Reckinger E, Armour KL, Watkins NA, Clark MR, de Groot PG, Farndale RW, Ouwehand WH, Williamson LM. 2004. The effect of recombinant IgG antibodies against the leucine-33 form of the platelet beta3 integrin (HPA-1a) on platelet function. *Thromb Haemost* 91: 743-54
145. Proulx C, Filion M, Goldman M, Bradley A, Devine D, Decary F, Chartrand P. 1994. Analysis of immunoglobulin class, IgG subclass and titre of HPA-1a antibodies in alloimmunized mothers giving birth to babies with or without neonatal alloimmune thrombocytopenia. *Br J Haematol* 87: 813-7
146. Kapur R, Kustiawan I, Vestrheim A, Koeleman CA, Visser R, Einarsdottir HK, Porcelijn L, Jackson D, Kumpel B, Deelder AM, Blank D, Skogen B, Killie MK, Michaelsen TE, de Haas M, Rispens T, van der Schoot CE, Wuhrer M, Vidarsson G. 2014. A prominent lack of IgG1-Fc fucosylation of platelet alloantibodies in pregnancy. *Blood* 123: 471-80

147. Wuhrer M, Porcelijn L, Kapur R, Koeleman CA, Deelder A, de Haas M, Vidarsson G. 2009. Regulated glycosylation patterns of IgG during alloimmune responses against human platelet antigens. *J Proteome Res* 8: 450-6
148. Kapur R, Della Valle L, Sonneveld M, Hipgrave Ederveen A, Visser R, Ligthart P, de Haas M, Wuhrer M, van der Schoot CE, Vidarsson G. 2014. Low anti-RhD IgG-Fc-fucosylation in pregnancy: a new variable predicting severity in haemolytic disease of the fetus and newborn. *Br J Haematol* 166: 936-45
149. Stafford P, Ghevaert CJG, Campbell K, Proulx C, Smith G, Williamson LM, Ranasinghe E, Watkins NA, Huntington JA, Ouwehand WH. 2008. Immunologic and structural analysis of eight novel domain-deletion β 3 integrin peptides designed for detection of HPA-1 antibodies. *Journal of Thrombosis and Haemostasis* 6: 366-75
150. Husebekk AJ, S; Oian, P; Aune, B; Dahl, LB; Skogen, B;. 1997. Screening for anti-HPA 1a antibodies during pregnancy - feasibility and interpretation of results. *Blood* 90: 3084-
151. Jaegtvik S, Husebekk A, Aune B, Oian P, Dahl LB, Skogen B. 2000. Neonatal alloimmune thrombocytopenia due to anti-HPA 1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. *BJOG* 107: 691-4
152. Maslanka K, Guz K, Zupanska B. 2003. Antenatal screening of unselected pregnant women for HPA-1a antigen, antibody and alloimmune thrombocytopenia. *Vox Sanguinis* 85: 326-7
153. Killie MK, Husebekk A, Kjeldsen-Kragh J, Skogen B. 2008. A prospective study of maternal anti-HPA 1a antibody level as a potential predictor of alloimmune thrombocytopenia in the newborn. *Haematologica* 93: 870-7
154. Kjeldsen-Kragh J, Kim M, Killie MK, Husebekk A, Freedman J, Semple JW. 2009. In HPA 1a-immunized women the decrease in anti-HPA 1a antibody level during pregnancy is not associated with anti-idiotypic antibodies. *Haematologica* 94: 441-3
155. Yougbare I, Lang S, Yang H, Chen P, Zhao X, Tai WS, Zdravic D, Vadasz B, Li C, Piran S, Marshall A, Zhu G, Tiller H, Killie MK, Boyd S, Leong-Poi H, Wen XY, Skogen B, Adamson SL, Freedman J, Ni H. 2015. Maternal anti-platelet beta3 integrins impair angiogenesis and cause intracranial hemorrhage. *J Clin Invest* 125: 1545-56
156. Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, Damsky CH. 1997. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest* 99: 2139-51
157. Thirkill TL, Douglas GC. 1999. The Vitronectin Receptor Plays a Role in the Adhesion of Human Cytotrophoblast Cells to Endothelial Cells. *Endothelium* 6: 277-90
158. Douglas GC, Thirkill TL, Blankenship TN. 1999. Vitronectin receptors are expressed by macaque trophoblast cells and play a role in migration and adhesion to endothelium. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1452: 36-45
159. Killie MK, Kjeldsen-Kragh J, Husebekk A, Skogen B, Olsen JA, Kristiansen IS. 2007. Cost-effectiveness of antenatal screening for neonatal alloimmune thrombocytopenia. *Bjog* 114: 588-95
160. Bussel J. 2009. Diagnosis and management of the fetus and neonate with alloimmune thrombocytopenia. *Journal of Thrombosis and Haemostasis* 7: 253-7
161. Killie MK, Kjeldsen-Kragh J, Randen I, Skogen B, Husebekk A. 2004. Evaluation of a new flow cytometric HPA 1a screening method: A rapid and reliable tool for HPA

1a screening of blood donors and pregnant women. *Transfusion and Apheresis Science* 30: 89-92
162. Bugert P, McBride S, Smith G, Dugrillon A, Klüter H, Ouwehand WH, Metcalfe P. 2005. Microarray-based genotyping for blood groups: comparison of gene array and 5′-nuclease assay techniques with human platelet antigen as a model. *Transfusion* 45: 654-9
163. Skogen B, Bellissimo DB, Hessner MJ, Santoso S, Aster RH, Newman PJ, McFarland JG. 1994. Rapid determination of platelet alloantigen genotypes by polymerase chain reaction using allele-specific primers. *Transfusion* 34: 955-60
164. Killie MK, Salma W, Bertelsen E, Skogen B, Husebekk A. 2010. Quantitative MAIPA: Comparison of different MAIPA protocols. *Transfusion and Apheresis Science* 43: 149-54
165. Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. 1987. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood* 70: 1722-6
166. Campbell K, Rishi K, Howkins G, Gilby D, Mushens R, Ghevaert C, Metcalfe P, Ouwehand WH, Lucas G. 2007. A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation. *Vox Sang* 93: 289-97
167. Scheffer PG, Ait Soussan A, Verhagen O, Page-Christiaens G, Oepkes D, de Haas M, van der Schoot CE. 2011. Noninvasive fetal genotyping of human platelet antigen-1a. *BJOG: An International Journal of Obstetrics & Gynaecology* 118: 1392-5
168. Le Toriellec E, Chenet C, Kaplan C. 2013. Safe fetal platelet genotyping: new developments. *Transfusion* 53: 1755-62
169. Wienzek-Lischka S, Krautwurst A, Fröhner V, Hackstein H, Gattenlöhner S, Bräuninger A, Axt-Fliedner R, Degenhardt J, Deisting C, Santoso S, Sachs UJ, Bein G. 2015. Noninvasive fetal genotyping of human platelet antigen-1a using targeted massively parallel sequencing. *Transfusion* 55: 1538-44
170. Bussel JB, Zabusky MR, Berkowitz RL, McFarland JG. 1997. Fetal Alloimmune Thrombocytopenia. *New England Journal of Medicine* 337: 22-6
171. Overton TG, Duncan KR, Jolly M, Letsky E, Fisk NM. 2002. Serial aggressive platelet transfusion for fetal alloimmune thrombocytopenia: Platelet dynamics and perinatal outcome. *American Journal of Obstetrics and Gynecology* 186: 826-31
172. Van Den Akker ESA, Oepkes D, Lopriore E, Brand A, Kanhai HHH. 2007. Noninvasive antenatal management of fetal and neonatal alloimmune thrombocytopenia: safe and effective. *BJOG: An International Journal of Obstetrics & Gynaecology* 114: 469-73
173. Pacheco LD, Berkowitz RL, Moise KJJ, Bussel JB, McFarland JG, Saade GR. 2011. Fetal and Neonatal Alloimmune Thrombocytopenia: A Management Algorithm Based on Risk Stratification. *Obstetrics & Gynecology* 118: 1157-63
174. Giers G, Wenzel F, Stockschläder M, Riethmacher R, Lorenz H, Tutschek B. 2010. *Fetal alloimmune thrombocytopenia and maternal intravenous immunoglobulin infusion*. 1921-6 pp.
175. Rayment R, Brunskill SJ, Soothill PW, Roberts DJ, Bussel JB, Murphy MF. 2011. Antenatal interventions for fetomaternal alloimmune thrombocytopenia. *Cochrane Database Syst Rev*: CD004226
176. Bussel JB, Berkowitz RL, Hung C, Kolb EA, Wissert M, Primiani A, Tsaur FW, MacFarland JG. Intracranial hemorrhage in alloimmune thrombocytopenia: stratified management to prevent recurrence in the subsequent affected fetus. *American Journal of Obstetrics & Gynecology* 203: 135.e1-.e14

