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Human platelet antigen (HPA)-1a alloimmunization – Why only blame it on the platelets?

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Preface and acknowledgement

The work presented here is performed at the Immunology Research Group, Department of Medical Biology, UiT, the Arctic University of Norway, Tromsø. The work was started during medical school, and was continued from January 2013 to September 2014, and from September 2015 to April 2016, and last period from September 2019 to May 2020. Two periods were financed by Helse Nord. The last period was supported financially by Forskerlinja at the UiT, and has primarily been used to write and publish.

I honestly do not know why I started the research line of medical school. I did not know anything about research, I was not creative, I did never think new clever thoughts, and I had never been working in a lab. However, I have always wanted to do more. Maybe that is the reason. Immunology was also difficult to get a hand on in early medical school. All these different cells, the cytokines, the selection, the HLA; it was a mess. Starting a project in immunology I hoped to finally understand this seemingly inaccessible field of medicine and biology. Now, after all this time, I think I will never grasp just a bit of it. Despite the lack of understanding, however, it`s been real fun! And I also think that my work and my time in the Immunology research group in some way will make me a better clinical doctor. Thank you, Anne, for being that lecturer in medical school that made this field exciting. And thank you for always being that positive and encouraging, Anne. Thank you, Tor, for your extraordinary patience, your kindness, your everlasting high spirit, and for all clever thoughts and ideas; always an answer or a new question.
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II. Ahlen MT, Heide G, Husebekk A, Skogen BR, Kjeldsen-Kragh, Stuge TB. *The prevalence of HPA-1a alloimmunization and the potential risk of FNAIT depends on both the DRB3*01:01 allele and associated DR-DQ haplotypes. Scand J Immunol 2020*;92:e12890. doi:10.1111/sji.12890. Shared first authorship, Ahlen and Heide contributed equally to this publication.

III. Heide G, Stuge TB, Skogen BR, Husebekk A, Ahlen MT *The DR7-DQ2 haplotype in a native Norwegian population. Scand J Immunol 2013 May; 77(5):429.* (Letter to the editor). doi: 10.1111/sji.12031

IV. 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 and may have significance for placenta development: A laboratory study using an in vitro model. Reprod Biol Endocinol, 2017 Apr 21;15(1):28*. doi: 10.1186/s12958-017-0245-6

Abbreviations

AH	Ancestral Haplotype
AMIS	Antibody-mediated Immune suppression
APC	Antigen-presenting cell
CD	Cluster of differentiation
CTB	Cytotrophoblast
DC	Dendritic cell
ECM	Extracellular matrix
EV	Extracellular vesicle
EVT	Extravillous trophoblast
FcRn	Neonatal Fc receptor
FNAIT	Fetal and Neonatal Alloimmune Thrombocytopenia
GP	Glycoprotein
GVHD	Graft Versus Host Disease
HDFN	Hemolytic Disease of the Fetus and Newborn
HLA	Human leukocyte antigen
HPA	Human platelet antigen
ICH	Intracranial hemorrhage
IFN	Interferon
IL	Interleukin
ITG	Integrin
ISBT	International Society of Blood Transfusion
IVIg	Intravenous Immunoglobulin
KIR	Killer-cell Immunoglobulin-like Receptor

LILRB	Leukocyte immunoglobulin-like receptor subfamily B member 1
mHAgs	minor Histocompatibility Antigens
MHC	Major Histocompatibility complex
MP	Microparticle
MV	Microvesicle
NBMDR	Norwegian Bone Marrow Donor Registry
NK	Natural Killer Cell
RBC	Red Blood Cell
Rh D	Rhesus D
SMC	Smooth Muscle Cell
SNP	Single Nucleotide Protein
ST	Syncytiotrophoblast
TNF	Tumor Necrosis Factor
TPH	Transplacental Hemorrhage
TRALI	Transfusion-related acute lung injury
uNKc	uterine Natural Killer cell
vWF	von Willebrand Factor

Thesis summary

Pregnancy is an immunological paradox in which the semi-allogenic fetus is not rejected by the mother's immune system. Immunization against the Human platelet antigen (HPA)-1a antigen takes place in 1:1,000 pregnancies, as a break of this tolerance. The HPA-1a antigen is a platelet antigen, and platelets have naturally been the subject of focus regarding both the cause and the effect of HPA-1a alloimmunization. HPA-1a immunization in pregnancy has been seen as a cause of feto-maternal transfer of platelets during pregnancy and at delivery and the consequences of thrombocytopenia have been seen as an effect of anti-HPA-1a antibodies. However, it is known that the HPA-1a antigen is not platelet specific and that the antigen is expressed also in fetal cells in the placenta and in fetal endothelium. Placental debris is shed into the maternal circulation due to placental turnover during pregnancy, so HPA-1a in the placenta is a potential source of antigen for alloimmunization. In addition, HPA-1a in both the placenta and in the endothelium of the fetus are potential places for the effect of anti-HPA-1a-antibodies. In recent years, there has been increasing focus on the potential differences in anti-HPA-1a antibodies against the β3 integrin itself and the β3 integrin in complex with αV or αIIβ. The differences in specificities of anti-HPA-1a antibody antibodies made in HPA-1a negative women may reflect differences in antigen source. We show here that HPA-1a-specific T cells are activated by antigen associated with αVβ3 from trophoblast in the same way as antigen associated with αIIbβ3 from platelets and that the HPA-1a antigen may be found in plasma of HPA-1a negative women pregnant with an HPA-1a positive fetus in the form of extracellular vesicles and that these vesicles may be of placental origin, suggesting that the source of antigen in HPA-1a immunization also may be placental extracellular vesicles. We also show that the anti-HPA-1a antibody 26.4 binds αVβ3 integrin and affects adhesion and migration in a trophoblast cell line.

The production of IgG anti-HPA-1a antibodies is dependent on a T cell response, which in turn is dependent on presentation of the antigen in a major histocompatibility complex (MHC), and the DRA/DBR3*0101 MHC class II molecule has been shown to present HPA-1a antigen to T cells. In addition, several other human leukocyte antigen (HLA) alleles have been suggested or shown to be associated with immunization, albeit in conflicting reports, and the close linkage between genes in the MHC complex makes it difficult to interpret results on single alleles. We aimed to more precisely define which alleles have a role in HPA-1a immunization and here find that the prevalence of HPA-1a immunization is higher when the *DRB3*01:01* allele is associated with the DR3-DQ2 haplotype and that the *DQB1*02* allele may also play a role.

There is a further need to more precisely select women most at risk of immunization for both follow up and treatment. Both the definition of risk factors in women and the knowledge of when and how the immunization occurs – which our work contributes to –are important for follow up and to decide how and when to treat with potential prophylaxis.

Introduction

Fetal and neonatal alloimmune thrombocytopenia: an overview

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition in which fetal and neonatal platelets are depleted as a cause of anti-platelet antibodies made by the mother during pregnancy. The antibodies are produced because of differences in platelet antigen type between the mother and the fetus. Antibodies cross the placenta and destroy the baby's platelets, making the baby thrombocytopenic and at risk of bleeding. Common clinical signs are skin bleeding with petechias and ecchymosis. A more concerning and rarer clinical consequence is, however, intracranial haemorrhage (ICH), with possible severe sequela, including death.

In 75–85% of the cases in Caucasians, FNAIT is caused by a difference in the human platelet antigen (HPA)-1 system between the mother and the fetus and the production of anti-HPA-1a antibodies by the mother (Figure 1) [1-3]. HPA-1a alloimmunization is the most common cause of both severe thrombocytopenia in fetuses and newborns and ICH in term newborns [4]. The HPA-1 system is defined by a leucine/proline polymorphism in position 33 of the β3 integrin (GPIIIa) [5] which makes a heterodimer together with αIIb (GP IIb) on platelets, forming the fibrinogen receptor. The β3 integrin is, however, also found on other cell types – for example, together with αV, forming the vitronectin receptor [6, 7].

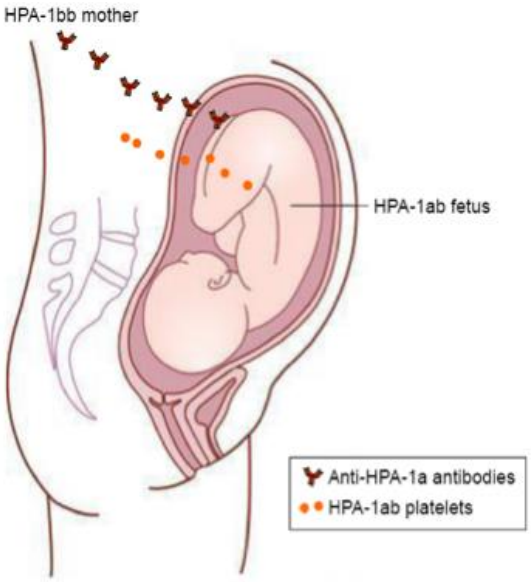

Figure 1. Overview of the pathomechanism in HPA-1a alloimmunization during pregnancy

Two percent of Norwegian pregnant women are HPA-1a negative and thereby at risk of being immunized during pregnancy with an HPA-1a positive fetus [8]. About 10 % of HPA-1a negative women develop anti-HPA-1a antibodies [8-11], and the making of antibodies does not necessary lead to thrombocytopenia in the fetus. FNAIT caused by HPA-1a antibodies is found in 1:1,000-1,200 newborns [8, 10]. ICH is reported to occur in some FNAIT cases due to anti-HPA 1a antibodies [8, 9, 12], and most of those cases already occur in utero [13, 14]. HPA-1a immunization is also associated with reduced birth weight in baby boys [15]. There is currently no screening for the condition, and the condition is underdiagnosed in routine clinical practice [16]. In addition, there is currently neither specific treatment nor prophylaxis for the condition. The ongoing treatments available are general and differ between countries. Without any treatment, the thrombocytopenia resolves within some weeks after birth [17].

Immunization is clearly associated with the HLA allele *DRB3*01:01* [8, 10, 18]. The molecular mechanism of this association is now also well known: the HPA-1a peptide fits well into the peptide-binding groove of HLA-DRA/DRB3*0101 and is presented to HPA-1a specific T cells [19-21]. Also, other HLA alleles are proposed to be associated with immunization, albeit without explanation beyond a statistical allele association.

In this thesis, I will argue that, despite recent advances, our understanding of the pathogenesis of FNAIT remains limited, and there are several missing links.

The making of the placenta and the role of β3 integrin

The haemochorial placenta is formed when fetal-derived cells (trophoblast) invade a modified layer of the maternal uterus, the decidua, in early pregnancy (Figure 2) [22]. Placenta is a temporary but highly specialized organ and is responsible for the normal, or physiological, exchange between the developing fetus and the mother during pregnancy. The villous trophoblasts line the villi in the placenta with the inner layer of cytotrophoblasts (CTB) and the outer layer of syncytiotrophoblasts (ST). Placenta represents the closest proximity of the maternal and fetal circulations [23], and at term, the ST line an area of about 11–13 m^2 in a normal placenta [24]. A subset of cytotrophoblasts (CT) in anchoring villi aggregate into cell columns that attach to the uterine wall and invade the uterine wall (extravillous cytotrophoblast, EVT) and its blood vessels (endovascular cytotrophoblast) as far as the first third of the myometrium [25]. Endovascular trophoblast cells displace both the endothelial cells lining the artery and the smooth muscle cells (SMCs). Spiral

arteries are maternal arteries that supply the placenta with blood, and remodelling of these arteries is necessary for an adequate blood supply to the placenta. The replacement of SMCs makes a low–resistance system allowing continued blood flow to the placenta and the fetus.

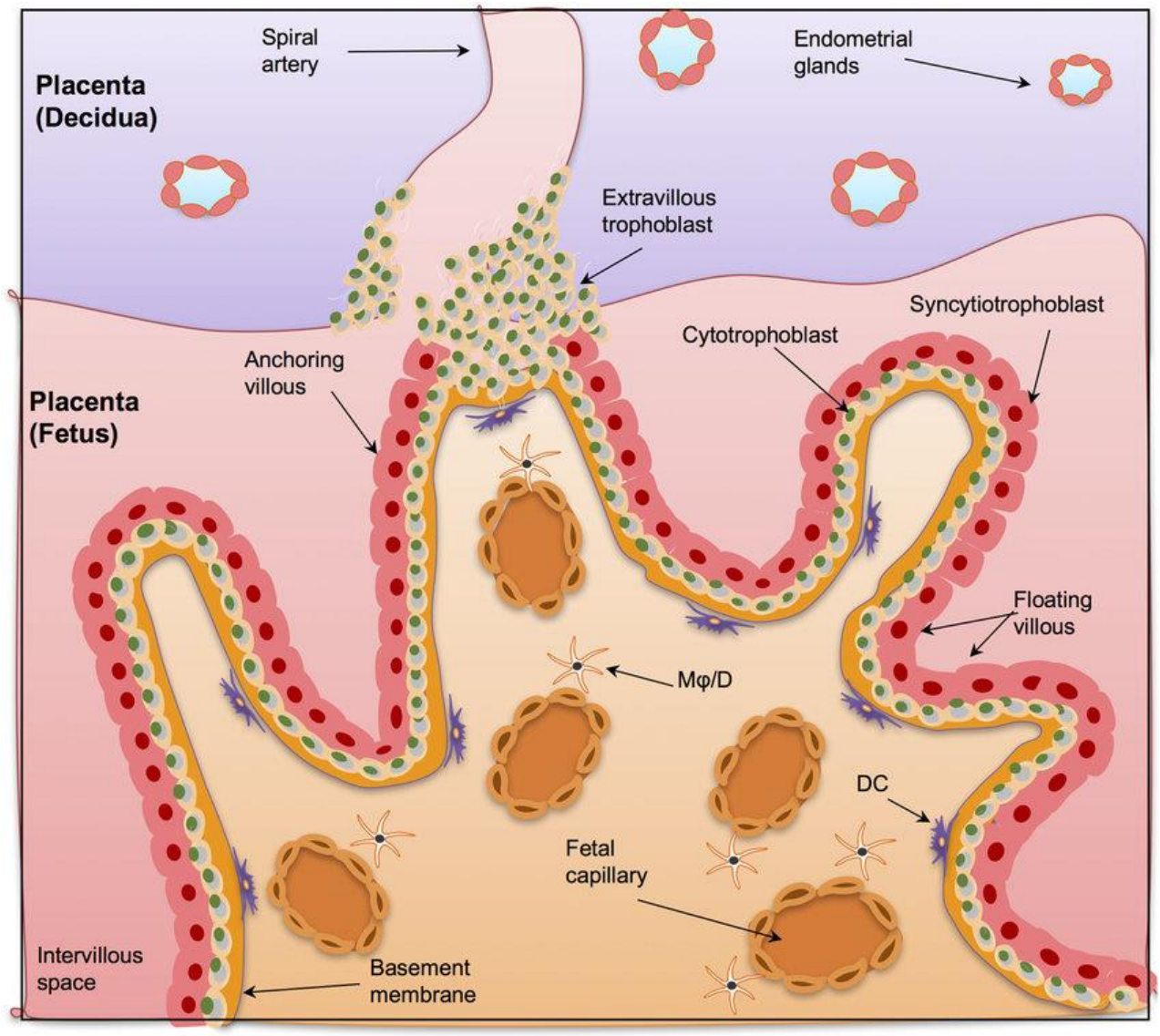

Figure 2. Graphic presentation of the feto-maternal interface. Mφ/D = macrophage/dendritic cells; DC = dendritic cells. The figure is reprinted with permission from Leòn-Juàrez et al., 2017 [26], doi: 10.1093/femspd/ftx093.

Bowen and Hunt (2000) [27] present a review with focus on integrins and their presence and function from fertilization, at implantation, and during placental development. The integrin most of interest to describe here is one involving the β3 integrin: the vitronectin (αVβ3) receptor. Human oocytes express β3 integrin, and in the very earliest phase of implantation, αVβ3 is released from spermatozoa after the acrosome reaction, and the released vitronectin further promotes the

attachment between sperm and egg and sperm aggregation. An anti-αVβ3 antibody with blocking characteristics, however, does not affect sperm-egg binding [27]. αVβ3 is also one of the receptors considered to be particularly important during the initial process of implantation [27] [28]. Human uterine epithelium express αVβ3, and the expression rises with the rise in progesterone during the window of implantation [27]. There is naturally little knowledge about the in vivo integrin expression by human trophoblast cells at the time of implantation. Examination of mouse trophectoderm, however, showed that it expresses αVβ3 during the initial stages of apposition and adhesion [27]. Exposure of hormones and cytokines during pregnancy may influence both the expression and activation of the αVβ3 integrin. The activation of αVβ3 integrin was, for example, reduced in response to IFNγ in endothelial cells, and oestrogen both with and without progesterone downregulated the integrin in an endometrial adenocarcinoma cell line [29].

Cells at the terminal end of the trophoblastic column express the αVβ3 integrin, which may facilitate invasion and development of the placenta. Primary CTs that mediate invasion through Matrigel and collagen need the αVβ3 and α1β1 to promote migration [30], and αVβ3 and -β1 integrins also mediate adhesion of human CT to endothelial cells in vitro, suggesting that these cells may have similar effects on endovascular trophoblast adhesion and migration within the uterine arteries in vivo [30]. αVβ3 integrin is even enhanced on CT that has invaded the uterine wall and maternal vasculature [25].

Other indications of the importance of αVβ3 in placentation are that lack of expression of β3 in the human epithelium has been associated with unexplained infertility and that in preeclampsia the upregulated expression of integrin subunits α1β1 or αVβ3 in cytotrophoblasts does not occur. Whether this is a cause or an effect of preeclampsia, however, is not known [27].

Alloimmune responses

Both the fetus and the placenta may be described as allografts, and because the fetus may be an allograft to the mother, pregnancy could be seen as a state of transplantation. Adaptive immune responses are the most important threat to the success of transplantation of a grafted tissue or organ between individuals. Transplants between allogeneic individuals are rejected because of T cell responses in the recipient against the polymorphic MHC molecules on the transplant, where differences in these major alloantigens can cause acute rejection due to direct recognition by host T cells. However, rejection may also appear in MHC-identical grafts as a cause of differences in other polymorphic antigen systems: minor histocompatibility antigens (mHAgs). mHAgs may pose a

barrier to transplantation when they are presented together with specific class I and class II MHC molecules. Such antigens may cause rejection of the allograft, both acute and chronic, as well as graft-versus-host disease (GVHD) [31].

HPA-1a immunization is an allo-response, and "immunization" refers to the production of anti-HPA-1a antibodies. The potential harmful effect the condition has on the fetus is caused by the antibodies. However, the production of these antibodies in HPA-1a negative women is also dependent on a T helper cell response to the same antigen, which is further dependent on the presentation of the antigen in a specific MHC-complex. This is a key event in the immune response that results in IgG production.

Another well-known situation in which alloimmune responses may arise, which also resembles the situation in HPA-1a alloimmunization, is through transfusion of red blood cells, which may cause alloimmune responses against antigens in the AB0 or Rhesus (Rh) system. The human Rh locus consists of two related genes, encoding the D, Cc, and Ee blood group antigens. D is the major cause of Rh incompatibility, and it is estimated that 15–17% of Caucasians do not express the D antigen and are hence Rh negative [32] and at risk of immunization when exposed to the D antigen. The D antigen is strongly immunogenic [32]. Despite that, however, the incidence of primary RhD immunization also depends on the dose of RhD-positive red cells. Fifteen percent of women at risk will show a primary immune response against the D antigen after the intravenous delivery of 1 mL RhD-positive red cells. A secondary immune response, however, may occur after exposure of as little as 0.03 mL RhD positive red cells [32]. As a consequence, the disease only develops when the RhD negative mother has had a significant transplacental haemorrhage (TPH). The average TPH occurring at delivery is, however, less than 1 mL of whole blood [32]. During pregnancy, small fetal bleeds may lead to sensitization, followed by a larger volume, leading to immunization, at delivery [32-34]. An antibody-based prophylaxis against Rhesus immunization has been available for several years (1965–1970). Early studies of the vaccine effect the AMIS effect) more than 50 years before the description of the anti-D effect found that the antigen-specific IgG antibody can suppress the antibody response to particulate antigens. Despite both the overall use and obvious effect of this vaccine – the immunomodulatory effect it induces is not well described. However, several hypotheses exist [35].

Alloantigens

Alloantigens are antigens presented only in some individuals of a species that are capable of inducing the production of alloantibodies by individuals who lack the antigen. The polymorphic MHC antigens responsible for transplant rejection, encoded within the MHC complex, have already been mentioned as such alloantigens. Other possible alloantigens are the human platelet antigens and other mHAgs. T cells specific for different fetal mHAgs [31, 36] can be found in multiparous women.

Human platelet antigens

Human "platelet alloantigens are defined by alloantibodies directed against genetically determined molecular variations of proteins or carbohydrates on the platelet membrane" [37]. The human platelet alloantigens can be divided into two groups: type I platelet alloantigens that consist of antigen systems also found on other cell and tissue types – for example, MHC molecules – and type II platelet alloantigens that consist of platelet specific antigens [37]. Platelet-specific alloantigens are defined as all protein alloantigens expressed on the platelet membrane except those encoded by genes of the MHC [38]. However, many previously considered platelet specific antigens are also detected on other cells and tissues and are not platelet specific.

The human platelet antigen nomenclature system was adapted in 1990 by the ISBT platelet working party [38] and aims to categorize human platelet specific protein alloantigens (HPAs): HPAs are formed due to genetic cell variants of glycoproteins on the platelet surface [39]. The antigen system is numbered in order of discovery with the higher frequency antigen designated "a" and the lower frequency antigen designated "b" [40]. In almost all of the serologically defined antigens, the difference between high and low frequency alleles is defined by a single amino acid substitution [37]. Twelve antigens are clustered into six bi-allelic groups [39]. For the six bi-allelic HPA systems, SNP typing on large numbers of DNA samples has also provided information on allele frequencies, with significant differences occurring between populations.

To date, a total of 35 HPA antigens have been designated in 29 systems (HPA 1-29) [40-47], detected on six different glycoproteins (GPs): ITGB3, ITGA2B, ITGA2, GPIBA, GP1BB, and CD109 (https://www.versiti.org/medical-professionals/precision-medicine-expertise/platelet-antigen-database#hpa-database) that may cause alloimmune complications. For the current list, see http://www.ebi.ac.uk/ipd/hpa/table1.html. Most of the defined HPA systems are present on the

αIIbβ3 integrin (fibrinogen receptor) [48]. Alloimmunization in pregnancy against recognized but low-frequency HPAs, not resolved by standard typing, accounts for only a few FNAIT cases [39].

Placenta antigens

mHAgs are also expressed in syncytiotrophoblast and in trophoblast debris shed from the placenta [49]. Hence, the placenta may be a source of mHAgs exposed to the mother during pregnancy.

Integrin β3 is, in addition to being part of the fibrinogen receptor (αIIbβ3), also part of the vitronectin receptor (αVβ3) found on different cells types, including trophoblast cells [7, 30, 50, 51]. In the placenta of week 10–18, the β3 integrin is found in distal columns CT, interstitial placental bed, endovascular placental bed, and the maternal endothelium [25]. In sections of second trimester (18–22 weeks) placenta, αVβ3 was weakly expressed or not detected by antibody staining on villous CT or in the initial layers of cell columns. However, strong staining for αVβ3 was detected on CT within the uterine wall and in uterine vasculature. In addition, β3 integrin has been detected on placental syncytiotrophoblast microvilli [50-52].

Immunology of the placenta

Pregnancy is an immunological paradox in which the semi-allogenic transplant – the fetus – is not rejected by the immune system of the mother. The mother tolerates the fetus and lets it develop for 9 months despite the fact that she is naïve to its paternally derived antigens, is not immunosuppressed and is still likely capable of making a response to invading pathogens.

Medawar, in 1953, postulated three general mechanisms which make the uterus an immune-privileged site during pregnancy: the induction of tolerance in the mother for paternal derived antigens, the construction of an anatomical and physiological barrier between the mother and the fetus, and a reduced expression of alloantigens by fetal cells [53]. However, these three mechanisms have later been challenged.

The anatomical arrangement of the haemochorial placenta gives many possibilities for exposure of maternal blood and immune cells to trophoblast cells, trophoblast debris and fetal cells, and their antigens [54]. As described, the area of ST in a normal term placenta in contact with maternal blood is large [55], and migration of extracellular trophoblast proceeds deep into the myometrium of the mother. Fetal cells are exposed to the maternal circulation and thereby to the maternal peripheral

leucocytes through both extravillous trophoblast in contact with maternal tissue and blood and villous ST in contact with maternal blood.

The expression of paternal MHC on the surface of trophoblast cells could, in theory, predispose the placenta to serve as a target of an anti-fetal allo-response. However, both ST and EVT cells lack HLA class II [56] and classical HLA class I A and B on the cell surface [57, 58] and are thereby in a way protected against an immune response against foreign HLA polymorphisms. EVTs do still express the class I protein HLA-C, which in principle is polymorphic. HLA-C in the placenta, however, has reduced polymorphism compared to other class I molecules and is also only weakly expressed on EVTs [59].

The lack of a noticeable immune response to MHC incompatibility between mother and fetus, described above, could be explained by the lack of MHC class II expression and the low MHC class I expression in areas accessed by the maternal blood. Despite this, however, pregnancy can elicit lymphocyte responses to both major and minor histocompatibility antigens from the fetus [54]. HLA class I has been shown to be expressed on cells in the villous stroma, and Hofbauer cells, which are placental villous macrophages of fetal origin, also exhibit MHC class II proteins [54]. Furthermore, the lack of class II on trophoblast cells is challenged by Ranella et al. (2005) who report expression of both HLA-DR and HLA-DO intracellularly in trophoblasts [60]. In sum, there are several potential alloantigens available for the mother's immune system to react to: class I and II from fetus and villous stromal cells; HLA-C from EVTs; or minor antigens from trophoblast, villous stromal cells, or fetal cells that travers the placenta during pregnancy (microchimerism) [54]. "There is no longer doubt that maternal T and B cells are aware of fetal antigens, that they respond to the presence of the fetus, and that under normal circumstances they are tolerant to these antigens" [61]. The question, then, is no longer whether or not the mother is "aware" [62] of fetal allo-antigens, but what the cellular and molecular events leading to acceptance of the fetus are, regardless of their expression. In that respect, several mechanisms have been suggested to be of importance for the acceptance of the fetus, some of which are briefly explained in the following paragraphs.

Instead of the classical HLA class I, EVTs express HLA-G. For HLA-G, there are 50 alleles (per 2013). Due to alternative splicing and different connections with β2-microglobulin, 7 HLA-G

isoforms exist [63]. HLA-G is expressed mainly in membrane-bound form at the feto-maternal interface, in a few adult tissues, and in different cells such as activated monocytes and erythroid and endothelial precursors [64]. Soluble HLA-G is found in body fluids, such as plasma [65]. HLA-G binds the inhibitory receptors LILRB1, LILRB2, and KIR2b14, which are differentially expressed by immune cells. Binding of these receptors may have an effect both on effector- and antigen-presenting cells: inducing apoptosis of activated CD8 positive T cells, modulating the activity of NK cells and dendritic cells, blocking alloantigen-specific cytotoxic T cell response, and inducing expansion of regulatory T cell populations [63].

The different maternal leukocytes in the decidua are also under the influence of fetal cells. Briefly, these influences could generally be called tolerance-promoting. Non-classical HLA class I on EVTs act as modulators of Tc, uterine NK cells (uNKc), macrophages, and dendritic cells (DCs) [66], and both trophoblasts and maternal macrophages in decidua produce different potent immunoregulatory molecules [66-68]. The phenotype of uNK cells in the decidua also differ from those in peripheral blood. These cells express killer inhibitory receptors (KIRs) that are specific for HLA class I on EVTs. In addition, CD8 effector T cells in decidua are non-functional since the production of cytotoxic molecules is downregulated. This hinders cytolysis of EVTs by CD8 T cells [69].

The activation state of a DC is decisive for the outcome of the meeting between a DC and a naïve T cell [54], and anti-inflammatory factors like IL-10, progesterone, human chorionic gonadotropin (hCG), and oestradiol may induce tolerogenic dendritic cells [70]. T regs also accumulate in the decidua, where they are attracted by hCG, in contrast to the decrease in T regs in maternal circulation [69].

Reports conflict regarding the presence of lymphatic vessels in the endometrium and decidua [71]. Red-Horse et al. showed in 2006 that the human non-pregnant endometrium does not contain lymphatic vessels [72, 73], while pregnancy induces lymph angiogenesis in decidual parts of uterus, and trophoblast cells are found in close proximity to these vessels [72]. Volchek et al. (2010), however, showed that lymphatic vessels were abundant in non-decidualized hypersecretory endometrium during gestation, while the decidua was nearly always free of lymphatics. In particular, there were no lymphatic vessels in the areas of the spiral arteries, surrounded by decidual

stromal cells [71]. Among the explanations of the opposite findings in these two publications is the wide definition of the decidua by Red-Horse et al.

During pregnancy, the fetus is protected against disease by maternal antibodies. IgG antibodies of high concentration in the maternal circulation are transported to the fetus via the placenta [74]. The antibodies are endocytosed by the syncytiotrophoblast, bind the human neonatal Fcγ receptor (FcRn) in the acidic environment in the endosome and are further transported to the fetal side. The transport of antibodies happens predominantly in the third trimester and is important for protection of the fetus and newborn child until it makes a sufficient number of antibodies by itself: maternal-fetal transport of IgG via the FcRn in the syncytiotrophoblast provides passive immunity to the fetus. IgA and IgM are not transferred from the mother to the fetus because specific receptors in the placenta for these immunoglobulins do not exist [74].

Platelets and their expression of integrins in fetuses and newborns

Platelets are the primary effector of haemostasis, in addition to having a potentially wide range of other physiological and pathological effects [75]. The normal platelet count in adults is 150–450 $x10^9$/L. By between 18 and 21 weeks of gestation, platelet values in fetuses are reported to be 234 ± 57 [76], resembling the adult values for platelets. Israels et al. 2003 [77] also found that platelet counts in full term newborns are not different from adult values, and that mean platelet numbers for infants between 18 and 30 weeks of gestation are 250 [78, 79]. In even younger fetuses, between 10 and 17 weeks of gestation, platelet counts are reported to be somewhat lower: 159 ± 34 [76].

There is limited knowledge about the function of fetal platelets and the regulation of platelets within the developing fetus in vivo [80], but fetal and neonatal platelets have in general been characterized as hyporeactive. Studies of platelet aggregation have shown that the aggregation of neonatal platelets is lower than with adult platelets [81, 82]. In a recent review, however, Margraf et al. (2019) [80], reported that bleeding time in the newborn is similar to, or shorter than, bleeding time in adults.

Integrins, receptors for adhesive proteins, including αIIbß3, are present on fetal and cord blood platelets [82-84]. Some studies, including flow cytometric analyses, indicate that the number of

these receptors on both neonatal (term) and fetal platelets is similar to the number on adult platelets [85-87]. Gruel et al. (1986) found that αIIbβ3 appeared to be normally expressed on fetal platelets as early as 18 weeks of gestational age and that HPA-1 is expressed on platelets at the same frequency and quantity in fetuses between 18 and 26 weeks of gestation as in adults [87].