177. Van Den Akker ESA, Oepkes D, Brand A, Kanhai HHH. 2006. Vaginal delivery for fetuses at risk of alloimmune thrombocytopenia? *BJOG: An International Journal of Obstetrics & Gynaecology* 113: 781-3
178. te Pas A, Lopriore E, van den Akker EA, Oepkes D, Kanhai H, Brand A, Walther F. 2007. Postnatal management of fetal and neonatal alloimmune thrombocytopenia: the role of matched platelet transfusion and IVIG. *European Journal of Pediatrics* 166: 1057-63
179. Crowther C, Middleton P. 2000. Anti-D administration after childbirth for preventing Rhesus alloimmunisation. *Cochrane Database Syst Rev*: Cd000021
180. Bennardello F, Coluzzi S, Curciarello G, Todros T, Villa S. 2015. Recommendations for the prevention and treatment of haemolytic disease of the foetus and newborn. *Blood Transfusion* 13: 109-34
181. Jennings ER, Dibbern HH, Hodell FH, Monroe CH, Peckham NH, Sullivan JF, Pollack W. 1969. Prevention of Rh Hemolytic Disease of the Newborn. *California Medicine* 110: 130-3
182. Kumpel BM, Elson CJ. 2001. Mechanism of anti-D-mediated immune suppression--a paradox awaiting resolution? *Trends Immunol* 22: 26-31
183. Crow AR, Freedman J, Hannach B, Lazarus AH. 2000. Monoclonal antibody-mediated inhibition of the human HLA alloimmune response to platelet transfusion is antigen specific and independent of Fcgamma receptor-mediated immune suppression. *Br J Haematol* 110: 481-7
184. Siegrist CA. 2007. The Challenges of Vaccine Responses in Early Life: Selected Examples. *Journal of Comparative Pathology* 137, Supplement 1: S4-S9
185. Brinc D, Lazarus AH. 2009. Mechanisms of anti-D action in the prevention of hemolytic disease of the fetus and newborn. *Hematology Am Soc Hematol Educ Program*: 185-91
186. Getahun A, Heyman B. 2009. Studies on the mechanism by which antigen-specific IgG suppresses primary antibody responses: evidence for epitope masking and decreased localization of antigen in the spleen. *Scand J Immunol* 70: 277-87
187. Karlsson MC, Wernersson S, Diaz de Stahl T, Gustavsson S, Heyman B. 1999. Efficient IgG-mediated suppression of primary antibody responses in Fcgamma receptor-deficient mice. *Proc Natl Acad Sci U S A* 96: 2244-9
188. Chan PL, Sinclair NRS. 1973. Regulation of the immune response: VI. Inability of F(ab′)(2) antibody to terminate established immune responses and its ability to interfere with IgG antibody-mediated immunosuppression. *Immunology* 24: 289-301
189. Lubenko A, Williams M, Johnson A, Pluck J, Armstrong D, MacLennan S. 1999. Monitoring the clearance of fetal RhD-positive red cells in FMH following RhD immunoglobulin administration. *Transfus Med* 9: 331-5
190. Mollison PL, Crome P, Hughes-Jones NC, Rochna E. 1965. Rate of removal from the circulation of red cells sensitized with different amounts of antibody. *Br J Haematol* 11: 461-70
191. Jones NC, Mollison PL, Veall N. 1957. Removal of incompatible red cells by the spleen. *Br J Haematol* 3: 125-33
192. Kumpel BM. 2006. On the immunologic basis of Rh immune globulin (anti-D) prophylaxis. *Transfusion* 46: 1271-5
193. Armour KL, Smith CS, Ip NCY, Ellison CJ, Kirton CM, Wilkes AM, Williamson LM, Clark MR. 2014. Clearance of Human IgG1-Sensitised Red Blood Cells In Vivo in Humans Relates to the In Vitro Properties of Antibodies from Alternative Cell Lines. *Plos One* 9

194. Kumpel BM, Goodrick MJ, Pamphilon DH, Fraser ID, Poole GD, Morse C, Standen GR, Chapman GE, Thomas DP, Anstee DJ. 1995. Human Rh D monoclonal antibodies (BRAD-3 and BRAD-5) cause accelerated clearance of Rh D+ red blood cells and suppression of Rh D immunization in Rh D- volunteers. *Blood* 86: 1701-9
195. Kumpel BM. 2007. Efficacy of RhD monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. *Vox Sang* 93: 99-111
196. Olovnikova NI, Belkina EV, Nikolaeva TL, Chertkov IL. 1998. [Absence of relation between activity of mono- and polyclonal anti-rhesus immunoglobulins in in vitro and in vivo tests]. *Biull Eksp Biol Med* 125: 71-4
197. Woodrow JC, Clarke CA, Donohow WT, Finn R, McConnell RB, Sheppard PM, Lehane D, Roberts FM, Gimlette TM. 1975. Mechanism of Rh prophylaxis: an experimental study on specificity of immunosuppression. *British Medical Journal* 2: 57-9
198. Karlsson MCI, Getahun A, Heyman B. 2001. FcγRIIB in IgG-Mediated Suppression of Antibody Responses: Different Impact In Vivo and In Vitro. *The Journal of Immunology* 167: 5558-64
199. Tiller H, Killie MK, Chen P, Eksteen M, Husebekk A, Skogen B, Kjeldsen-Kragh J, Ni H. 2012. Toward a prophylaxis against fetal and neonatal alloimmune thrombocytopenia: induction of antibody-mediated immune suppression and prevention of severe clinical complications in a murine model. *Transfusion* 52: 1446-57
200. Ghevaert C, Herbert N, Hawkins L, Grehan N, Cookson P, Garner SF, Crisp-Hihn A, Lloyd-Evans P, Evans A, Balan K, Ouwehand WH, Armour KL, Clark MR, Williamson LM. 2013. Recombinant HPA-1a antibody therapy for treatment of fetomaternal alloimmune thrombocytopenia: proof of principle in human volunteers. *Blood* 122: 313-20
201. Kjeldsen-Kragh J, Ni H, Skogen B. 2012. Towards a prophylactic treatment of HPA-related foetal and neonatal alloimmune thrombocytopenia. *Current Opinion in Hematology* 19: 469-74 10.1097/MOH.0b013e328358f86c
202. Bai XF, Link H. 2000. Nasal tolerance induction as a potential means of immunotherapy for autoimmune diseases: implications for clinical medicine. *Clinical & Experimental Allergy* 30: 1688-96
203. Skripak JM, Nash SD, Rowley H, Brereton NH, Oh S, Hamilton RG, Matsui EC, Burks AW, Wood RA. 2008. A randomized, double-blind, placebo-controlled study of milk oral immunotherapy for cow's milk allergy. *J Allergy Clin Immunol* 122: 1154-60
204. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, Shreffler WG, Steele P, Henry KA, Adair M, Francis JM, Durham S, Vickery BP, Zhong X, Burks AW. 2009. Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* 124: 292-300, .e1-97
205. Mobs C, Slotosch C, Loffler H, Jakob T, Hertl M, Pfutzner W. 2010. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* 184: 2194-203
206. Patel D, Couroux P, Hickey P, Salapatek AM, Laidler P, Larche M, Hafner RP. 2013. Fel d 1-derived peptide antigen desensitization shows a persistent treatment effect 1 year after the start of dosing: a randomized, placebo-controlled study. *J Allergy Clin Immunol* 131: 103-9.e1-7
207. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, Steinman L. 2000. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-

controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med* 6: 1176-82

208. Koffeman EC, Genovese M, Amox D, Keogh E, Santana E, Matteson EL, Kavanaugh A, Molitor JA, Schiff MH, Posever JO, Bathon JM, Kivitz AJ, Samodal R, Belardi F, Dennehey C, van den Broek T, van Wijk F, Zhang X, Zieseniss P, Le T, Prakken BA, Cutter GC, Albani S. 2009. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum* 60: 3207-16
209. Hall AM, Cairns LS, Altmann DM, Barker RN, Urbaniak SJ. 2005. Immune responses and tolerance to the RhD blood group protein in HLA-transgenic mice. *Blood* 105: 2175-9
210. Stephen J, Cairns LS, Pickford WJ, Vickers MA, Urbaniak SJ, Barker RN. 2012. Identification, immunomodulatory activity, and immunogenicity of the major helper T-cell epitope on the K blood group antigen. *Blood* 119: 5563-74
211. Bakchoul T, Greinacher A, Sachs UJ, Krautwurst A, Renz H, Harb H, Bein G, Newman PJ, Santoso S. 2013. Inhibition of HPA-1a alloantibody-mediated platelet destruction by a deglycosylated anti-HPA-1a monoclonal antibody in mice: toward targeted treatment of fetal-alloimmune thrombocytopenia. *Blood* 122: 321-7
212. Ghevaert C, Wilcox DA, Fang J, Armour KL, Clark MR, Ouwehand WH, Williamson LM. 2008. Developing recombinant HPA-1a-specific antibodies with abrogated Fcgamma receptor binding for the treatment of fetomaternal alloimmune thrombocytopenia. *J Clin Invest* 118: 2929-38
213. Saleh M, Court W, Huster W, Shaw D, LoBuglio A. 1988. Effect of commercial immunoglobulin G preparation on human monocyte Fc-receptor dependent binding of antibody coated platelets. *Br J Haematol* 68: 47-51
214. Saleh MN, Court WS, LoBuglio AF. 1986. In vitro effects of gammaglobulin (IgG) on human monocyte Fc receptor function. I. Effect on monocyte membrane-associated IgG and Fc receptor-dependent binding of antibody-coated platelets. *Am J Hematol* 23: 197-207
215. Sesarman A, Vidarsson G, Sitaru C. 2010. The neonatal Fc receptor as therapeutic target in IgG-mediated autoimmune diseases. *Cell Mol Life Sci* 67: 2533-50
216. Nixon AE, Chen J, Sexton DJ, Muruganandam A, Bitonti AJ, Dumont J, Viswanathan M, Martik D, Wassaf D, Mezo A, Wood CR, Biedenkapp JC, TenHoor C. 2015. Fully human monoclonal antibody inhibitors of the neonatal fc receptor reduce circulating IgG in non-human primates. *Front Immunol* 6: 176
217. Liu LX, Nardi MA, Flug F, Karpatkin S. 1992. A monoclonal antibody (LK-4) which differentiates PLA1 from PLA2 platelet extracts but not intact platelets. *Thrombosis Research* 66: 309-20
218. Weiss EJ, Goldschmidt-Clermont PJ, Grigoryev D, Jin Y, Kickler TS, Bray PF. 1995. A monoclonal antibody (SZ21) specific for platelet GPIIIa distinguishes P1 A1 from Pl A2. *Tissue Antigens* 46: 374-81
219. Ryckewaert JJ, Schweizer B, Chapel A, Marguerie G. 1992. Production of anti-P1A monoclonal antibodies. *J Lab Clin Med* 119: 52-6
220. Griffin H, Ouwehand W. 1995. A human monoclonal antibody specific for the leucine-33 (P1A1, HPA-1a) form of platelet glycoprotein IIIa from a V gene phage display library. *Blood* 86: 4430-6
221. Okamoto N, Kennedy SD, Barron-Casella EA, Casella JF, Inoko H, Kickler TS. 1998. Identification of a human heavy chain antibody fragment directed against human platelet alloantigen 1a by phage display library. *Tissue Antigens* 51: 156-63

222. Proulx C, Chartrand P, Roy V, Goldman M, Décary F, Rinfret A. 1997. Human Monoclonal Fab Fragments Recovered from a Combinatorial Library Bind Specifically to the Platelet HPA-1a Alloantigen on Glycoprotein IIb–IIIa. *Vox Sanguinis* 72: 52-60
223. Smith K, Garman L, Wrammert J, Zheng N-Y, Capra JD, Ahmed R, Wilson PC. 2009. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. *Nat. Protocols* 4: 372-84
224. Radic MZ, Mascelli MA, Erikson J, Shan H, Weigert M. 1991. Ig H and L chain contributions to autoimmune specificities. *The Journal of Immunology* 146: 176-82
225. Chailyan A, Marcatili P, Tramontano A. 2011. The association of heavy and light chain variable domains in antibodies: implications for antigen specificity. *Febs j* 278: 2858-66
226. Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, Tumpey TM, Pappas C, Perrone LA, Martinez O, Stevens J, Wilson IA, Aguilar PV, Altschuler EL, Basler CF, Jr JEC. 2008. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature* 455: 532-6
227. Lanzavecchia A, Corti D, Sallusto F. 2007. Human monoclonal antibodies by immortalization of memory B cells. *Curr. Opin. Biotechnol.* 18: 523-8
228. Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A. 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 10: 871-5
229. Foung SK, Perkins S, Raubitschek A, Larrick J, Lizak G, Fishwild D, Engleman EG, Grumet FC. 1984. Rescue of human monoclonal antibody production from an EBV-transformed B cell line by fusion to a human-mouse hybridoma. *J Immunol Methods* 70: 83-90
230. Townsend SE, Goodnow CC, Cornall RJ. 2001. Single epitope multiple staining to detect ultralow frequency B cells. *J Immunol Methods* 249: 137-46
231. Carter AM. 2007. Animal Models of Human Placentation – A Review. *Placenta* 28, Supplement: S41-S7
232. Kilburn BA, Wang J, Duniec-Dmuchowski ZM, Leach RE, Romero R, Armant DR. 2000. Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. *Biol Reprod* 62: 739-47
233. Shiverick KT, King A, Frank H, Whitley GS, Cartwright JE, Schneider H. 2001. Cell culture models of human trophoblast II: trophoblast cell lines--a workshop report. *Placenta* 22 Suppl A: S104-6
234. Whitley GSJ, Cartwright JE. 2009. Trophoblast-mediated spiral artery remodelling: a role for apoptosis. *Journal of Anatomy* 215: 21-6
235. Kalkunte S, Lai Z, Tewari N, Chichester C, Romero R, Padbury J, Sharma S. 2008. In vitro and in vivo evidence for lack of endovascular remodeling by third trimester trophoblasts. *Placenta* 29: 871-8
236. Keogh RJ. 2010. New technology for investigating trophoblast function. *Placenta* 31: 347-50
237. Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, Lala PK. 1993. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 206: 204-11
238. Manyonda IT, Whitley GS, Cartwright JE. 2001. Trophoblast cell lines: a response to the Workshop Report by King et al. *Placenta* 22: 262-3
239. Hunkapiller NM, Fisher SJ. 2008. Chapter 12. Placental remodeling of the uterine vasculature. *Methods Enzymol* 445: 281-302