However, Margraf et al. (2019) note that αIIbβ3 is reduced in neonatal versus adult platelets [80]. Sitaru et al. (2005) [88] also analysed the expression of platelet glycoproteins and platelet activation markers both in cord blood and in peripheral venous blood through whole blood flow and found a lower level of αIIbβ3 expression on platelets from peripheral blood in term newborns as well as preterm infants compared to adults. Simak et al. (1999) [86] also found a significantly lower expression of αIIbβ3 in the membrane of resting neonatal platelets compared with adult platelets by examining full blood in flow cytometry. Thus, it is well documented that fetuses have relatively high concentration of platelets, also carrying β3 integrin already from early stages of pregnancy, while the amount of integrin per platelet is more uncertain.

Human leucocyte antigens (HLA) and HLA haplotypes

The MHC complex is located on chromosome 6 and contains several genes located close together (Figure 6). The complex consists of three basic groups of genes: class I, class II, and class III. Class I encodes for HLA-A, -B, and –C molecules, class II encodes for HLA-DR and –DQ-molecules, and class III encodes for molecules primarily involved in inflammation.

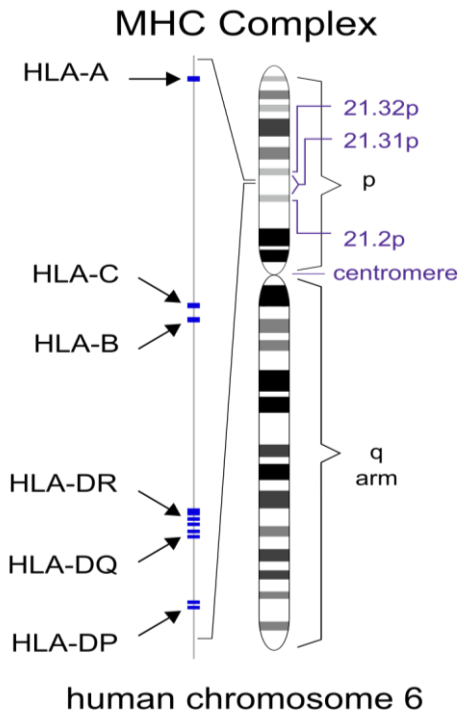

Figure 3. The MHC complex of chromosome 6. Commons.wikimedia.org

HLA class II molecules are proteins with one alpha- and one beta-chain coded for in the MHC class II region. Several allelic variants of DRB- and DQ alleles as well as of HLA-A, -B, and –C alleles give rise to the wide polymorphism in HLA molecules. An HLA-DQ molecule, for example, consists of an alpha chain encoded for in DQA, and one beta chain encoded for in DQB. This applies also to the DR molecules. However, for DR molecules the beta chain, in addition to being encoded for in DRB1, may also be encoded for by allelic variants of DRB3, 4, or 5, and most individuals carry such an extra DRB locus [89].

HLA-associations in HPA-1a alloimmunization

HPA-1a alloimmunization is strongly associated with *HLA-DRB3*01:01* [90-92]: over 90% of immunized women carry this allele, compared to only 27% in the general population (our own

data). In retrospective studies of HPA-1a alloimmunization, all or almost all immunized women were *DRB3*01:01* positive [10, 93]. The crystal structure of HLA-DRB3*0101 was resolved in 2007, with a modified HPA-1a peptide in the peptide-binding groove [21]. Parry et al. (2007), described structural studies of the DRA/DRB3*0101 molecule in complex with an N-terminal fibrinogen glycoprotein peptide which contains a Leu/Pro dimorphism [21]. Further, Ahlen et al. (2009) showed that isolated HPA-1a specific T cells from immunized women were DRA/DRB3*0101 restricted [94]. HPA-1a derived peptide binds the DRA/DRB3*0101 molecule, where the allogeneic residue L33 functions as an anchor residue [19]. Rayment et al. (2009) characterized T cell clones derived from several FNAIT mothers, confirmed that they respond to HPA-1a positive platelets, and defined their restricting class II molecule and epitope "core" [95].

The risk of HPA-1a immunization postpartum in *DRB3*01:01* positive women is 12.7%, compared to 0.5% in *DRB3*01:01* negative women [96]. Kjeldsen-Kragh et al. (2019) also demonstrated that the *DRB3*01:01* allele has a significant dose-dependent effect on maternal anti-HPA-1a levels, while the opposite trend is seen for neonatal platelet counts [97]. Wienzek-Lischka et al. (2017) did not, however, find any association between platelet count or ICH in the neonate and *DRB3*01:01* heterozygosity or homozygosity in the mother [91]. In summary, there is a clear HLA-*DRB3*01:01*-association in HPA-1a immunized women, there is a dose-dependent relationship with anti-HPA-1a alloantibody formation, and the association has a molecular explanation.

Làbbe et al. showed in 1992 that another HLA allele, *HLA-DQB1*02:01,* is also strongly associated with HPA-1a alloimmunization [18]. *DQB1*02:01* was found in 94% of immunized women compared to 40% in the control population, and the association of *DQB1*02:01* with HPA-1a immunization is one of the strongest found to date [18]. In addition, the *HLA-DQA1*05:01* allele was found in 86% of the immunized women. However, there are no reports of a function of DQA1*05/DQB1*0201 in relation to FNAIT. Neither are there any reports of FNAIT-associated T cell responses restricted by DQ2 molecules. The explanation for this association remains unknown.

A third HLA-allele, *DRB4*01:01,* has been proposed to have importance in the immunization against HPA-1a [98]. Loewenthal et al. (2013) found that 61% of HPA-1a immunized women in their study group carried this allele, compared to only 38% in the control group (women giving birth to thrombocytopenic children, not caused by anti-HPA antibodies). The *DRB4*01:01* allele (in

this case also including *DRB4*01:03*) was found more frequently in FNAIT cases compared to controls both with and without the presence of the *DRB3*01:01* allele [98]. By modelling the structure of DRA/DRB4*0101 and comparing it to the structure of DRA/DRB3*0101, the study concluded that DRA/DRB4*0101 also forms a binding cavity that might prefer binding L33 over P33, despite the lower avidity of the epitope compared to DRA/DRB3*0101, and that the *DRB4*01:01* allele thereby might be a contributor in the development of FNAIT, at least in combination with *DRB3*01:01* [98]. However, no difference in platelet number or in the response to treatment between having two or one associated alleles was found [98].

In contrast to the overrepresentation of *DRB4*01:01* in the above-mentioned study, Labbe et al. (1990) found a *DRB4*01:01* frequency of 41.7% in immunized women, compared to 70% in non-immunized women [18]. Wienzek-Lischka et al. (2017) also found that the frequency of *DRB4*01:01P* was lower in FNAIT cases compared to 100 healthy controls [91]. In addition, they did not find any association between the platelet count or ICH of the neonate and the absence or presence of *DRB4*01:01P* in the mother. In summary, the role and importance of the allele is still uncertain.

HLA haplotypes

HLA haplotypes are clusters of HLA gene alleles that are inherited together because of their close linkage on chromosome 6 [99]. An HLA-DR-DQ haplotype consist of a combination of specific allelic variants of DRB1, DQA1, and DQB1.

High-resolution typing of HLA-DR-DQ haplotypes in European Americans (2003) established new HLA haplotype frequency reference standards and established frequencies of the class II loci DRB1, DQA1, and DQB1 [100]. A total of 75 distinct DRB1-DQA1-DQB1 haplotypes were identified in the sample, and most of the common haplotypes were already known from previous studies on European populations [100]. Frequency distribution of the 75 haplotypes showed that as many as 59 haplotypes occurred at frequencies of less than 1%, including a total of 21 haplotypes that occurred only once and a further 6 that were seen only twice. Only 16 haplotypes occurred at a frequency >1% [100]. High resolution HLA haplotype frequencies derived from the US donor registry showed that there were 33 common DRB1 alleles, which accounted for 99.5% of all the DRB1 alleles; 15 common DQB1 alleles, which accounted for 99.9% of all DQB1 alleles; and 8 common DRB345 alleles, which accounted for 100% of the known alleles [101].

The *HLA-DRB3*01:01* allele is associated with DRB1*03, DRB1*11, DRB1*12, DRB1*13, and DRB1*14 alleles in different HLA-DR-DQ haplotypes [102, 103]. Among the 100 most common haplotypes from the US donor registry, the *DRB3*01:01* associated DRB1-alleles *DRB1*03:01* (0,06526), *DRB1*03:02* (0,00010), *DRB1*14:06* (0,00003), *DRB1*03:01* (0,00864), *DRB1*14:02* (0,00001), *DRB1*14:02* (0,00001), and *DRB1*13:01* (0,00035) are represented [101].

*DRB1*14* positive individuals from different US population groups with a single DRB3-associated DRB1 allele were selected for identification of the DRB3 association (that is, not DRB1*03, 11, 12, or 13) [104]. Of these, in the Caucasian population, 90% were *DRB1*14:01* positive, and only 2% were *DRB1*14:02* positive. The *DRB1*14:02* allele was also associated with *DRB3*01:01* [104] and comes with *DQB1*03:01* in the DR14-DQ3 haplotype, at low frequency [101]. Hence, the *DRB3*01:01*-associated DR14-DQ3 haplotype is very rare in Caucasians.

For DRB1*03, in 161 positive individuals from each of five US population groups, only 6 of 21 known alleles were detected, and Caucasians were found to be the least diversified, in whom only *DRB1*03:011* was observed [102]. Among only the Caucasoid population 23.4% were *DRB1*03* positive, and the allele came in the following combinations: *DRB1*03:011 + DRB3*01:01* in 83.1%, *DRB1*03:01 + DRB3*02:02* in 16.2%, and *DRB1*03:011 + DRB3*03:03* in 0.8% [102].

For DRB1*13 alleles the frequencies in Caucasians were found to be: 48.6% for *DRB1*13:01*, 40.0% for *DRB1*13:02*, 8.6% for *DRB1*13:03*, and 1.7% for *DRB1*13:05*. The most common allele, *DRB1*13:01*, came in the following combinations: *DRB1*13:01-DRB3*01:01*: 63%, and *DRB1*13:01-DRB3*02:02*: 37% (N = 51). The other *DRB1*13* alleles came in the following combinations: *DRB1*13:02-DRB3*03:01*: 100% (N = 37), *DRB1*1303-DRB3*01:01*: 91% (N = 11), and *DRB1*13:03-DRB3*02:02*: 9% (N = 11) [103].

HLA haplotypes in HPA-1a alloimmunization

The most important from the above section is that HLA alleles do not exist alone. One of the *DRB3*01:01* associated haplotypes is the DR3-DQ2 which also consist of *HLA-DQB1*02:01,* the other allele known to be associated with HPA-1a alloimmunization [18]. As already described, *DRB3*01:01* also occurs with *DRB1*13:01* on the same haplotype, and Wienzek-Lischka et al.

(2017) found that both these haplotypes have a similar effect on the case-control status [91]. *DRB1*03-DRB3*01:01* is, however, overrepresented among HPA-1a immunized women compared to the *DRB1*13-DRB3*01:01* haplotype [91]. The other DR-DQ haplotype containing DQ2 is the DR7-DQ2 haplotype, and DRB4*01 is again known to be associated with DR7-DQ2 [101].

HLA haplotypes in the Norwegian population:

When it comes to the Norwegian population, Spurkland et al. (1992) 30 years ago defined 24 different HLA-DR-DQ haplotypes considered as common haplotypes in the Norwegian population, with frequency ≥ 1% in the population [105]. More recently, Lande et al. (2018), identified 4779 NBMDR (Norwegian Bone Marrow Donor Registry) individuals recruited from Norwegian blood banks and genotyped for the loci HLA-A, -C, -B, -DRA, -DQB1, and -DPB1. The registry does not contain information regarding ethnicity [106]. The frequencies of different DR-DQ haplotypes of specific interest in this thesis were as follows: DR3-DQ2: 13.58%, DR7-DQ2: 5.8%, DR14-DQ5: 2.55% (14:01-05:01), DR13-DQ6: 13.57% (13:01-06:03: 7.67%, 13:02-06:04: 5.11%, 13:02-06:09: 0.61%), and DR14-DQ3: 0.16% (14:02-03:01) [106]. Some of the known *DRB3*01:01* associated haplotypes are found rarely or not at all in the Norwegian population [105-107].

Epidemiological studies indicate different rates of immune related diseases in Sami compared with non-Sami Norwegian population [107]. Genomic data for HLA class I and II loci in Norwegian Sami show that several alleles and haplotypes differ from the Norwegian population. For alleles and haplotypes of specific interest here, the *DRB1*03* allele was found at a frequency of 0.06 in Sami versus 0.124 in non-Sami, the *DQB1*02* allele was found at a frequency of 0.063 in Sami versus 0.179 in non-Sami, the DR3-DQ2 haplotype at a frequency of 0.06 in Sami versus 0.13 in non-Sami, and the DR7-DQ2 haplotype at a frequency of 0 in Sami versus 0.05 in non-Sami [107].

Conserved extended haplotypes/Ancestral haplotypes

The DR3-DQ2 haplotype is also part of the conserved extended haplotype or ancestral haplotype (AH) 8.1 [99]. The term "ancestral" suggests that this is "conserved, population-specific haplotypes of larger continuous DNA sequences derived with little change from an ancestor of all individuals now carrying all or part of the haplotype" [99]. The haplotypes are built up from genotypes of four major blocks: HLA (w-N, TNF cluster, complement gene cluster and HLA-DQ-DR, and the AH with the highest frequency in the Caucasian population is the 8.1 AH [108]. The most characteristic

constituents in the four major blocks are HLA-B8, TNFα, mono-S-RCCX, and HLA-DQ2-DR3 genotypes [108]. The existence of antigen non-specific control of immune response related to this AH has been demonstrated in mice. Also, in humans, a similar pattern has been suggested to be represented by the HLA-B8, DR3 haplotype, and the 8.1 AH is unique in its association with a number of immune pathological diseases [99] and cancer [109].

Several polymorphic areas are documented within the TNF gene cluster. Polymorphism in the TNF promoter region has been observed to result in differences in the rate of gene transcription, and in the rate of protein production. Some of the polymorphisms are carried by the 8.1 AH [99]. There are, for example, reports of higher systemic levels of TNFα in healthy individuals with this haplotype [110]. However, the direct influence of AH 8.1 on TNFα-level is debated [110]. The biological functions of the TNFα is also varied and complex. In the local acute situation, the production of TNF alpha is beneficial, while systemic or protracted exposure to TNFα may be harmful [110].

Fetal-maternal microchimerism and extracellular vesicles in pregnancy

"Microchimerism refers to a small number of cells or DNA harboured by one individual that originated in a genetically different individual" [111]. During the course of a normal pregnancy, trafficking of cells occurs trans-placentally from the fetus to the mother and in the opposite direction. Both intact cells and DNA of fetal origin have been detected in maternal circulation [112, 113]. By the second trimester, from 1 to 5 fetal cells/mL are found in maternal venous blood [40]. And trophoblasts are probably the major contribution of both fetal cells and DNA detected in maternal blood.

The extracellular space of multicellular organisms contains a large number of membrane-limited vesicles called extracellular vesicles, microparticles, microvesicles, extracellular particles, and so on. Extracellular vesicles (EVs) are defined in different ways by size and by their origin. Gyorgy et al. [114] include exosomes, activation- or apoptosis-induced microvesicles or microparticles (MPs), and apoptotic bodies in the group of EVs. EVs also include other vesicular structures such as large apoptotic bodies, exosome-like vesicles, and membrane particles [114].

Exosomes are vesicles of 50–100 nm in diameter generated by exocytosis of multivesicular bodies, while microvesicles are larger extracellular membrane vesicles of 100–1,000 nm in diameter formed by the release of budding/ blebbing of the plasma membrane of cells [114, 115]. The vesicles retain proteins from the cell of origin, along with cytosolic contents. Apoptotic bodies are made by the release from blebs of cells undergoing last stage of apoptosis and range from 1–5 μm in size.