240. Solly K, Wang X, Xu X, Strulovici B, Zheng W. 2004. Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev Technol* 2: 363-72
241. Sanz C, Freire C, Alcorta I, Ordinas A, Pereira A. 2001. Platelet-specific antibodies in HLA-immunized patients receiving chronic platelet support. *Transfusion* 41: 762-5
242. Yver A, Homery MC, Fuseau E, Guemas E, Dhainaut F, Quagliaroli D, Beliard R, Prost JF. 2012. Pharmacokinetics and safety of roledumab, a novel human recombinant monoclonal anti-RhD antibody with an optimized Fc for improved engagement of FCγRIII, in healthy volunteers. *Vox Sanguinis* 103: 213-22
243. Béliard R. 2006. Monoclonal anti-D antibodies to prevent alloimmunization: lessons from clinical trials. *Transfusion Clinique et Biologique* 13: 58-64
244. Stasi R. 2010. Rozrolimupab, symphobodies against rhesus D, for the potential prevention of hemolytic disease of the newborn and the treatment of idiopathic thrombocytopenic purpura. *Curr Opin Mol Ther* 12: 734-40
245. Robak T, Windyga J, Trelinski J, von Depka Prondzinski M, Giagounidis A, Doyen C, Janssens A, Alvarez-Roman MT, Jarque I, Loscertales J, Rus GP, Hellmann A, Jedrzejczak WW, Kuliczkowski K, Golubovic LM, Celeketic D, Cucuianu A, Gheorghita E, Lazaroiu M, Shpilberg O, Attias D, Karyagina E, Svetlana K, Vilchevska K, Cooper N, Talks K, Prabhu M, Sripada P, Bharadwaj TP, Naested H, Skartved NJ, Frandsen TP, Flensburg MF, Andersen PS, Petersen J. 2012. Rozrolimupab, a mixture of 25 recombinant human monoclonal RhD antibodies, in the treatment of primary immune thrombocytopenia. *Blood* 120: 3670-6
246. Beliard R, Waegemans T, Notelet D, Massad L, Dhainaut F, Romeuf CD, Guemas E, Haazen W, Bourel D, Teillaud JL, Prost JF. 2008. A human anti-D monoclonal antibody selected for enhanced FcγRIII engagement clears RhD+ autologous red cells in human volunteers as efficiently as polyclonal anti-D antibodies. *British Journal of Haematology* 141: 109-19
247. Avent ND, Liu W, Scott ML, Jones JW, Voak D. 2000. Site directed mutagenesis of the human Rh D antigen: molecular basis of D epitopes. *Vox Sang* 78 Suppl 2: 83-9
248. Scott M. 1996. Rh serology--coordinator's report. *Transfus Clin Biol* 3: 333-7
249. Jones J, Scott ML, Voak D. 1995. Monoclonal anti-D specificity and Rh D structure: criteria for selection of monoclonal anti-D reagents for routine typing of patients and donors. *Transfus Med* 5: 171-84
250. Kjaersgaard M, Aslam R, Kim M, Speck ER, Freedman J, Stewart DI, Wiersma EJ, Semple JW. 2007. Epitope specificity and isotype of monoclonal anti-D antibodies dictate their ability to inhibit phagocytosis of opsonized platelets. *Blood* 110: 1359-61
251. Jefferis R. 2009. Glycosylation as a strategy to improve antibody-based therapeutics. *Nat Rev Drug Discov* 8: 226-34
252. Iida S, Misaka H, Inoue M, Shibata M, Nakano R, Yamane-Ohnuki N, Wakitani M, Yano K, Shitara K, Satoh M. 2006. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcgammaRIIIa. *Clin Cancer Res* 12: 2879-87
253. Kapur R, Della Valle L, Sonneveld M, Hipgrave Ederveen A, Visser R, Ligthart P, de Haas M, Wuhrer M, van der Schoot CE, Vidarsson G. 2014. Low anti-RhD IgG-Fc-fucosylation in pregnancy: a new variable predicting severity in haemolytic disease of the fetus and newborn. *British Journal of Haematology* 166: 936-45
254. Siberil S, de Romeuf C, Bihoreau N, Fernandez N, Meterreau JL, Regenman A, Nony E, Gaucher C, Glacet A, Jorieux S, Klein P, Hogarth MP, Fridman WH, Bourel D, Beliard R, Teillaud JL. 2006. Selection of a human anti-RhD monoclonal antibody for