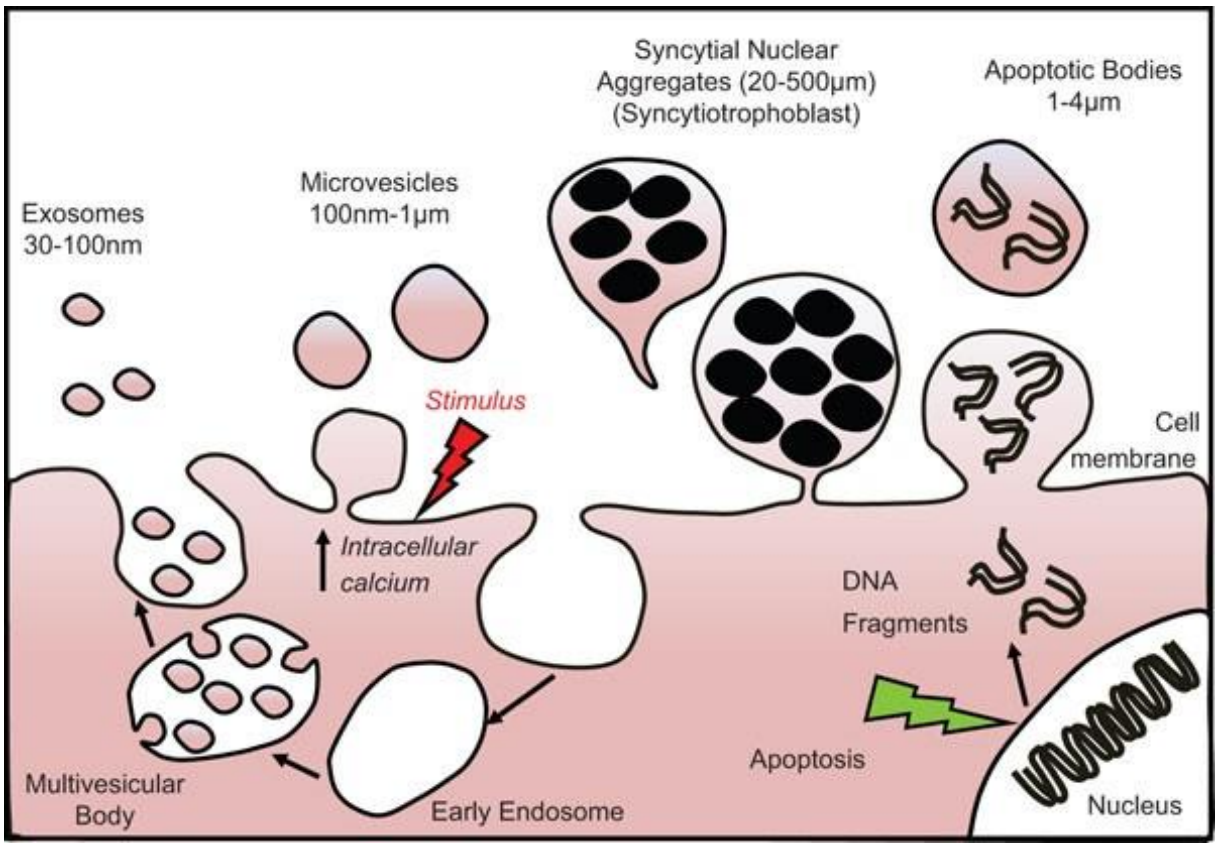

Figure 4. Extracellular vesicles shed from the placenta.
Reused from Tannetta, D. et al., *Extracellular vesicles and reproduction–promotion of successful pregnancy*. Cell Mol Immunol **11,** 548–563 (2014). https://doi.org/10.1038/cmi.2014.42

Formation of EVs has been observed in different kinds of cell types including endothelial cells, platelets, leukocytes, smooth muscle cells [116], erythrocytes [117], cancer cells [118], and trophoblast cells [119]. They are released from cells under stress and several stimuli may lead to or inhibit the formation of microvesicles. A database of the proteomes of EVs has recently been established (vesiclespedia, http://microvesicles.org/#).

The placenta produces a wide variety of extracellular vesicles (Figure 4) [120]. The largest vesicles consist of syncytial nuclear aggregates. Those vesicles were already reported in 1893, when found trapped in cadaver lungs of pregnant women. Syncytial aggregates range from 20 µm up to 100 µm in size, contain hundreds of nuclei [121], and are found in maternal peripheral blood of pregnant women as early as 6 weeks of gestation [122]. The MVs are drained via the uterine vein into the maternal circulation. Lok et al. (2008) found that placenta-derived MVs ranged from 1.5 to 3% of the total number of microparticles isolated from maternal blood [123]. Microvesicles arise from budding of the plasma membrane [115] and therefore contain surface proteins from the cells that they are derived from. Placental microvesicles may arise from ST, villous CTs exposed to the maternal blood following denudation, and from EVTs [120]. One third of STs have blebs on their surface, suggested to be a source of trophoblast microvesicles [124]. The amount of MVs in maternal plasma has been reported to increase along with increasing gestation, and to reach the highest level at 36 weeks of gestation [123, 125]. Exosomes are also shed in large quantities from the ST in normal pregnancies, and the number of shed exosomes also increases with increasing gestational age [126].

EVs also carry immunomodulatory proteins, by example HLA-G5 and FasL, that may contribute to apoptosis or reduced activity of T cells during pregnancy [127-130]. In addition, Syncytin-1 is expressed on EVs from placenta, which reduces the secretion of proinflammatory TNFα and IFNγ from leukocytes [131].

Anti-HPA 1a antibodies

Anti-HPA-1a antibodies are heterogeneous with respect of specificity of binding to β3 integrin. Hence, binding sites for these antibodies will differ between and within individuals, which will further influence the effect of the antibodies. Some anti-HPA-1a antibodies bind only to the β3 chain, while others recognize complex epitopes formed by αIIb and β3 [132-134]. Antibodies against the latter will affect platelets, while antibodies which bind the β3 chain will potentially affect all cells with combinations of heterodimers, including the β3 integrin. Bayat et al. (2019) demonstrated that HPA-1a antibody subtypes currently used as standards for antibody testing also differ with regards to binding specificity [135], where "AP3" binds the β3 subunit independently of the α subunit, while "Gi5" and "23C6" bind the αIIbβ3 and αVβ3 heterodimers respectively. In

addition, a recent study by Santoso et al. (2016) demonstrates that anti-HPA-1a antibodies only reacting with the αVβ3 complex were present in serum from mothers of ICH positive but not ICH negative FNAIT cases [136]. The binding of these antibodies to endothelial cells was further shown to reduce adhesion of the endothelial to vitronectin, lead to cell anoikis, and to interfere with angiogenesis [136]. Yougbare et al. (2015) also demonstrated in mice that anti-β3 antibodies produced in mice may induce ICH in pups by impairing angiogenesis rather than by thrombocytopenia [137].

The natural history of HPA-1a alloimmunization

A large prospective screening study conducted in Norway, consisting of about 100,000 pregnant women, showed 2.1% HPA-1a negative women in the population [8], which resembles the frequency of HPA-1a negativity also in other Caucasian populations [1, 9, 138].

Studies of Caucasian populations of women have shown somewhat different frequencies of anti-HPA-1a antibody formation in women at risk: from 6 to 12% [8-10]. Anti-HPA-1a alloantibodies can be found in primipara women and are detected already at 17 weeks of gestation [10]. Turner et al. (2010) also found that 5 of 25 women developed antibodies in second and third trimester at their first pregnancy [16]. Kamphuis et al. (2010) report, in their review, that several women were found to have antibodies even in the first trimester [139]. However, all women in the last study were multiparous, and more typically, anti-HPA-1a antibodies were detected around week 20 of pregnancy [140].

Only one of three delivered infants of HPA-1a negative women with detectable anti-HPA-1a antibodies is thrombocytopenic [9]. However, severe thrombocytopenia can occur as early as 16 weeks of gestation, and there is a relation between antibody level and the severity of thrombocytopenia in the newborns [11, 140]. Jin et al. (2019) report in a retrospective study that as many as 60% of FNAIT cases were first pregnancies [14].

Anti-HPA-1a antibodies are also associated with reduced birth weight in baby boys [15]. The most feared complication of FNAIT is, however, ICH, which is reported to occur in up to 26% of retrospective FNAIT cases due to anti-HPA-1a immunization [13], while a review of prospective studies reports a frequency of about 10% [139]. The only established predictor for ICH secondary to

FNAIT is a history of ICH in a previous, affected sibling [4], and most reports agree that pregnant women with a previous child with ICH belongs to the highest risk group [141]. If the previously affected sibling had ICH, the next affected fetus is likely to have early, severe thrombocytopenia and in utero ICH in the absence of effective treatment [142].

The mortality of ICH varies somewhat in the literature. Spencer and Burows (2001), describe a mortality of 7% [13], while another literature review, of 246 FNAIT cases, found that 10.2% died due to ICH and 15% of the children developed neurological sequelae [143]. Psailia et al. (2009) also examined the outcome for 40 cases of ICH in children with immune thrombocytopenia. Fifty percent of the children survived with full recovery, 25% survived with neurological sequelae, and 25% died [144], while Tiller et al. (2013) found that one third of the children died within 4 days after delivery and 53% of the children survived with severe neurological disabilities [145]. In any case, the outcome of FNAIT-related ICH may in some cases be catastrophic. More children also died when signs of bleedings other than petechiae and ecchymoses were present, and petechiae were reported in fewer patients with ICH than without ICH [144].

An observational study of cases from an international multicentre registry characterized pregnancies where the fetus or neonate was diagnosed with FNAIT and suffered from ICH [145]. HPA-1a alloimmunization was found to be the cause in 91% of the ICH cases. Most of the ICH bleedings occurred by the end of the second trimester, and the end clinical outcome was devastating for most cases. About half of the bleedings occurred before 28 gestational weeks and often affected the first-born child (63%), and as many as 67% of the bleedings started before 34 gestational weeks. The mothers were primigravida in 27% of index cases. This differs from the finding by Jin et al. (2019), who report a frequency of first pregnancies in ICH cases of more than 70% [14]. No cases of intrapartum ICH bleedings were confirmed. Antenatal treatment was not given in most cases of fetal and neonatal ICH.

Treatment

Treatment available for HPA-1a immunization in pregnancy is only general and differs between countries. In Norway, HPA-1a immunized women are monitored throughout pregnancy, children of HPA-1a alloimmunized women are taken by planned caesarean section at about 38–39 weeks of

pregnancy, and compatible platelets are prepared for transfusion when the baby is born [146]. In addition to this, women who in a previous pregnancy have carried or given birth to children with ICH are also treated with intravenous immunoglobulins (IVIg) during the next pregnancy.

One way to treat the condition during pregnancy, which is used in some countries other than Norway, is to perform fetal blood sampling during pregnancy and transfuse platelets in utero when the platelet counts are low. There is, however, an increased risk of fetal loss as a result of these procedures. A fetal loss risk associated with the serial of fetal blood sampling and platelet transfusions is reported to be of 5.5–6% per pregnancy [12, 147], indicating the clear need for further development of less invasive approaches [147].

Other available treatments avoid risk to the fetus from blood sampling by only treating the mother with IVIg with or without high-dose steroids [148]. Treatment during pregnancy consists of weekly maternal infusions of IVIg beginning at 12–20 weeks of gestation, with or without later addition of steroids, continuing until delivery [3, 149]. The response of these treatments is reported to be uncertain by Kaplan et al. (1998) [150]. Kamphuis and Oepkes (2011), however, report almost 100% success with IVIg treatment against FNAIT, with only one or two reported cases of fetal ICH despite IVIg treatment [141]. Tiller et al. (2013), found that IVIg treatment failed to prevent ICH in only 2 of 19 (11%) cases [145]. Compared with historical data reporting a 79% risk of ICH recurrence in FNAIT, their data also indicate that IVIg is effective in preventing ICH, as is also reported by Yougbare et al. (2015) in mice [137]. The latter group has also shown previously in mice that IVIg downregulates both maternal and the neonatal anti-platelet antibody levels and that, in addition to the effect on the antibodies, the FcRn can be blocked by IVIg [137]. In summary, IVIg appears to be able to downregulate maternal antibodies both by blocking the Fc receptor on trophoblasts and by reducing the antibody titre.

Rayment et al., 2011 [151], did a Cochrane review on the effect of corticosteroid versus IVIg and found that the relative risk of death was not significantly different between the two treatment groups and that there was no significant difference between mean platelet count at birth [148] or mean change in fetal platelet count between pre-treatment and birth in the two groups. In summary, both IVIg and prednisone can be used as first line treatment of FNAIT in case of no peripartum haemorrhage in an affected sibling when the pre-treatment fetal platelet count is >20. IVIg in combination with prednisone is, however, more effective in raising the fetal platelet count than IVIg

alone in high-risk pregnancies when the fetal platelet count is <20 or the affected sibling suffered ICH [151]. The most important course of action for a neonate with FNAIT and severe thrombocytopenia also treated with IVIg during pregnancy is to increase the platelet count as soon as possible with compatible platelets [152]. There is no additional gain in adding IVIg postnatally [152].

In absence of a specific treatment for the condition, the Norwegian screening study chose to do caesarean sections a few weeks before term to avoid the potential increased risk of ICH by vaginal delivery. The safety of vaginal delivery on pregnancies with FNAIT was evaluated through a prospective data collection [153]. In FNAIT pregnancies with a thrombocytopenic sibling without ICH, vaginal delivery was *not* associated with neonatal intracranial bleeding. Only four of the neonates, however, had platelet counts lower than 50.

Aims of thesis

Our research group has the privilege to cooperate closely with the National Reference Laboratory of Advanced Platelet Immunology. We also benefit from material from a large prospective screening study in Norway between 1995 and 2004. As a result of the screening study and many years of work, our group has also developed HPA-1a specific T cell clones and an HPA-1a specific human monoclonal antibody ("26.4"), useful for further studies on the pathogenesis of FNAIT. Our understanding of the pathogenesis of this disease, however, still remains somewhat limited, and there are still many missing links in this large puzzle.

One of the questions still not fully answered is why some women at risk of immunization (HPA-1a negative women carrying HPA-1a positive foetuses) are immunized while others are not. In the context of introducing a screening for the condition and develop treatment in particular, there is a need to more precisely detect the women most at risk of being immunized and in need for treatment. In that respect, different HLA alleles have been suggested and shown to be associated with immunization and may be used as risk markers. However, some of these results are contradictory. In addition, the close linkage between genes in the MHC complex makes it difficult to define which of the associations have an impact. The first aim of this study is therefore to investigate the actual contribution of different HLA alleles already suggested to be associated with immunization and to determine whether carrying different DR-DQ haplotypes associated with the *HLA-DRB3*01:01* allele results in different risks of immunization.

Missing links may also be found in the placenta, as HPA-1a immunization during pregnancy is a break of tolerance against a fetal antigen. In addition, the HPA-1a antigen is present on trophoblast cells. The next aim of this thesis is to determine whether HPA-1a specific T cells can also be activated by HPA-1a antigen from the placenta, as a possible source of antigen during pregnancy. Determining this will have an impact on when to give a potential prophylaxis and will demonstrate that pregnant women actually produce an immune response against their fetus under certain conditions.

The third missing link focused on here is the association between the formation of anti-HPA-1a antibodies in the mother and reduced birth weight in baby boys. Our third aim is to determine whether anti-HPA-1a antibodies may affect adhesion, migration, and invasion of trophoblast cells in vitro, as a model for extravillous trophoblast function during placentation.

Summary of papers

Paper I

The role of Placenta-derived Human Platelet Antigen-1a in HPA-1a alloimmunization. Manuscript.

Heide G, Ahlen MT, Husebekk A, Skogen BR, Stuge TB.

HPA-1a alloimmunization is in most cases believed to occur in connection with feto-maternal haemorrhage, where fetal platelets enter the maternal circulation. The highest volume of maternal blood entering the fetal circulation takes place at delivery.