therapeutic use: impact of IgG glycosylation on activating and inhibitory Fc gamma R functions. *Clin Immunol* 118: 170-9
255. Kanda Y, Yamane-Ohnuki N, Sakai N, Yamano K, Nakano R, Inoue M, Misaka H, Iida S, Wakitani M, Konno Y, Yano K, Shitara K, Hosoi S, Satoh M. 2006. Comparison of cell lines for stable production of fucose-negative antibodies with enhanced ADCC. *Biotechnol Bioeng* 94: 680-8
256. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 17: 176-80
257. Imai-Nishiya H, Mori K, Inoue M, Wakitani M, Iida S, Shitara K, Satoh M. 2007. Double knockdown of alpha1,6-fucosyltransferase (FUT8) and GDP-mannose 4,6-dehydratase (GMD) in antibody-producing cells: a new strategy for generating fully non-fucosylated therapeutic antibodies with enhanced ADCC. *BMC Biotechnol* 7: 84
258. Yaari G, Vander Heiden JA, Uduman M, Gadala-Maria D, Gupta N, Stern JN, O'Connor KC, Hafler DA, Laserson U, Vigneault F, Kleinstein SH. 2013. Models of somatic hypermutation targeting and substitution based on synonymous mutations from high-throughput immunoglobulin sequencing data. *Front Immunol* 4: 358
259. Uduman M, Yaari G, Hershberg U, Stern JA, Shlomchik MJ, Kleinstein SH. 2011. Detecting selection in immunoglobulin sequences. *Nucleic Acids Res* 39: W499-504
260. Passacquale G, Vamadevan P, Pereira L, Hamid C, Corrigall V, Ferro A. 2011. Monocyte-Platelet Interaction Induces a Pro-Inflammatory Phenotype in Circulating Monocytes. *PLoS ONE* 6: e25595
261. Deckmyn H, Vanhoorelbeke K, Peerlinck K. 1998. Inhibitory and activating human antiplatelet antibodies. *Baillieres Clin Haematol* 11: 343-59
262. Dettke M, Dreer M, Höcker P, Panzer S. 2001. Human platelet antigen-1a antibodies induce the release of the chemokine RANTES from human platelets. *Vox Sanguinis* 81: 199-203
263. Lapierre Y, Rigal D, Adam J, Josef D, Meyer F, Greber S, Drot C. 1990. The gel test: a new way to detect red cell antigen-antibody reactions. *Transfusion* 30: 109-13
264. Chan A, Wong HF, Chui CH, Wong L, Cheng G. 1996. The impact of a gel system on routine work in a general hospital blood bank. *Immunohematology* 12: 30-2
265. Meyer O, Agaylan A, Schonemann C, Kiesewetter H, Salama A. 2008. Application of the particle gel agglutination assay in the typing of single human leucocyte antigens. *Tissue Antigens* 71: 157-9
266. Abou-Chaker K, Meyer O, Salama A. 2009. Rapid typing of the human neutrophil antigen 1a by the particle gel agglutination assay. *Tissue Antigens* 73: 242-4
267. Evenson DA, Stroncek DF, Pulkrabek S, Perry EH, Radford J, Miller JS, Verfaillie C. 1995. Posttransfusion purpura following bone marrow transplantation. *Transfusion* 35: 688-93
268. Curtis BR, Bussel JB, Manco-Johnson MJ, Aster RH, McFarland JG. 2005. Fetal and neonatal alloimmune thrombocytopenia in pregnancies involving in vitro fertilization: A report of four cases. *American Journal of Obstetrics and Gynecology* 192: 543-7
269. Ward MJ, Pauliny J, Lipper EG, Bussel JB. 2006. Long-term effects of fetal and neonatal alloimmune thrombocytopenia and its antenatal treatment on the medical and developmental outcomes of affected children. *Am J Perinatol* 23: 487-92
270. Burton GJ, Fowden AL. 2015. The placenta: a multifaceted, transient organ. *Philos Trans R Soc Lond B Biol Sci* 370: 20140066
271. Jeronimo M, Azenha C, Mesquita J, Pereira DF. 2014. A rare manifestation of neonatal alloimmune thrombocytopaenia. *BMJ Case Rep* 2014

272. Althaus J, Weir EG, Askin F, Kickler TS, Blakemore K. 2005. Chronic villitis in untreated neonatal alloimmune thrombocytopenia: an etiology for severe early intrauterine growth restriction and the effect of intravenous immunoglobulin therapy. *Am J Obstet Gynecol* 193: 1100-4
273. Tchakarov A, Coffey A, Tatevian N. 2013. Neonatal Alloimmune Thrombocytopenia Associated with Massive Chronic Intervillositis: A Case Report and Review of the Literature. *Pediatric and Developmental Pathology* 16: 32-4
274. Barker DJP. 2004. The developmental origins of well-being. *Philosophical Transactions of the Royal Society B: Biological Sciences* 359: 1359-66
275. Barker DJ. 2006. Adult consequences of fetal growth restriction. *Clin Obstet Gynecol* 49: 270-83
276. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. 2008. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* 359: 61-73
277. Murphy MF, Hambley H, Nicolaides K, Waters AH. 1996. Severe fetomaternal alloimmune thrombocytopenia presenting with fetal hydrocephalus. *Prenat Diagn* 16: 1152-5
278. Labarrere CA, Hardin JW, Haas DM, Kassab GS. 2015. Chronic villitis of unknown etiology and massive chronic intervillositis have similar immune cell composition. *Placenta* 36: 681-6
279. Tamblyn JA, Lissauer DM, Powell R, Cox P, Kilby MD. 2013. The immunological basis of villitis of unknown etiology – Review. *Placenta* 34: 846-55
280. Lee J, Romero R, Xu Y, Kim J-S, Topping V, Yoo W, Kusanovic JP, Chaiworapongsa T, Hassan SS, Yoon BH, Kim CJ. 2011. A Signature of Maternal Anti-Fetal Rejection in Spontaneous Preterm Birth: Chronic Chorioamnionitis, Anti-Human Leukocyte Antigen Antibodies, and C4d. *PLoS ONE* 6: e16806
281. Boyd TK, Redline RW. 2000. Chronic histiocytic intervillositis: a placental lesion associated with recurrent reproductive loss. *Human Pathology* 31: 1389-96
282. Moffett A, Hiby SE, Sharkey AM. 2015. The role of the maternal immune system in the regulation of human birthweight. *Philos Trans R Soc Lond B Biol Sci* 370: 20140071
283. Italiano JE, Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. 2008. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 111: 1227-33
284. Sato Y, Fujiwara H, Konishi I. 2010. Role of platelets in placentation. *Med Mol Morphol* 43: 129-33
285. Sabrkhany S, Griffioen AW, oude Egbrink MGA. 2011. The role of blood platelets in tumor angiogenesis. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1815: 189-96

Supplemental data I

Mariana Eksteen, Heidi Tiller, Tor B. Stuge

Efforts to isolate several HPA-1a-specific memory B cells

Several attempts have been made to isolate a number of HPA-1a-specific memory B cells from several HPA-1a-immunized in pregnancy women. Various B cell markers, isolation methods and EBV transformation modes have been probed. The overview of these attempts presented in table bellow.

	Donor	Date of thawing PBMCs	Cell count	Number of cells sorted by MACS	Number of cells sorted by FACS	Detection of anti-HPA-1a Abs by MAIPA/ELISA*	selection of HPA-1a-producing B-lymphoblasts
1	**D18**	09.03.2009	$40x10^6$	$CD22^+$ $3x10^6$ cells	$CD22^+IgM^-IgA^-IgD^-$ 100,000 cells	27 BL cultures pos by MAIPA (6 strong pos)	Platelet-bound BL FACS and LDA, **Clone 26.4,** cells dividing slow with declining secretion of anti-HPA-1a Abs
2	**D19**	13.06.2010	$25x10^6$	$CD22^+$ 800,000 cells	$CD22^+IgM^-IgA^-IgD^-$ 100,000 cells	17 BL cultures pos by ELISA (5 strong pos)	Platelet-bound BL FACS and LDA; 1 clone pos by MAIPA, MAIPA and FC neg after a few days of culture
3	**D4**	12.08.2010	$40x10^6$	$CD22^+$ $2x10^6$cells	$CD22^+IgM^-IgA^-IgD^-$ 534,000 cells	33 BL cultures pos by ELISA (1 strong pos)	Platelet-bound BL FACS and LDA; 1 clone pos (D4BL14.31), MAIPA and FC neg after a few days of culture
4	**D19**	13.09.2010	$45x10^6$	$CD22^+$ $2,3x10^6$ cells; IgG^+ 600,000 cells	$CD27^+IgM^-IgA^-IgD^-$ 95,000 cells	2 BL cultures strong pos by MAIPA	Platelet-bound BL FACS and LDA; 2 clones pos, MAIPA and FC neg after a few days of culture
5	**D8**	14.10.2010	$6x10^6$	IgG^+70,000 cells, $GPIIbIIIa\text{-}FITC^+$ (few cells)	no FACS sorting	2 BL cultures strong pos by MAIPA	Platelet-bound BL FACS and LDA; 2 clones pos, MAIPA and FC neg after a few days of culture
6	**D8**	12.01 2011	$9x10^6$	$CD22^+$ 1,200,000cells	$IgM^-IgA^-IgD^-$ 60,000 cells	All BL cultures neg by MAIPA	-

7	**D8**	09.02.2011	$10x10^6$	IgG+ switched B cells, 100,000	no FACS sorting	All BL cultures neg by MAIPA	-
8	**D8**	10.03.2011	$9x10^6$	IgG+ switched B cells, 100,000	no FACS sorting	1 BL culture pos by MAIPA, neg after a few days of culture	-
9	**D8**	28.03.2011	$7x10^6$	$CD22^+$ 750,000 cells	CD22+ IgMAD- 120 wells CD22+IgG+ 60 wells	All BL cultures neg by MAIPA	-
10	**D8**	08.04.2011	$9x10^6$	CD22+700,000 cells, cultured 4 days with CpG and IL2 (up to $1x10^6$)	CD22+IgMAD-, 150,000 cells, culture in EBV sup 24h, wash, plate. No cultures!	All BL cultures neg by MAIPA	-
11	**D18**	15.08.2011	$25x10^6$	CD22+ 10^6 cells	$CD22^+IgM^-IgA^-IgD^-$ 40,000 cells	All BL cultures neg by MAIPA	-

MACS – magnetic-activated cell sorting; FACS – fluorescence activated cell sorting; LDA – limiting dilution assay; FC – flow cytometry; BL – B-lymphoblastoid cells; pos – positive; neg – negative.
*In-house ELISA assay has been developed for screening of BL cultures for anti-HPA-1a IgG antibodies.

Supplemental data II

Functional characterization of the mAb 26.4 IgG1 and IgG3

Mariana Eksteen and Tor B. Stuge

Aim

To test the capacity of the mAb 26.4, IgG1 and IgG3, to induce antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).

Materials and Methods

CDC and ADCC

The CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Madison, WI) was used to analyze the capacity of mAb 26.4, IgG1 and IgG3 to induce ADCC and CDC *in vitro.* The assay quantitatively measures release of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis. Chinese hamster ovary (CHO) cells expressing integrin β3 (as part of the αIIbβ3 heterodimer) were used as target cells (kindly provided by Prof. Sentot Santoso, Institute of Clinical Immunology and Transfusion medicine, Giessen, Germany). Human IgG1 and IgG3 of irrelevant specificities were used as assay negative controls (I 5154 and I 5654 respectively, Sigma). Pooled IgG isolated from plasma of HPA-1a immunized women was probed as assay positive control.

For ADCC assay, NK cells were isolated from donor PBMCs using NK cell isolation kit and LS columns (Miltenyi Biotec, Germany). Isolated NK cells were incubated with CHO cells at effector/target (E/T) ratio 5/1 in presence of antibodies for 4 h at 37 °C, 5% CO_2 humidified atmosphere. For CDC assay, human serum was used as a source of complement. Blood from a healthy HPA-1a-positive volunteer was allowed to clot at room temperature for 60 min and then centrifuged at 900*g* for 20 min. Serum was harvested and then used either fresh or stored at -80°C. Serum inactivated at 56 ^{o}C for 20 min was used as a negative control. Target cells were seeded at 2 x 10^4 cells per well in triplicate sets in a round-bottom 96-well culture plate and incubated with 30% of human serum in presence of antibodies for 2 hours. To determine the

release of LDH the absorbance was recorded at 492 nm. The absorbance values were corrected and the results calculated according to the manufacturer's instructions.

Results and discussion

mAb 26.4, IgG1 and IgG3, did not activate NK cells or complement, neither did pooled IgG from HPA-1a immunized women (Figures 1 and 2). An assay positive control was lacking – a human mAb specific for platelet antigen that is known to induce ADCC and CDC. Without such a control the results are not reliable.

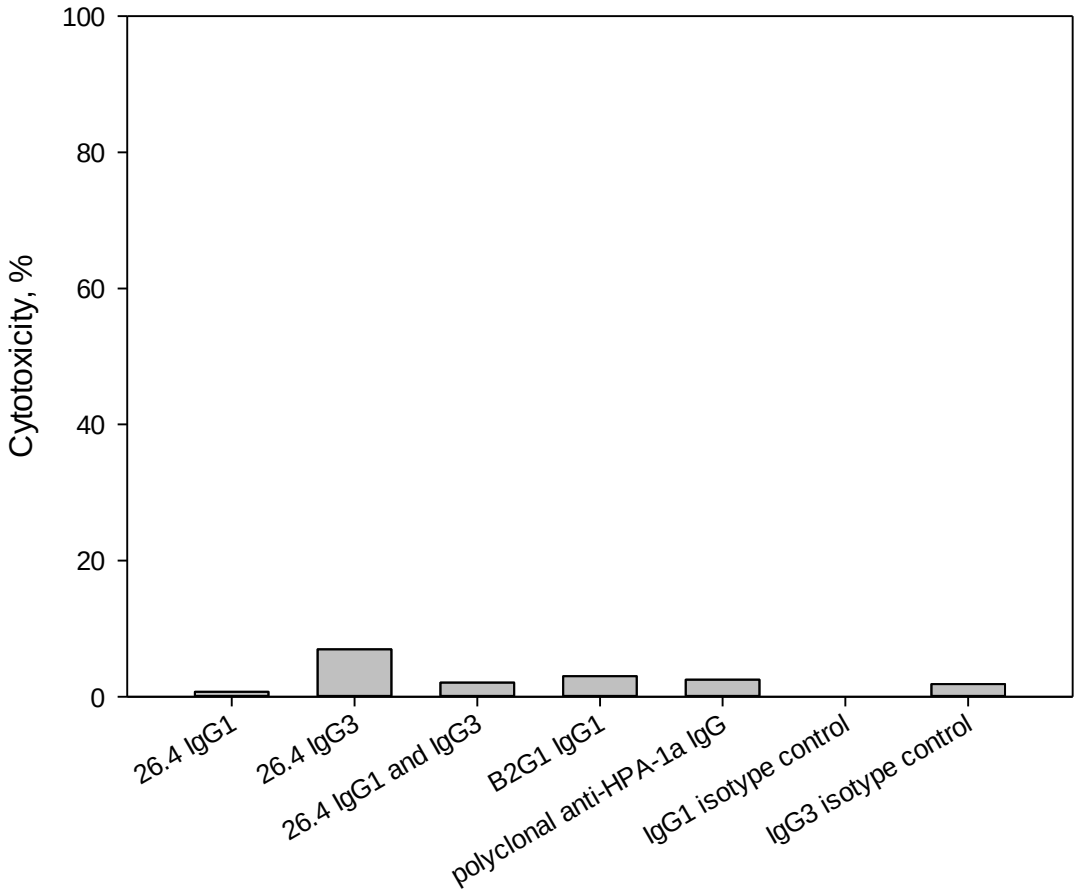

Figure 1. CDC results. CHO cells were incubated with various concentrations of 26.4 (0.01, 0.1, 1 and 10 µg/ml) followed by addition of human serum as a source of complement, and incubated for 2h. CDC activity was determined by measuring released LDH. Only data with the highest 26.4 concentration shown.