However, it is not known whether the number of platelets entering the maternal circulation during pregnancy and at delivery is high enough to cause alloimmunization, and although most women in prospective studies seem to be alloimmunized during or after delivery, a considerable percentage of women are already immunized during their first pregnancy. Since the HPA-1a antigen that causes HPA-1a alloimmunization is found also on trophoblast cells during pregnancy and these cells are indeed in direct contact with the maternal circulation and immune system, both as intact cells and trophoblast derived vesicles shed into the maternal circulation, it is possible that these cells are also responsible for alloimmunization during pregnancy.

Here, we show that HPA-1a antigen from αVβ3 on trophoblast cell lines activates HPA-1a-specific T cells in the same way as antigen from platelets, mostly from αIIbβ3, when processed and presented by *HLA-DRB3*01:01*-positive monocytes. The activation of HPA-1a-specific T cell clones was dependent on the amount of antigen available for the antigen-presenting monocytes. We also show that HPA-1a specific T cells may be activated by HPA-1a antigen-positive extracellular vesicles from plasma of HPA-1a positive individuals and pregnant women, and that fetal HPA-1a positive vesicles can be detected in maternal plasma of HPA-1a negative women pregnant with an HPA-1a-positive fetus. Some of these HPA-1a positive fetal vesicles seem to be of placental origin. However, we failed to show that fetal HPA-1a positive isolated vesicles from maternal plasma of an HPA-1a negative woman activate specific T cells. Possible reasons for the lack of activation are discussed in the paper.

Papers II and III

The prevalence of HPA-1a alloimmunization and the potential risk of FNAIT depends on both the DRB3*01:01 allele and associated DR-DQ haplotypes. Regular article.

Ahlen MT, Heide G, Husebekk A, Skogen BR, Kjeldsen-Kragh, Stuge TB.

The DR7-DQ2 haplotype in a native Norwegian population. Scand J Immunol 2013 May; 77(5):429, Letter to the editor.

Heide G, Stuge TB, Skogen BR, Husebekk A, Ahlen MT

The association between the *HLA-DRB3*01:01* allele and HPA-1a alloimmunization is well known, as over 90% of immunized women carry this allele. The antigen with leucine in position 33 fits into the peptide-binding groove of the MHC molecule, and leucine functions as an anchor residue for binding to the MHC complex. It is also shown that our isolated HPA-1a-specific T cell clones are restricted by the HLA-DRA/DRB3*0101 molecule.

Another HLA allele, *HLA-DQB1*02*, has also been shown to be associated with HPA-1a alloimmunization, However, HPA-1a specific T cell clones restricted by this molecule have not been found, and the molecular explanation for this association is not known. *HLA-DRB3*01:01* is associated with different HLA-DR-DQ haplotypes in the Norwegian population. The DR3-DQ2, which also consist of *DQB1*02:01*, is one of them. *HLA-DQB1*02:01* could therefore be associated with HPA-1a alloimmunization simply due to close linkage to the *HLA-DRB3*01:01* allele. Also, *DRB4*01* has been suggested to be associated with HPA-1a alloimmunization. This allele is also in linkage disequilibrium with DR7-DQ2, the other known DR-DQ haplotype that has the *DQB1*02* allele.

We genotyped 213 random *DRB3*01:01* positive donors from the general population, and 157 *DRB3*01:01* positive HPA-1a alloimmunized women and show that the DR3-DQ2 haplotype is overrepresented in this group of immunized women compared to the general population. Most important, the prevalence of immunization in HPA-1a negative women is twice as high for women carrying DR3-DQ2 versus DR13-DQ6 as their *DRB3*01:01* associated haplotype. This also

indicates that the association with *DQB1*02* is due to linkage with *DRB3*01:01*. However, the other DR-DQ haplotype involving *DQB1*02*, DR7-DQ2 was also seen more frequently among *DRB3*01:01* positive immunized women compared to the general population. The results suggest that the DQ2 allele is not simply acting in synergy with the *DRB3*01:01* allele in enhancing the chance of HPA-1a alloimmunization. We also find that *DRB4*01:01* is infrequent but overrepresented in *DRB3*01:01*-positive women. This association may be coincidental due to the close linkage to the DR7-DQ2 haplotype.

Paper IV

Anti-Human Platelet Antigen (HPA)-1a antibodies affect trophoblast functions and may have significance for placenta development: A laboratory study using an in vitro model. Regular article.

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Anti-HPA-1a alloantibodies seem to not only affect the number of platelets in the fetus. Maternal anti-HPA-1a antibodies are also associated with clinically significant reduced birth weight in newborn boys and with ICH. The integrin β3, carrying the HPA-1 system, not only forms a heterodimer with αIIb, it also combines with αV, forming the vitronectin receptor. The vitronectin receptor, αVβ3, is expressed on various cell types, including trophoblast cells. It has been proposed that the binding of anti-HPA-1a antibodies to antigens expressed on invading trophoblast cells can affect the development of placenta, and consequently lead to reduced placenta function and be a cause of reduced birth weight.

We aimed here to examine whether interaction of anti-HPA-1a antibodies with antigens on trophoblast cells could affect adhesion, migration, and invasion of extravillous trophoblast cells, by using an in vitro model with human anti-HPA-1a antibodies and the extravillous trophoblast cell line (HTR8/SVneo). The xCELLigence system was used to assess the possible effect of anti-HPA-1a antibodies on adhesion and migration of HTR8/SVneo cells. Specially designed chambers pre-coated with Matrigel were used to assess the effect of anti-HPA-1a antibodies on the invasive capacity of the cells.

Anti-HPA-1a antibodies partially inhibit adhesion, migration, and the invasive capacity of HTR8/SVneo cells. Our findings suggest that anti-HPA-1a antibodies may affect the development of placenta.

Discussion

General discussion

Immunization: what and how, where, and when?

We start at the very beginning of HPA-1a-immunization. An unknown alloantigen is not sufficient by itself to start an alloimmunization process. Alloimmunization requires both a certain amount of the foreign antigen, some kind of trigger or danger signal, and that the immune system be well enough equipped to make a response. Activation of naive T cells requires properly activated antigen presenting cells (APCs), and one of the missing factors in HPA-1a immunization during pregnancy is a pro-inflammatory signal. Such a signal is required for maturation of APCs, which in turn express key co-stimulatory molecules that allow proper activation of naive T cells. Only subsequent to such activation could the same T cells interact with HPA-1a specific B cells, causing the B cells to differentiate into anti-IgG secreting plasma cells [154]. We do not know whether the form of the antigen matters when it comes to immunization, nor what the secondary, or pro-inflammatory, signal in the primary response is.

Regarding HPA-1a immunization, the theory has been that immunization takes place following feto-maternal bleeding during pregnancy or at delivery, where the antigen, in the form of platelets, enter the maternal circulation. The argument for antigen entrance at delivery might hold for those HPA-1a negative women being immunized at birth when the greatest feto-maternal bleeding takes place. In RhD immunization, immunization takes place only after a significant trans-placental haemorrhage (TPH), and the average TPH at delivery is less than 1 mL of whole blood [32]. If we assume, as also reviewed by Curtis [3], a normal fetal platelet count at 18 weeks of gestation and assume that platelets do express HPA-1a as early as 18 weeks of gestation [40], an absolute maximum of 4 $x10^8$ platelets with HPA-1a antigen will be present for the mother's immune system at delivery [3]. If RhD antigen is given intravenously to RhD negative individuals, a primary immune response occurs in only 15% of the individuals after 1 mL [32]. It is not known whether the amount of platelet HPA-1a antigen entering the maternal circulation at delivery is sufficient to cause alloimmunization or how much HPA-1a antigen is needed for immunization to occur.

For those women immunized during their first pregnancy, the exposure to HPA-1a antigen may also come from small repetitive haemorrhages during pregnancy with fetal blood platelets. The amount of platelet antigen entering the maternal circulation from haemorrhages *during* pregnancy will, however, be even smaller than by the bleeding at parturition. Since it is uncertain if the highest exposure of antigen seen at delivery may cause HPA-1a immunization, it is even more uncertain if repetitive small amounts of blood platelets entering the maternal circulation during pregnancy is enough antigen for an anti-HPA-1a response to occur. The source of antigen then, may be something else than, or in addition to platelets. In that respect, the HPA-1a antigen from αVβ3 integrin on trophoblast cells is also a potential source of the minor histocompatibility antigen HPA-1a.

HPA-1a antigen is exposed to the mother`s circulation during pregnancy by the expression of β3 integrin on ST and EVTs [25, 155] or on shed trophoblast material in form of extracellular vesicles, in addition to platelets. Since large amounts of placental cell debris, including from ST, are released in different forms and sizes into the maternal circulation during pregnancy [156], the mother may be exposed to foreign β3 integrin in large amounts during the whole pregnancy. The β3 integrin, as part of the αVβ3, the vitronectin receptor, on trophoblast debris and cells has been proposed as an antigen source in HPA-1a alloimmunization. However, it has not been demonstrated that the antigen from this receptor, on trophoblast cells, activates HPA-1a-specific T cells. In this project, we have shown using β3 integrin from a trophoblast cell line that HPA-1a specific T cell clones are also activated with HPA-1a antigen from the vitronectin receptor in extravillous trophoblast cells, when processed and presented by *DRB3*01:01* positive monocytes. The activation of HPA-1a specific T cell clones depends only on the amount of the antigen, and the T cells act in the same way regardless of if the antigen source is the vitronectin or the fibrinogen receptor. We also show that sorted HPA-1a positive EVs, either alone or bound to dynabeads, when phagocytized, processed, and presented by macrophages to T cells, also activate HPA-1a specific T cells. We did not succeed, however, in demonstrating that the same specific T cells also could be activated by HPA-1a positive EVs of fetal origin in plasma of HPA-1a negative pregnant women. We speculate whether this could be because the antigen amount in the experiments were too low, as we only immunoprecipitated HPA-1a positive vesicles from a maximum of 4 mL plasma of each pregnant woman. The number of fetal vesicles in maternal plasma will naturally be very low compared to maternal HPA-1a positive platelet vesicles that we show activates specific T cells. It was also difficult to measure the amount of antigen immune-precipitated, as working with extracellular

vesicles pose some technical challenges that will be discussed later. In addition, the density of HPA-1a antigen on ST, and therefore also on debris from these cells, compared to the antigen density on the platelet surface is not known but is probably low and most probably much lower than on platelets [50, 51, 155].

Experiments with EVs might be affected by the choice of which women to analyse plasma from. HPA-1a positive fetal cells or EVs from plasma of HPA-1a negative immunized women would possibly be coated by circulating IgM and complement and removed from circulation [32] and thereby no longer be available to the maternal immune system; or to experimenters to isolate for further experiments. We therefore chose to use plasma from HPA-1a negative women with low or zero antibody titers for our experiments. Vesicles from non-immunized HPA-1a negative women, however, may differ from vesicles in immunized HPA-1a negative women in regards to their potential to start an immune response.

Given that large amounts of placental material are shed into the maternal circulation during pregnancy and that the β3 integrin is present in parts of this material, there should be plenty of antigen available for immunization in *all* HPA-1a negative women carrying HPA-1a positive fetuses, despite the likely low antigen density on the surface. The question then relates to why not all these women are already immunized during pregnancy. The exposure to the immune system to low doses at a time could lead to tolerance in the mother rather than a crucial antibody response. It is also believed that placental EVs are part of the communication between the fetus and the mother [120]. There is, however, little consensus regarding the effects of the vesicles on immune cells. This may be due to different methods used to prepare vesicles in vitro, due to different characteristics of the vesicles and to differences in the immune cells examined. The primary mechanism of clearance of microvesicles is believed to be phagocytosis [157-159], and vesicles produced in different ways by different causes may again have different impact on the phagocytizing macrophages. Abumaree et al. (2006) showed that shed trophoblasts were phagocytized in vitro by activated monocytes from the U937 cell line [160] and propose that apoptotic trophoblast material induces an anti-inflammatory response as it induces IDO and IL-10 secretion by the monocytes [160]. The authors further show that primary macrophages derived from peripheral blood also could phagocytose apoptotic trophoblasts from term placenta and induce the same anti-inflammatory response. In addition to this, uptake of apoptotic trophoblast material led to reduced cell surface expression of HLA class II and ICAM-1 and reduced expression of CD80, CD86, CD40, and B7H3 on the

macrophage surface [161], which again may lead to low expression of potential alloantigens to specific T cells and further lead to antigen-specific tolerance rather than activation of the T cell. Others, however, have shown that MVs may induce pro-inflammatory phenotypes of macrophages [162]. In that respect, studies presented here have also found [124] that phagocytosis of apoptotic extracellular vesicles results in a downregulation of activation surface receptors on monocytes, while phagocytosis of necrotic debris leads to an up regulation of the same receptors and the activation of monocytes. β3 negative mice injected with LPS and other bacterial or viral antigens and transfused with wild type β3 platelets produced higher levels of anti-β3 antibodies compared to controls [163]. Mouse pups of immunized β3 negative female mice exposed to the same antigens were more thrombocytopenic compared to pups born to uninfected controls [163]. Hence, both the amount of antigen taken up at one time, the source of antigen, and the form and presentation of the antigen may be of importance for HPA-1a immunization.

There are several feto-maternal interfaces, which again raises the question of where the potential immunization against placental antigens actually occurs. Immunization may happen locally in the decidua where the antigen is available for the mother both on ST and on invasive extravillous trophoblast cells and where maternal immune cells are also present. However, the existence of lymph node–like structures in the decidua has never been shown. The characteristics of the maternal leukocytes in the decidua is likely also both phenotypic and functionally different from other leukocytes [69]. It is also debated whether there are draining lymph nodes from the decidua [71, 72]. In addition to the local interface, as a second feto-maternal interface, a disruption of the trophoblast membrane integrity, as could occur from trauma, infections, or other placental abnormalities, may also give rise to intact fetal blood cells in the maternal circulation [3, 164]. By a short disruption of trophoblast integrity, fetal cells may transfer to the maternal circulation, and by 36 weeks of gestation, 100% of women have detectable fetal cells in their circulation [3]. These fetal antigens are most likely to accumulate in the maternal spleen [54]. Also, for trophoblast debris shed into the maternal circulation that is small enough to be passed the lung capillaries, the logical place for potential antigen presentation and activation of specific cells is the spleen.

Finally, when are pregnant women immunized? In the Norwegian prospective screening study, 75% of the women were immunized in connection with delivery [8], which again means that 25 of the women were immunized during pregnancy. Other studies, however, report a considerably higher

percentage of immunization in primigravida women [2, 10, 14, 16]. In that respect, anti-HPA-1a antibodies have been detected in primiparas women as early as 17 weeks of gestation [3, 10]. As such, the pregnant women making these antibodies have to be exposed to HPA-1a during early pregnancy, in prior undetected or failed pregnancies, or through blood transfusion. The antigen may, however, be present for the maternal immune system in the form of αVβ3 from the onset of implantation, during the implantation process, and after the placenta is established [27, 28].

HLA-alleles and haplotypes

Since anti-HPA-1a antibodies are produced, FNAIT must result from an immune response involving activation of both T and B lymphocytes. For a B cell to differentiate into an antibody-producing plasma cell, it needs to interact with the same antigen complex as a T cell specific for the same complex [154]. Most humoral responses that involve the switch of isotype from IgM to IgG, IgA, and IgE are dependent on T cell support, and only antibodies of IgG type can be transported over the placenta in pregnancy [154], being either protective or harmful to the fetus. In that case, T cell activation is crucial for the antibody response in HPA-1a immunization, and the T cell response is dependent on antigen presentation in an MHC complex.

Almost all HPA-1a alloimmunized women carry the MHC class II *HLA-DRB3*01:01* allele. Pooled prospective data shows that the odds ratio for becoming anti-HPA-1a immunized is 0.03 for HPA-1a negative/*DRB3*01:01* negative women compared to HPA-1a negative/*DRB3*01:01* positive women [165]. Recognition of antigen by a T cell is dependent on MHC restriction. The strong association between the MHC class II allele *DRB3*01:01* and the production of anti-HPA-1a antibodies is solid evidence for the involvement of DRA/DRB3*0101-restricted maternal CD4 T cell responses in the development of these antibodies, and further FNAIT [154]. The DRA/DRB3*0101 molecule is important in presenting the HPA-1a antigen to specific T cells [20]. Hence, the association of this allele has a clear molecular explanation. Binding of the HPA-1a peptide to the peptide-binding groove of the DRA/DRB3*0101 molecule is mediated by three anchor residues, where a small hydrophobic side chain of the Leu33 fits in the small P9 hydrophobic pocket [21]. The anchoring of Leu33 allows for a broader repertoire of HPA-1a-specific T cells, and different HPA-1a specific T cell clones have been isolated from one single HPA-1a immunized woman [166]. Substitution of the Leu33 with a valine or an isoleucine, which also have a small hydrophobic side chain, could also stimulate HPA-1a specific T cells [166].

PBMC from HPA-1a immunized women with β3 peptides containing the Leu33 residue has been shown to result in enhanced T cell proliferation compared to stimulation with peptides containing the Pro33 residue [167], and the generation of clonal HPA-1a-specific CD4 T cell lines has been reported [20, 95]. However, there is still no formal evidence that these T cells are directly involved in supporting antibody responses [154].

Through a literature search of prospective FNAIT studies, the risk of becoming immunized postpartum for HPA-1a negative women was estimated at 12.7% in *DRB3*01:01* positive women, compared to 0.5% in *DRB3*01:01* negative women [96]. However, the analysis did not allow analysis for nulliparous and multiparous women separately. The results were mainly based on results from the Norwegian screening and intervention study, where antibodies were detected in 210 of 1,990 women, and only 1,182 of 1,990 (65%) women were examined for antibodies postpartum [96].

In published studies, there seems to be a closer association between *DRB3*01:01* and immunization in retrospective cases compared to prospective cases, with an association close to 100% in retrospective studies [8, 10, 16, 91, 93]. Our data from prospectively included immunized women show a close but somewhat lower association to the allele. This may point to an effect of the *DRB3*01:01* allele to the severity of the disease. In that case, Kjeldsen-Kragh et al. (2020) find that 3 of 64 newborns with severe thrombocytopenia of *DRB3*01:01* positive mothers suffered ICH, while none of the 18 newborns of *DRB3*01:01* negative mothers were severely thrombocytopenic [165]. In retrospective cases, however, the mother's *DRB3*01:01* carrier status did not significantly influence the risk of having a child with ICH [165], nor did Wienzek-Lischka et al. (2017) find that the platelet counts in the neonates was dependent on the dose of *DRB3*01:01 [91]*. This study was, however, based on retrospective cases referred to a reference laboratory with generally very low platelet counts, indicating a selection towards more severe cases, and a potential dose effect may therefore have been missed.

It is also shown that there is a dose-effect of *DRB3*01:01* in HPA-1a immunization: Women homozygous for this allele have higher antibody-titre and lower platelet count than women with only one *DRB3*01:01* allele [97]. The authors presume that APCs with double dose of DRA/DRB3*0101 are able to bind more of the antigen than heterozygous cells, thereby offering a more efficient activation of the HPA-1a-specific T cells, and that this response also offers more

efficient help to HPA-1a-specific B cells and results in higher antibody levels. Another potential explanation for a dose-effect of *DRB3*01:01* may be that the increased expression of DRA/DRB3*0101, increasing the density of the peptide and HLA-complex on the cell surface of homozygous APCs, supports a more productive synapse, resulting in more efficient antigen-specific T and B cell activation [168]. The minimum number of TCR-peptide-HLA interactions required for T cell activation has been debated [168]. Other studies have shown increased levels of DRB3 transcripts in homozygous cells [169], and binding measurements of HPA-1a to B-LCLs have shown that homozygous *DRB3*01:01* cells bind more HPA-1a peptide on their surface than heterozygous cell lines [166].

Other alleles shown and suggested to be associated with HPA-1a immunization, are *DQB1*02:01* and *DRB4*01:01* [18, 98]. There may be synergistic effects of having two or more alleles on enhancing the chance of immunization. The different HLA molecules coded for by these alleles may cause different T-cell responses, restricted by different MHC molecules coded for by these alleles, acting together, and the activation of more T cells could result in a stronger immune response [154]. Because of the strong linkage disequilibrium between genes in the HLA complex, and the haplotype blocks the HLA alleles are inherited with, however, it is difficult to determine which genes are primarily involved in the associated disease and which genes are only secondarily involved only because of their linkage [170]. This again makes it difficult to discover possible mechanisms. As such, an association with an HLA allele may not be caused by that specific allele itself but rather by another allele or genetic element that is inherited on the same haplotype as the specific allele. HLA complex genes, however, most often hold the strongest genetic predisposition, and some HLA complex genes seems to be almost necessary, but not sufficient, for the most strongly HLA-associated diseases to develop [170], as is the case for *DRB3*01:01* in HPA-1a alloimmunization.

Loewenthal et al. (2013) found that 13 of 23 immunized women carried both *DRB3*01:01* and *DRB4*01:01* (57%), compared to none in the control group [98]. However, the frequency of *DRB3*01:01* among women in the control group was already as low as 2 of 24 women, giving a very low probability of having both alleles from the beginning. The authors also show, using modeling, that the DRA/DRB4*0101 molecule is able to bind the HPA-1a antigen, albeit with much lower affinity than the DRA/DRB3*0101. Only those peptides that can form stable complexes with MHC molecules have the potential to activate T cell responses [154], and it is not

shown by cellular experiments that peptides in complex with this molecule may activate HPA-1a specific T cells. Also, the association to this allele is contradictory, as Labbe et al. (1992) found that this allele was actually underrepresented in immunized women compared to non-immunized women [18]. Others have also found similar results: that this allele is not overrepresented in immunized women [91, 92]. The discrepancy between studies regarding *DRB4*01:01* may be explained by typing resolution. The allele may, however, also be linked to other gene elements, which favours immune response to the HPA-1a alloantigen [101], and thereby only be associated by the close linkage to other MHC gene elements.

In continuation of the above, the most probable allele to be associated with immunization, from our haplotype data, in addition to *DRB3*01:01*, is the *DQB1*02* allele. Thorsby et al. (1997), suggest that genes that to a major extent are responsible in HLA-associations are the genes encoding the peptide-presenting HLA molecules [170], and T cells specific to HPA-1a and restricted by HLA-DRA*05/DQB1*02 is not shown. We speculated that the already known association with *DQB1*02* only was a result of the allele being part of the DR3-DQ2 haplotype, which is one of the DR-DQ haplotypes that *DRB3*01:01* is in close linkage to. As expected, the DR3-DQ2 haplotype was overrepresented in *DRB3*01:01* positive women compared to a control group of *DRB3*01:01* positive individuals from the general population. The association with this haplotype accounted for about 80% of the *DQB1*02* association. However, the other DR-DQ haplotype carrying the *DQB1*02* allele, DR7-DQ2, was also overrepresented in already *DRB3*01:01* positive immunized women compared to the *DRB3*01:01*-positive random population. This suggest that *DQB1*02* actually has a function other than antigen presentation in immunization against HPA-1a.

Regarding *DRB4*01:01*, we found that this allele was actually overrepresented in already *DRB3*01:01* positive immunized women, although not frequently present. For all of the *DRB4*01:01* positive women in this group, however, the allele came in combination with the DR7-DQ2 haplotype. In the random population, the *DRB4*01:01* allele was distributed on more associated DR-DQ haplotypes. Many individuals were also DR7-DQ2 positive without carrying the *DRB4*01* allele at the same time (data not shown). Hence, although the *DRB4*01:01* molecule has been shown to possibly bind the HPA-1a antigen with low affinity, the association with this allele may be explained by the association with the DR7-DQ2 haplotype in HPA-1a immunization. Indeed, the *DQB1*02* allele is also overrepresented in immunized women in the work by Loewenthal et al. (2013) [98]. However, the possible association between the DR7-DQ2 haplotype

and *DRB4*01:01* is not mentioned in their study. Although DQA1*05/DQB1*02-restricted HPA-1a specific T cells have not been shown to exist, the existence of such cells cannot be ruled out.

The last allele positively associated with immunization is *DRB1*13* [91, 92]. Regarding the association both to *DRB1*13* and the DR13-DQ6 haplotype, we show that this association is positive because of the linkage to *DRB3*01:01*, and not by the DRB1*13 allele or the DR-DQ haplotype in itself. We also show that the prevalence of immunization in *DRB3*01:01* positive women carrying DR3-DQ2 is twice as high as in women carrying DR13-DQ6, and that the prevalence for immunization actually is lower in *DRB3*0101*-DR13-DQ6 positive women than in *DRB3*01:01* positive women in general. We therefore also argue that DRB13*01 seems to have no function in HPA-1a immunization.

The role of other genetic elements must also be considered, as DR-DQ haplotypes also may be parts of extended ancestral haplotypes (AH), also including the MHC part III of the MHC complex on chromosome 6. This part of the complex does, by example, contain the part encoding TNFα. AH 8.1, also including DR3-DQ2, is considered to be an "inflammatory haplotype". A decreased life-expectancy of 8.1AH carrier women has been found: they have an increased susceptibility for many autoimmune diseases, and an association of the AH8.1 class II marker alleles with ovarian cancer and breast cancer has also been reported [109]. The AH 8.1, among other alleles contain a TNF SNP (TNF-308A) possibly associated with higher levels of TNFα in haplotype-positive individuals [110]. The role of additional genetic elements in the mentioned haplotype cannot be ruled out.

Effect of anti-HPA-1a alloantibodies

During placentation extravillous trophoblast cells invade the maternal tissue, including the endometrium, the myometrium, and the spiral arteries. Thus, the fetal trophoblast cells invades deep into the maternal tissue, and some cells also displace the maternal endothelial cells and smooth muscles cells lining the spiral arteries. All these processes are necessary for the development of a functional placental unit. In summary, it seems that αVβ3 is an important integrin throughout various steps of the making of the placenta, even though depletion of β3 via gene mutation does not stop the reproduction process [27]. By hindering some of these processes, one can speculate that the placenta does not function adequately, and that the exchange between the mother and the fetus may

be non-optimal. We have shown that anti-HPA-1a antibodies, by the clone "26.4", do inhibit adhesion and migration of the extravillous trophoblast cell line HTR8/SVneo in vitro. Since it has been shown that anti-HPA-1a antibodies causes significantly reduced birth weight in boys, we suggest that the inhibition of adhesion, migration, and invasion by extravillous trophoblast cells during placentation may be one of the causes of this. However, for the anti-HPA-1a antibodies to affect placentation, they have to already be present in sufficient amounts during the first trimester, when placentation takes place. Anti-HPA-1a alloantibodies are detected already at week 17 [10] in primipara women, potentially in time to influence the very last part of placentation but likely too late for the antibodies to affect the making of the placenta in a significant way. Other pregnant women, however, already have anti-HPA-1a antibodies at the start of pregnancy, after immunization at an earlier occasion, that may affect the making of the placenta. The influence of antibodies on trophoblast cells were only measured in vitro in our study, by a trophoblast cell line. The next step will be to evaluate if the same effect holds also for primary isolated trophoblast cells and if different conditions alter the antibody effect.

The antibodies are of IgG type and are therefore transported over the placenta and bind the β3 integrin on fetal platelets but also bind other cells than platelets that also carry the HPA-1a epitope. Other possible mechanisms for anti-HPA-1a antibodies to affect the fetus may be through binding of the β3 integrin on fetal endothelial cells. Both platelet-expressed αIIbβ3 and endothelial- and tumor cell-expressed αVβ3 participate in angiogenesis, tumor growth, and metastasis [171]. Blocking αVβ3, the vitronectin receptor, suppresses endothelial migration during angiogenesis [172]. In a mouse model, Yougbare et al. (2015) found that anti-β3 antibodies induce ICH in αIIb-/- pups without thrombocytopenia [137]. They further showed that anti-β3 antibodies both inhibited angiogenic signalling, induced endothelial cell apoptosis, and decreased the vessel density in affected brains and retinas. Most importantly, the data suggested that the impairment of angiogenesis is the critical cause of ICH in FNAIT, rather than thrombocytopenia [137]. In that respect, Santoso et al. (2016) demonstrated anti-HPA-1a antibodies that only reacted with the αVβ3 complex in serum from mothers of ICH positive but not from ICH negative FNAIT cases [136], resulting both in reduced adhesion of endothelium to vitronectin, cell anoikis, and interference with angiogenesis [136]. It that case, is it also interesting that more HPA-1a alloimmunized patients died when signs of bleedings *other* than petechiae and ecchymoses were present together with ICH and that petechiae were reported in fewer patients with ICH than without ICH [144]. Also, the positive effect of IVIg on ICH does not necessary reflect the response in platelet numbers. In all studies

reporting on IVIg in the treatment of FNAIT, about 20% of the fetuses did not seem to respond in respect of platelet numbers – their platelet counts remained below 50 [141]. Taken together, anti-HPA-1a antibodies may be directed only against β3, against the αIIbβ3 complex, or against the αVβ3 complex [93, 133, 134, 136, 137], and thereby bind to and affect different cells and tissues, resulting in very different clinical effects in the fetus. This variation of anti-HPA-1a antibodies may also reflect the primary antigen source in HPA-1a alloimmunization. In that respect, it seems that antibodies directed against the αVβ3 integrin have more serious clinical effects, in forms of ICH and maybe growth restriction, than antibodies directed only against the αIIbβ3 integrin.

Is there a need for treatment, and who should be treated?

There is currently no specific treatment for the condition. As described, the treatment available is more general and the approach also differs among countries. For severely alloimmunized women, there are clear benefits of high dose IVIg administration, where IVIg reduces the risk of ICH in subsequent pregnancies [145]. IVIg also reduces the level of circulating alloantibodies in mice [137]. In RhD-immunization, this is thought to be mainly due to the negative feedback following high total circulating maternal IgG levels [32]. Further benefits of IVIg therapy may include interference with transplacental transfer of maternal antibodies by saturation of the trophoblastic Fc receptor [32]. However, inhibition of transfer of antibodies to the fetus may also have negative effects as the fetus also is protected against foreign antigens by antibody transfer from the mother by this receptor. In addition, treatment with IVIg has potential adverse effects [173].

ICH caused by HPA-1 immunization also happens in primigravida, as shown by Tiller et al. [145], and will not be recognized before the treatment with IVIg is too late to initiate. Severe HPA-1a and FNAIT is underdiagnosed in the absence of routine screening [16]. Undoubtedly, even if the number of children born with ICH not diagnosed during pregnancy will be low, for those women and children exposed, the outcome for the fetus or newborn could be catastrophic. This clearly indicates a need to identify pregnancies at risk.

Wilson and Jungners (1968) state nine criteria for screening, also referred to as the WHO criteria [174]. Murphy et al. (2002) [175] review these criteria in light of HPA-1a immunization and FNAIT. Among those criteria are the following (criteria in italics): *The natural history of the condition should be known.* Regarding the natural history in the mother, HPA-1a negative women are known to be at risk, and the percentage of HPA-1a negative women in the population is known.