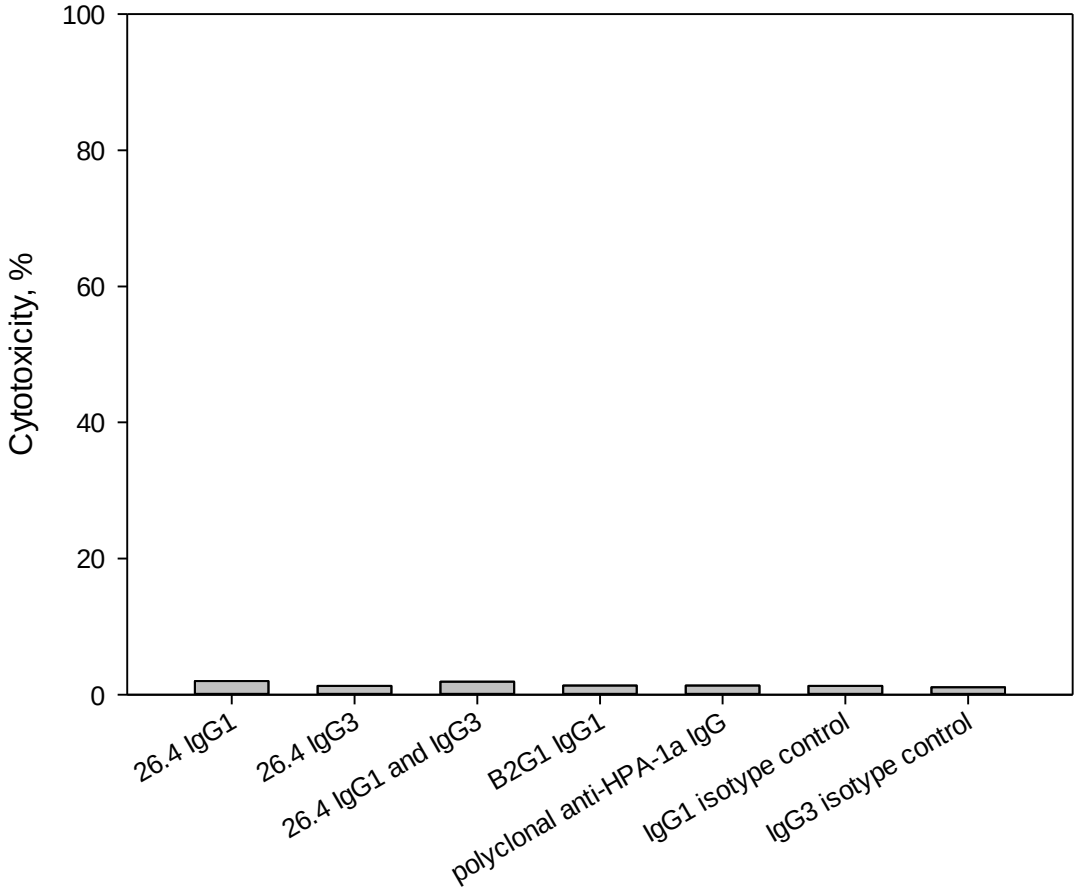

Figure 2. ADCC results. CHO cells were incubated with various concentrations of 26.4 followed by addition of NK cells at E/T ratio 10/1, and were incubated for 4 h. ADCC activity was determined by measuring released LDH. Only data with the highest 26.4 concentration shown.

Supplemental data III

mAb 26.4 epitope characterization

Mariana Eksteen and Tor B. Stuge

Aim

To test whether 26.4 epitope is constraint to PSI domain or extends to several domains of integrin β3. For this purpose, the domain-deletion peptide ELISA technique has been employed.

Materials

Antibodies

Generation and characterization of mAb 26.4 has been described previously (1). The following mAbs were used as controls: integrin β3-specific murine mAbs, clones Y2/51 (Beckman Coulter, Pasadena, CA) and SZ21 (Dako, Glostrup, Denmark); integrin αIIb-specific mAb clone SZ22 (Beckman Coulter, Pasadena, CA); human mAb specific to HPA-1a, clone B2G1, isolated from maternal B cells of a case of FNAIT using phage display (2) and produced recombinantly (3) (kindly provided by Cedric Ghevaert, Department of Hematology, School of Clinical Medicine, University of Cambridge, UK). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-human IgG (*Jackson ImmunoResearch* Laboratories, West Grove, PA) were used as secondary antibodies.

Recombinant domain-deletion peptides

The following peptides were used: ΔβA-Leu33, ΔβA-Pro33, PSI-Leu33, and GPVI (hDID2) as a negative control (peptides kindly provided by Rosey Mushens, International Blood Group Reference Laboratory, NHS Blood and Transplant, Filton, Bristol, UK; Winnie Chong, Department of Histocompatibility and Immunogenetics, NHS Blood and Transplant, Colindale Avenue, London, UK; Willem H Ouwehand, University of Cambridge & Wellcome Trust Sanger Institute, NHS Blood and Transplant, UK). Cloning, expression and purification of the recombinant domain-deletion peptides with calmodulin (CaM) tag was described previously (4). CaM-binding peptide N9A coupled to BSA was kindly provided by Peter Smethurst and

Nicola Foad (Department of Haematology, University of Cambridge, NHS Blood and Transplant Centre, Long Road, Cambridge CB2 0PT, UK).

Methods

Integrin β3 domain-deletion peptide ELISA was performed as reported previously (5). Briefly, the β3 peptides were immobilized to ELISA plates via CaM-binding peptide N9A coupled to BSA (6). Murine and human mAbs were used at concentrations of 1 and 10 µg/ml. MAb binding was detected by HRP-conjugated goat-anti-mouse IgG or HRP-conjugated goat-anti-human IgG. Absorbance at 492 nm was read on a microplate photometer *(*Multiskan EX, Thermo Scientific, Waltham, MA). Each sample was tested in duplicate and average absorbance values were used to generate the graphs (Figure 1 and 2). GraphPad Prism 5 software (San Diego, CA) was used to analyze and present the data.

Results

Binding of the murine mAbs, clones Y2/51 and SZ21, to domain-deletion peptides was used as an assay control. MAb Y2/51 at concentrations of 1 and 10 µg/ml bound the multi-domain peptide ΔβA, Leu33 and Pro33 variants (Figure 1). mAb SZ21 at 1 µg/ml bound to ΔβA-Leu33, and binding to ΔβA-Pro33 and PSI-Leu33 generated relatively low response. mAb SZ21 at 10 µg/ml bound multi-domain peptides ΔβA, independently on Leu33 or Pro33 variant, as well as a single-domain peptide PSI-Leu33. None of the mAbs bound to the control peptide GPVI (hDID2). These results are consistent with the results published previously (4). mAb SZ22 (specific to αIIb, CD41) was used as a murine mAb negative control and did not bind neither of the peptides (data not shown).

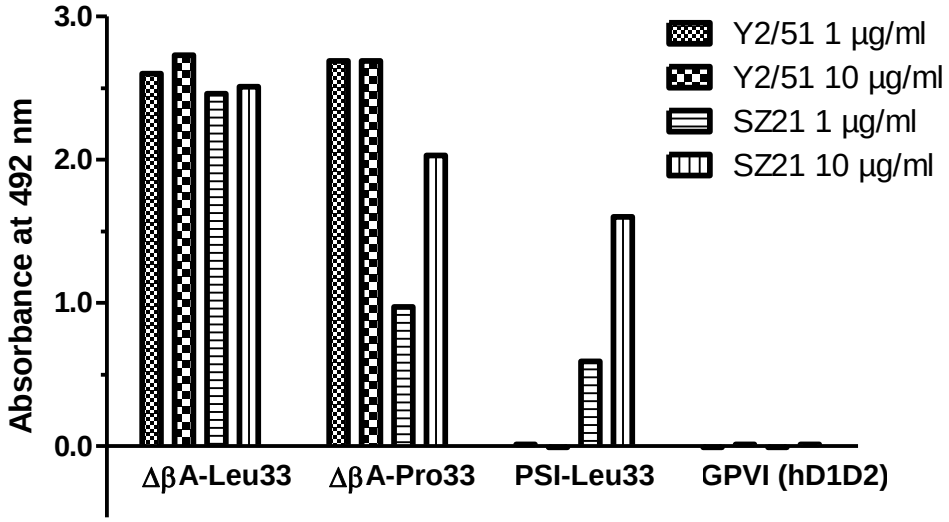

Figure 1. Reactivity of murine mAbs specific to integrin β3 with recombinant integrin β3 domain-deletion peptides analyzed by ELISA. Representative of the two independent experiments.

mAb 26.4 bound exclusively to the multi-domain peptide ΔβA-Leu33; no binding to the ΔβA-Pro33, single-domain peptide PSI-Leu33 or peptide negative control was observed (Figure 2). mAb B2G1 had an identical binding pattern, consistent with the previously published results (4).

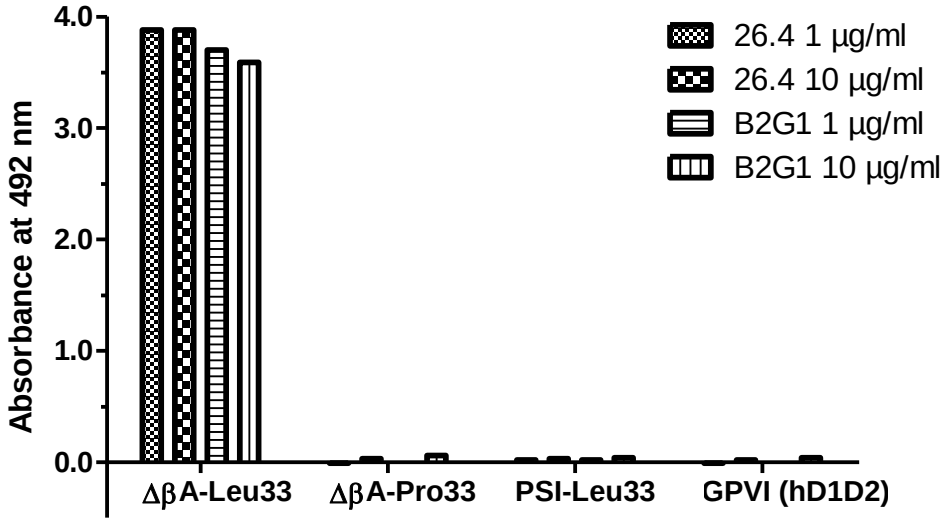

Figure 2. Reactivity of human mAbs specific to HPA-1a with recombinant integrin β3 domain-deletion peptides analyzed by ELISA. Representative of two independent experiments.

Conclusion

The results suggest that the mAb 26.4 epitope in not constraint to PSI domain, but extends to several domains of integrin β3.

References

1. Eksteen M, Tiller H, Averina M, Heide G, Kjaer M, Ghevaert C, Michaelsen TE, Ihle O, Husebekk A, Skogen B, Stuge TB. 2015. Characterization of a human platelet antigen-1a-specific monoclonal antibody derived from a B cell from a woman alloimmunized in pregnancy. *J Immunol* 194: 5751-60
2. Griffin H, Ouwehand W. 1995. A human monoclonal antibody specific for the leucine-33 (P1A1, HPA-1a) form of platelet glycoprotein IIIa from a V gene phage display library. *Blood* 86: 4430-6
3. Garner, Smethurst, Merieux, Aeby, Smith, Armour, Scott, Williamson, Metcalfe, Goodall, Clark, Rigal, Schawaller, Ouwehand. 2000. A rapid one-stage whole-blood HPA-1a phenotyping assay using a recombinant monoclonal IgG1 anti-HPA-1a. *British Journal of Haematology* 108: 440-7
4. Stafford P, Ghevaert CJG, Campbell K, Proulx C, Smith G, Williamson LM, Ranasinghe E, Watkins NA, Huntington JA, Ouwehand WH. 2008. Immunologic and structural analysis of eight novel domain-deletion β 3 integrin peptides designed for detection of HPA-1 antibodies. *Journal of Thrombosis and Haemostasis* 6: 366-75
5. Abou-Chaker K, Meyer O, Salama A. 2009. Rapid typing of the human neutrophil antigen 1a by the particle gel agglutination assay. *Tissue Antigens* 73: 242-4
6. Smethurst PA, Joutsi-Korhonen L, O'Connor MN, Wilson E, Jennings NS, Garner SF, Zhang Y, Knight CG, Dafforn TR, Buckle A, MJ IJ, De Groot PG, Watkins NA, Farndale RW, Ouwehand WH. 2004. Identification of the primary collagen-binding surface on human glycoprotein VI by site-directed mutagenesis and by a blocking phage antibody. *Blood* 103: 903-11

Supplemental data IV

Molecular modeling of the mAb 26.4 Ig variable region and protein-protein docking

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Aim

To predict the mAb 26.4 paratope and epitope on integrin β3 using available software.

Materials and Methods

The antigen

Subunit β3 of the αIIβb3 integrin (HPA-1a; L33). Structure of complete ectodomain of integrin αIIbβ3 is available in Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do, code: 3FCS).

The antibody

Human mAb specific for HPA-1a, clone 26.4 (1).

Software used for docking

Internal Coordinate Mechanics (ICM) software (MolSoft L.L.C., San Diego, CA)

Results and discussion

Prediction of antibody variable domains structure

Two models based on the canonical structure method were constructed using the PIGS web server (prediction of immunoglobulin structure, http://biocomputing.it/pigs/, (2)). The modeling of extremely long H3 loops, such as the one present in this antibody, is in general unreliable. Such loops might as well undergo to conformational changes upon antigen binding. We therefore decided to produce two versions of the models, one complete with all the loops, the second containing only the stems of the H3 loop proximal to the antibody framework. In the

latter case, we would reconstruct the whole loop after a preliminary docking of the partial model with the antigen. Using the proABC web server (3) the probability of each residue of the antibody to be in contact with the antigen was predicted.

Protein-protein docking

Selection of interaction regions. The information on the antibody residues in possible contact with the antigen was used when setting up the docking project, but the docking results showed that the software did not follow the constraints; in many cases, the loop was not even in contact with the antigen.

Protein flexibility. Two step protein-protein docking protocol in ICM: 1) Rigid body-docking simulations, 2) Side-chain refinement of ligand-binding residues. Due to the high degrees of flexibility in proteins, the proteins were kept rigid during docking (it is computationally unfeasible to keep them flexible). Only the first step was performed due to the lack of complexes worth refinement.

Future work

X-ray crystallization of the mAb26.4 Fab part and antibody-antigen complex will be probed.

References

1. Eksteen M, Tiller H, Averina M, Heide G, Kjaer M, Ghevaert C, Michaelsen TE, Ihle O, Husebekk A, Skogen B, Stuge TB. 2015. Characterization of a human platelet antigen-1a-specific monoclonal antibody derived from a B cell from a woman alloimmunized in pregnancy. *J Immunol* 194: 5751-60
2. Marcatili P, Rosi A, Tramontano A. 2008. PIGS: automatic prediction of antibody structures. *Bioinformatics* 24: 1953-4
3. Olimpieri PP, Chailyan A, Tramontano A, Marcatili P. 2013. Prediction of site-specific interactions in antibody-antigen complexes: the proABC method and server. *Bioinformatics* 29: 2285-91