*HLA-DRB3*01:01* is known to increase the risk of immunization; however, the PPV is only 35%. We also know that about 10% of HPA-1a negative women produce anti-HPA-1a antibodies. *There should be a recognizable latent or early symptomatic stage.* There is a no defined interval between the detection of antibodies in the mother and bleeding signs in the fetus. *Accepted treatment should be recognized.* As discussed earlier, there is no clear approach to antenatal treatment for the first affected pregnancy, and the treatment methods differ between countries including for children where there is a known previously affected pregnancy, and there is currently no specific treatment for the condition. *There should be a policy on who should be treated.* In the case of HPA-1a immunization and FNAIT, this criterion will need further discussion and more precise definition. *Diagnoses and treatment should be cost-effective.* Different research societies have tried to evaluate the effect of a potential screening by objectively defining costs and health consequences of a hypothetical screening [143] [175]. *Case-finding should be a continuous process.* This criterion will demand a continues national screening of all pregnant women.

In summary, some criteria will still have to be fulfilled to defend the introduction of a screening program. The most important criterion to meet is the development of a safe treatment method for the condition. In addition, it will be beneficial to more precisely define which women who are most at risk of immunization. While waiting for the screening criteria to be fulfilled, the condition will still be under-diagnosed.

A study is ongoing to offer treatment in the form of a hyperimmune anti-HPA-1a IgG prophylaxis for the prevention of HPA-1a immunization, resembling RhD prophylaxis [176]. Plasma from HPA-1a immunized female donors was collected in the NAITgam donation program. Rallybio acquired this NAITgam orphan drug program from Prophylix AS in June 2019 and has proceeded with clinical trials. The success in making this prophylaxis will also demand the introduction of a screening programme for the condition. In that case, and in continuation of the topic of the above paragraph, there is a need to more precisely identify women most at risk of being immunized, who will then be selected for further follow up and treatment. Equally important will be to select women with a very low risk of immunization who are not in need of further follow up and treatment. It is currently known that the *DRB3*0101* allele is associated with immunization. Kamphuis et al. (2010) found that there is "no HPA-1a immunization" in *DRB3*0101* negative women in 98.1% of *DRB3*0101* negative cases [139], suggesting that *DRB3*01:01* negative HPA-1a negative women may not need of further follow up, or treatment. As described above, pooled prospective data also shows an odds ratio for becoming immunized at 0.03 for HPA-1a negative/*DRB3*01:01* negative

women compared to HPA-1a negative/*DRB3*01:01* positive women [165]. The positive predictive value (PPV) of *DRB3*01:01* positivity, however, is low (26%) [139]. In addition, in retrospective studies the *DRB3*01:01* carrier status of pregnant women does not significantly influence the risk of having a child with ICH in severely thrombocytopenic children [165]. All studies that could narrow the selection of women for follow up are of value. Our HLA-haplotype study is therefore of value considering what HLA-associations to further focus on.

Another quite different approach to therapy is to induce tolerance to the HPA-1a antigen. It may be possible to tolerize potentially platelet-reactive lymphocytes. The balance between activation and tolerization of T cells depends both of the physical nature of the antigen, the state of the MHC, and the route of antigen administration. Tolerization is the result of clonal depletion of T cells and activation of regulatory T cells. Induction of tolerance to a particular antigen is more efficient prior to the immune system's first encounter with the specific antigen [154]. Sukati et al. (2005), suggest that a peptide including the immunodominant β3 epitope mapped may be used as a basis for the induction of tolerance to the HPA-1a antigen [177]. It has been shown in rodent models, that a peptide containing a dominant T cell epitope can prevent responses to the corresponding antigen when given by a tolerogenic mucosal route [177].

Last, we know that in most HPA-1a negative women pregnant with an HPA-1a positive fetus, even in combination with *DRB3*01:01* positivity in the mother, anti-HPA-1a alloimmunization does not happen. Placental debris and extracellular vesicles may have characteristics that make them harmless under normal conditions. Increased knowledge of fetal extracellular vesicles in maternal circulation, their characteristics, and their impact on both the mother's antigen-presenting cells and T cells may not only help in understanding the break of tolerance in HPA-1a immunized women but also in the development of treatment for HPA-1a negative women.

Methodological considerations

In vitro activation of HPA-1a specific T cell clones

In vitro cell culture systems can never completely mimic biological environments and systems, and in vivo T cells will always be under the influence of many more factors and cells than the cells in the cell culture system. This is also the case for HPA-1a specific T cell clones isolated from peripheral blood of HPA-1a alloimmunized women. However, the HPA-1a specificity and HLA-DRA/DRB3*0101 restriction also holds for in vivo functions of the T cells.

HPA-1a specific T cell clones were activated in our study by the presentation of HPA-1a antigen by *DBR3*01:01* positive monocytes both from trophoblast and platelets. To ensure that the antigen amount given to monocytes for processing and presentation was high enough to cause activation of the specific T cell clones used, the trophoblast cells were lysed and β3 integrin was immune-precipitated on epoxy dynabeads before being given to monocytes. In that way, we were able to measure and estimate the relative antigen amount actually immune-precipitated from both antigen sources for comparison. Macrophages are able to phagocytize both dynabeads and latex beads [178], and the principal receptor to govern phagocytosis is the FcR. The macrophages will process the uptake and present peptides in MHC on the surface. Phagocytosis of dynabeads can be visualized by a light microscope, as the dynabeads are brown, and some monocytes may be fully packed with brown beads. The indirect proof of phagocytosis is, however, the activation of the T cell clones, which requires phagocytosis, processing of the phagocytised material, and presentation of peptide by the monocytes. The monocytes cannot process dynabeads, and it is possible that the phagocytosis of numerous beads will somehow reduce the capacity of monocytes to process and present antigens, in addition to potentially also causing activation of the APCs. Since in this case the goal was to compare activation of T cells by HPA-1a antigen from platelets and trophoblast cells, and the same approach in respect of numbers of beads and volumes was used for both these cell types, we avoided these problems. The different T cell clones used reacted different to antigens with respect to the amount of antigen needed for activation and the degree of activation measured by production of TNFα or IFNγ. However, the clones did not differ between antigens from trophoblast and platelets.

The overall goal of this project was to demonstrate that HPA-1a antigen from trophoblast cells is able to activate HPA-1a specific T cell clones in the same way as HPA-1a antigen from platelets, following the idea that trophoblast debris, forming extracellular vesicles in different forms and sizes, carries the antigen and is therefore a potential carrier of antigens to monocytes. To demonstrate that HPA-1a positive placental microvesicles in maternal HPA-1a negative plasma also activate HPA-1a specific T cell clones would be optimal and was the main goal. We were not able to demonstrate that fetal microvesicles isolated from maternal plasma activate HPA-1a specific T cells. However, we were able to demonstrate that HPA-1a positive vesicles isolated from HPA-1a positive plasma may activate HPA-1a specific T cells, when taken up, processed and presented by monocytes, which also indirectly demonstrates that macrophages phagocytize microvesicles. In addition, we demonstrate that there are HPA-1a positive vesicles in plasma of HPA-1a negative women pregnant with an HPA-1a positive fetus.

We were able to sort fetal HPA-1a vesicles from maternal HPA-1a negative plasma both by FACS and by anti-HPA-1a coupled dynabeads and tried to activate T cell clones by this material, without success. We suggest that the reason for this is that the amount of antigen isolated is too small for the T cells to be activated. T cells were activated by antigen bound to beads from 4 mL plasma of an HPA-1a positive individual ($5x10^5$ beads) and by $6x10^4$ sorted positive vesicles directly from an HPA-1a positive individual. The fetal HPA-1a positive vesicles sorted and the number of vesicles bound to beads from HPA-1a negative pregnant plasma were expected to be very low, as also shown by the detection of very few HPA-1a positive events in plasma of HPA-1a negative women with HPA-1 positive fetuses. β3 expression on non-platelet vesicles in plasma is also probably much lower than on platelet vesicles. Unfortunately, we did not find a good approach to conclude on the amount of antigen given to T cells in those experiments, in part because the total amount of material working with was low. By using vesicles from HPA-1a positive plasma, however, we are able to isolate a sufficient number of vesicles for activation. Also, since it is proposed that vesicles made under different conditions is able to make different responses in phagocytizing monocytes, it could be of interest to sort extracellular trophoblast vesicles in equal parts, by example, of necrotic and apoptotic vesicles to evaluate the potential different effect on activation of T cells. Because of technical challenges when working with microvesicles, and limited access to pregnant plasma, this was not implemented in this project.

Working with T cell clones, and also with other living cells such as monocytes and platelets, is also challenging. The T cell clones are not transformed T cells. Hence, they will lose their ability to divide and they have limited life-time, and have to be re-stimulated and expanded regularly [166]. It is time consuming and challenging to keep the cells going, and to have cells available and healthy at the time of experiments. Monocytes also had to be isolated prior to each experiment, and was used fresh, or after 3 days in culture. The need for monocytes being *DRB3*01:01* positive and HPA-1a negative was also somewhat limiting for the frequency of performing experiments.

Technical challenges when working with extracellular vesicles

Extracellular vesicles come in many different forms and sizes, and the isolation protocols used in the literature seem also to differ greatly. Our main question was to answer whether fetal extracellular material shed into maternal circulation from the placenta could activate HPA-1a specific T cells when taken up, processed, and presented by maternal monocytes. For our purpose, and also to be able to characterize the vesicles in flow cytometry, we chose not to isolate the smallest vesicles. Also, we assumed that the microvesicles shed from ST would be the vesicles with most antigen on the surface as they are made by blebbing of the membrane of the host cell. Our isolation method was therefore easy and only included centrifugation at 17,000 x g, after removing platelets with centrifugation in two steps. Electron microscopy and flow cytometry analysis showed that vesicles were isolated by this approach. The HPA-1a positive vesicles were further isolated from all the other vesicles either by incubation with 26.4-coupled dynabeads or by sorting 26.4-positive vesicles in the FACS.

Characterization of the isolated vesicles in flow cytometry proved challenging, and during the time of the project, the field also has developed significantly. One of the challenges is to be sure that the vesicles analysed are indeed single events. The term “swarm detection” describes the fact that several particles are detected as one by the flow cytometer. This could involve several small vesicles being detected as one. There are, however, methods for demonstrating single particle analysis. By serial dilutions of a sample of single particles, the number of positive events will halve while the fluorescence intensity of positively stained particles should remain consistent [179]. We stained different dilutions of vesicles in our experiments, showing that the mean fluorescence intensity remained stable with the decrease of vesicles per ml. Goergens et al. (2019) attempted to optimize the use of imaging flow cytometry for the detection of single extracellular vesicles by

using CD63eGFP-labelled extracellular vesicles as a reference material [180] and found that within a range of $2x10^6$ and $3x10^7$ extracellular vesicles per ml there is a linear decrease with higher diluted samples while the mean fluorescence intensity values remain stable; within this concentration range, mostly single extracellular vesicles were detected. All our experiments were also done with low flow/min and with few events (EVs) per ml. If the main goal in our flow experiments with vesicles was to demonstrate that fetal HPA-1a positive vesicles can be found in plasma of an HPA-1a negative pregnant woman, however, swarm detection would not influence the result. If we rather were to say anything about the amount of such vesicles isolated, swarm detection could potentially have had an influence. In addition, when staining for several surface markers with two or more specific antibodies, what appears to be one vesicle positive for two surface markers, may actually be two separate vesicles, each positive for one of the two markers. Again, in our experiments, we did not primarily seek to quantify the number of vesicles. In addition, we used only HPA-1a as a positive surface marker and CD41 and CD14 as negative markers, avoiding the potential problem of two vesicles positive for each positive marker appearing as one vesicle positive for both markers.

Detection of events that are not truly vesicles could also be due to other particles from buffers, or immunoprecipitated antibodies, appearing as vesicles by analysis. Very large differences between individual antibodies in terms of antibody-related background has been described [180]. Antibody in buffer should be one of the controls. Others have also used filtration of both buffers and antibodies to avoid the risk of false positive vesicles by bigger particles in the buffers [181]. Staining for each antibody should be shown individually, and isotype controls are not suitable to control for false positive events in EV flow cytometry [180]. The experiments with vesicles were done some years ago. In presenting our results, however, we have tried to follow the MIFlowCyt-EV guidelines published in 2020 [182]. This is a framework for the minimum information that should be provided regarding the flow analysis of extracellular vesicles, developed by a working group of researchers working with extracellular vesicles in flow cytometry, built on both Minimum Information for Studies of EVs guidelines (MISEV) [183] and Minimum Information about a Flow Cytometry experiment standard (MIFlowCyt) [184]. The use of this guideline has been of great help in analysing and presenting flow cytometry results.

By using another method, we could also potentially determine how many vesicles are needed for activation of an HPA-1a specific T cell. A given number of positive vesicles from antigen positive plasma, primarily of platelet origin, causing activation of T cells will probably be too few in the

form of trophoblast vesicles due to lower protein expression on the surface. In addition to the assumed low expression of HPA-1a antigen on ST microvesicles, we also expect that the expression will differ between trophoblast vesicles, reflecting the areas with or without the expression of the antigen on the mother cell, and this is an obvious challenge when trying to detect so few fetal HPA-1a positive vesicles in maternal plasma.

> The "amount of fluorescence light a flow cytometers detector receives is dependent on the fluorophore's emitted power (brightness), the number of fluorescent molecules being illuminated, the flow cytometer`s laser intensity, and the characteristics of the optical components between the fluorophore and the detector" [179].

Larger EVs' expression of multiple copies of the antigen of interest will enable separation from the auto-fluorescent population. As EVs decrease in diameter along with the surface area with limited expression of the antigen, the fluorescent populations will decrease [179]. The instruments will not be capable of determining the protein expression of smaller EVs. Hence, the EVs characterized in our experiments were not the smallest microvesicles, and the fluorescence intensity was low, both because the vesicles were small and because the antigen is expressed at a low level. For fetal HPA-1a positive vesicles in HPA-1a negative pregnant plasma that we show, assumed to be of placental origin, the fluorescence intensity was low (much lower than the intensity from platelet vesicles). In addition, the intensity varied between vesicles, as expected.

The extravillous cell line HTR8/SVneo as a model cell line

To evaluate if anti-HPA-1a antibodies affect trophoblast function we used the extravillous trophoblast cell line HTR8/SVneo. We hypothesized that anti-HPA-1a antibodies somehow affect trophoblast function and thereby alter placentation in HPA-1a alloimmunized women, since anti-HPA-1a antibody formation was associated with significantly reduced birth weight in boys [15]. Invasion of the uterus wall starts by implantation of the blastocyst and continues until remodelling of the spiral arteries [23]. During this period, the migration and invasion of extracellular trophoblast cells are important for developing a functional placenta for further nourishment and growth of the fetus. We needed a cell line that was isolated from the time of placentation, when invasion takes place. Graham et al. (1993) [185] established a first trimester human trophoblast cell line, the HTR8/SVneo, by transfecting normal first trimester trophoblast cells with a plasmid containing the gene for the simian virus 40 (SV40) large T antigen (Tag). Expression of different surface integrins also differ along gestation. To be able to evaluate the potential effect of our anti-HPA-1a antibody, the cell line used also necessarily needed to express the β3 integrin on the surface. HTR8/SVneo express β3 integrin together with αV, forming the vitronectin receptor, and we have shown

previously that our anti-HPA-1a antibody, "26.4", binds the HPA-1a epitope on this receptor with even better affinity than to the fibronectin receptor on platelets [186]. The problems with this cell line, also discussed by others, is that it does not quite resemble extravillous trophoblast cells in all aspects, as it for example does not express the extravillous cell marker HLA-G and also shows low expression of the vitronectin receptor. In addition, the cell line may have altered its characteristics after several passages in culture.

HLA allele typing, and what is what in associations with disease?

As already discussed, the general problem with HLA and haplotype associations in disease is to interpret the result for single HLA alleles because of the close linkage between genetic elements in the MHC complex. In that respect, the *DQB1*02* allele has actually been associated with HPA-1a immunization for several years [18]. Despite this, however, and probably because of the lack of a molecular explanation of the association, it seems that the association is believed to be found because of the close linkage to the *DRB3*01:01* allele. In fact, this was also our assumption when starting the HLA project. To more precisely define which alleles will increase or decrease the risk of immunization, our preferred control group will be a group of HPA-1a negative women not immunized during or after pregnancy. Only such a control group will give the possibility to say anything about the risk of immunization by carrying specific alleles. In the absence of such a control group, we compared only individuals that were positive for the allele already known to be truly associated with immunization, *DRB3*01:01*. By comparing only those individuals, we were able to examine what other alleles that also might be associated. And our results seem to point to an actual effect of the *HLA-DQB1*02* in HPA-1a immunization. Before the potential effect is demonstrated, however, the actual association will be debated. As is also the case for *DRB4*01:01*. In addition, results from the Norwegian screening study, together with our own results, also gave us the possibility to calculate the prevalence of HPA-1a immunization when carrying different *DRB3*01:01* associated DR-DQ haplotypes.

The results we present for haplotypes were "interpreted using the set of alleles known at the start of the project" [100], over 10 years ago. Presenting and publishing data now, the methods we have used for HLA typing are both old and time-consuming. New HLA alleles are detected all the time as are new combinations of HLA DR-DQ haplotypes. Despite the fact that many of these alleles are very rare and only found in one population in one lab, we cannot rule out that by using old HLA

methods we may have mistyped some of our participants in the HLA study and lost one rare HLA allele. However, it is not likely that this will have affected the interpretation of our main results and our main questions in the study.

We have used published known DR-DQ haplotypes in the Norwegian population [105, 106] and focused on the most common ones in assembling the haplotypes of each individual, based on typing of single alleles. New HLA haplotype frequency reference standard in European Americans (2003) revealed that the frequency distribution of the 75 haplotypes was right-skewed and that only 16 haplotypes occurred at a frequency >1% [100], defending our approach for assembling haplotypes. The authors also measured the probability of an accurate allele assignment for the DQ portion of the haplotype if only the DRB1 allele had been typed (that is, the probability of assigning the most commonly occurring DQ haplotype for each DRB1 allele). As an example, the *DRB1*0301* allele appears at a frequency of 13.19%, and exists in two DRB1-DQA1-DQB1 haplotypes, and the frequency of the most common of these is 99.6%. The *DRB1*1301* allele appears at a frequency of 5.66%, existing in four haplotypes, and the frequency of the most common is 98.1% [100].

Our control population consisted of random blood donors from the University Hospital of North Norway, Tromsø, and the ethnicity of those donors was not known. The Norwegian population is predominantly Caucasian. However, Tromsø is the town in Norway with most self-registered Sami people. Without using much time and space to discuss the criteria of being registered as Sami in Norway (there is no such official register), it is possible that a significant number of Sami among our control population may have affected the haplotype distribution in the control group. We know that frequencies of HLA alleles and haplotypes in Sami differ from the Norwegian population [107]. Also, a lower total number of alleles were detected in the Sami at some loci: HLA-B, -C, -DRB1, which could be a result of genetic drift. Linkage disequilibrium between HLA loci was generally also found to be higher in Sami than in non-Sami Norwegian – which is also a sign of genetic drift and isolation in the Sami [107]. Both the frequency of *HLA-DQB1*02* and *HLA-DRB3*01:01* in our control populations, however, matches the frequency of these alleles in other Caucasian populations [101].

Concluding remarks

HPA-1a alloimmunization in pregnancy is related to platelets, both considering the cause and the effect of the immunization. However, we should not only blame the platelets. The HPA-1a antigen is not dangerous for women at risk without the wrong MHCs able to present the antigen to T cells crucial for the antibody response in HPA-1a immunization. We have shown that exactly what DR-DQ haplotype the *DRB3*01:01* comes with also influences the prevalence of immunization. It may also be dangerous for the fetus that the antigen is also presented on trophoblasts, that the HPA-1a antigen is visible for the mother during the whole pregnancy in forms of extracellular vesicles, and that the anti-HPA-1a antibodies affect the trophoblast cells during important periods of placentation and bind to endothelial cells and cause ICH during pregnancy. It may be that the platelets and the thrombocytopenia in itself are not excessively dangerous.

Future perspective

A new prospective study including screening of all pregnant women would benefit from HLA typing of all HPA-1a negative women, including those not immunized. This will help to more precisely define which HLA-alleles actually make a difference in immunization, and may help both in revealing the immune mechanisms in HPA-1a immunization and in detecting women most at risk, both for immunization and for ICH. To be able to say anything about the risk of immunization due to carrying the different HLA alleles, the study should also include the gravida- and para-history of all women prior to their entry into the study, and all women should be followed for several years. A new prospective study should also preferentially include detection of antibodies both against β3 integrin, αIIbβ3 integrin complex, and αVβ3 integrin complex.

The potential effect of anti-HPA-1a antibodies on trophoblast function may further be studied in vitro both using different first trimester trophoblast cell lines in addition to first trimester trophoblast cells isolated from pregnant women. Considering the growing knowledge of the variety of anti-HPA-1a antibody-specificities, both antibodies against the β3 integrin, the αIIbβ3 complex, and the αVβ3 complex should be studied.

To answer if HPA-1a positive fetal vesicles in plasma of HPA-1a negative women carrying HPA-1a fetuses activate T cells will probably be possible only by increasing the amount of isolated HPA-1a positive vesicles, further using the same T cell assays as already described. It would be helpful to somehow be able to estimate the ratio of the β3 integrin expression on microvesicles of both platelet and trophoblast origin and to calculate the approximate number of trophoblast vesicles that express the same amount of integrin as one platelet vesicle. Characterization of other markers on HPA-1a positive fetal vesicles in plasma, in addition to definition of vesicle size will also be important to evaluate potential variable effect of different types of vesicles on immune cells, and will likely need an approach other than flow cytometry.

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Title page

Placenta-derived antigen in HPA-1a alloimmunization

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Abstract

Antibodies against human platelet antigen (HPA)-1a are the most common cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT) in Caucasians. HPA-1a is located on β3 integrin, as part of αIIbβ3, on platelets, and alloimmunization may be initiated by transplacental hemorrhage and release of fetal platelets into the maternal blood stream during pregnancy or in connection with delivery. However, it is not known whether the number of platelets entering the maternal circulation during pregnancy and at delivery is high enough to cause alloimmunization, and a considerable percentage of women are already immunized during their first pregnancy. Since the HPA-1a antigen that causes HPA-1a alloimmunization is found also on trophoblast cells, αVβ3, during pregnancy and these cells are indeed in direct contact with the maternal circulation and immune system, both as intact cells and trophoblast derived vesicles shed into the maternal circulation, it is possible that these cells are also responsible for alloimmunization during pregnancy.

We have isolated β3 integrin from a trophoblast cell line, and show that HPA-1a antigen from αVβ3 on trophoblast cell lines activates HPA-1a-specific T cells in the same way as antigen from platelets, mostly from αIIbβ3, when processed and presented by *HLA-DRB3*01:01*-positive monocytes. By isolating extracellular vesicles from plasma, we also show that HPA-1a specific T cells may be activated by HPA-1a antigen-positive extracellular vesicles from plasma of HPA-1a positive individuals. Analysis in flow cytometry of isolated extracellular vesicles show that fetal HPA-1a positive vesicles can be detected in maternal plasma of HPA-1bb women pregnant with an HPA-1ab fetus, and some of these HPA-1a positive fetal vesicles seem to be of placental origin.

Introduction

Antibodies against human platelet antigen (HPA)-1a are the most common cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT) in Caucasians [1]. Antibodies cross the placenta, bind fetal platelets and cause thrombocytopenia in the fetus or newborn baby, in most severe cases with intracranial hemorrhage or death as consequences [2].
HPA-1a antigen is localized on integrin β3 as part of glycoprotein αIIbβ3 (fibronectin receptor) on platelets [3]. The initial immune response that results in FNAIT is not fully described. For instance, as integrin β3 is expressed also by other types of cells in addition to platelets, it is not known which of these tissues the antigen that gives rise to the activation of immune cells is derived from. Neither is it known what triggers HPA-1a alloimmunization in pregnancy. One possibility is that HPA-1a alloimmunization is initiated by transplacental hemorrhage and release of fetal platelets into the maternal blood stream in connection with delivery. However, a substantial proportion of cases of alloimmunization have been reported to occur in primigravida [1, 4], suggesting that alloimmunization can occur during pregnancy. An alternative source of alloantigen stems from the placenta, from fetal trophoblasts. Trophoblasts express αVβ3 (vitronectin receptor) [5, 6], and material from such cells is shed to the maternal circulation during pregnancy. In this respect, a recent study demonstrate anti-HPA 1a antibodies only reacting with the αVβ3 complex in serum of HPA-1a negative women with ICH FNAIT cases [7], indicating an immune response against HPA-1a associated with the vitronectin receptor rather than the fibrinogen receptor.
Extravillous trophoblast cells, which are of fetal origin, invade the maternal tissue during placentation. In addition, the outer layer of trophoblasts, the syncytiotrophoblast, is bathed in maternal blood. Moreover, relatively large amounts of shed material of trophoblast origin, also containing integrin β3, is released into the maternal circulation in increasing amounts as the placenta grows [8]. Thus, there seems to be plenty of relevant trophoblast derived alloantigen already present in the maternal circulation *during* pregnancy that can account for immunization. The constant exposure of the trophoblast-derived integrin β3 alloantigen to the maternal immune system during pregnancy suggests that this may also be one of the antigen sources in HPA-1a alloimmunization, also resulting in anti-HPA-1a antibodies against the αVβ3 complex.

A key event in immune responses involving the differentiation of an antigen specific B cell into an IgG producing plasma cell, is the activation of T helper cells that respond to the same specific antigen complex. Previously it was shown that DRA/DRB3*0101-restricted HPA-1a-specific CD4+ T cells could be isolated from women who had given birth to a child affected with FNAIT [9], and that these cells respond specifically to platelet derived antigen as well as synthetic peptides. We aim here to show that HPA-1a antigen derived from placental tissue, when processed and presented by *HLA-DRB3*01:01*-positive monocytes, may activate HPA-1a-specific T cells, in the same way as HPA-1a antigen from platelets, and that placental extracellular vesicles expressing the HPA-1a antigen can be isolated from maternal plasma.

Methods

The project was approved by the Regional Committee for Medical Research Ethics, North Norway, approval no. 2012/1649. The approval expired before preparation of the manuscript.

Donors

An HPA-1bb, *HLA-DRB3*01:01* positive male donor, donated peripheral blood for isolation of monocytes. Buffy coats from random donors donating blood at the Blood Bank at the University Hospital of North Norway were used for isolation of HPA-1aa, -1ab, and -1bb platelets, and red blood cells (RBC).

EDTA blood from random donors were used for isolation of plasma and plasma-derived extracellular vesicles. Plasma from HPA-1a-negative and HPA-1a-positive pregnant women were used for isolation of plasma-derived extracellular vesicles.

HPA-1a-specific DRA/DRB3*01:01-restricted T cell clones previously established from HPA-1a alloimmunized pregnant women [9] were used for T cell studies.

Antigen sources

Platelets

Platelets were isolated from buffy coats of random donors. Blood were transferred to 50 mL centrifugal tubes and centrifuged at 155xg for 15 minutes without acceleration or brakes. Platelet rich plasma (PRP) were collected, diluted in PBS, centrifuged at 200xg for 15 minutes once more without acceleration or brakes, and the diluted plasma on top of RBC and cells were collected. Platelets were further washed twice in PBSA 0,2 % and resuspended in medium for cell culture experiments, or other suitable buffers for lysis or freezing. The HPA-1 genotype of the buffy coat donors were detected by isolating DNA from 200 μL of the buffy coat before isolating of platelets, and further genotyping as described elsewhere [10].

Cell lines

The extravillous cell line HTR8/SVneo was kindly provided by Dr. Charles Graham [11]. This is an immortalized human trophoblast cell line from first trimester villous explants used as a model for extravillous trophoblast cell function. The cell line expresses integrin β3, but does not express the EVT marker HLA-G on its cell surface.

Another trophoblast cell line, TCL-1, established from term placenta, was kindly provided by Dr. Satoru Takeda Tokyo, Japan [12]. This cell line expresses both extravillous trophoblast cell markers integrin β3 and HLA-G on the cell surface.

Isolation of placental extracellular vesicles (ECVs) from plasma

Plasma was centrifuged at 300xg to remove cells, and further twice at 1,600xg to remove platelets, and the platelet poor plasma (PPP) was cryopreserved at -70 °C until use. After rapid thawing in 37 °C water bath, the plasma was centrifuged in 1.5 mL tubes at 2,000xg to remove remaining platelets and macrovesicles, and further at 17,000xg for 25 minutes at 4°C to pellet extracellular vesicles. Filtrated PBS (fPBS) (0.2 um) was used as buffer, and the pellet was resuspended in 1mL fPBS and washed once (17,000xg for 15 minutes). The supernatant was removed by pipetting, and the pellet was resuspended in 0.2 μm fPBS or T cell medium (TCM; IMDM 10 % FBS, 4 % HPA-1a negative human serum, and penicillin-

streptomycin) before staining for flow cytometry, preparation for electron microscopy, or further experiments in cell culture. Sterile fPBS was used as a buffer for analysis further involving cell culture.

EDTA blood from new donors were centrifuged for 1,500xg in 15 minutes without brake. PPP was collected and centrifuged at 2,000xg in 10 minutes x2 at 4 °C, to remove platelets, before further isolation of extracellular vesicles, as described above.

Lysis of platelets, TCL-1, and HTR8/SVneo trophoblast cells

HTR8/SVneo cells, TCL-1 cells, and isolated platelets were washed twice in cold PBS, suspended in 3 mL RIPA buffer (R0278, Sigma Aldrich) together with protease inhibitor (cOmplete Tablets Mini, Roche), pushed through a 23m syringe 5 times, and centrifuged at 10,000xg for 20 minutes. The lysates were transferred to new Eppendorf tubes and kept at 4ºC for short storage, or at -20ºC for long time storage.

Immunoprecipitation of β3-integrin with dynabeads

Epoxy M-270 dynabeads (Invitrogen, cat.no. 143.11D) were coated with an anti-β3-integrin antibody; clone SZ21 (Beckman Coulter), PM 6/13 (Sigma Aldrich), EPR2417Y (Abcam), AP-3, or 26.4 (in-house mAb 10 µg/1 mg beads) to a finale concentration of 10 mg/ml. The β3-integrin from cell lysates, or integrin β3-positive ECVs, was immunoprecipitated by incubating antibody-coated beads at 4ºC on a tilter overnight (or for 4 hours) with cell lysates of different dilutions or with a suspension of isolated ECVs. The lysate or suspension was removed, and the beads were resuspended in RIPA buffer, fPBS, or T cell medium for further analysis.

Antigen presentation: Isolation of PBMCs and monocytes

For isolation of PBMCs peripheral EDTA blood was diluted 1:2 in PBS, layered over lymphoprep (Axis-Shield, United Kingdom), and centrifuged for 15 minutes at 800xg, with slow acceleration and without breaks. PBMCs were collected from the interface and washed twice in 40 mL PBS 0.2 % BSA. Cells were pelleted by centrifugation at 350xg for 4 minutes,

suspended in FBS 10 % DMSO and frozen until used for isolation of monocytes, or used for isolation of monocytes directly.

Monocytes were isolated prior to each experiment by suspending up to 10^8 PBMCs in 1.2 mL FBS, mix with 800 μL irradiated RBC from a random donor and 100 μL Rosette Sep Human Monocyte Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC). After incubation at room temperature for 20 minutes, the mix was diluted in 2 mL PBS, layered on top of 3 mL Lymphoprep, and centrifuged at 800xg for 15 minutes with slow acceleration and without brakes. Monocytes were collected at the interface, washed twice in PBS 0.2 % BSA and resuspended in medium.

Detection of antigen

Western Blot

Platelets (HPA-1aa;$16x10^8$ cells) and HTR8/SVneo cells ($20x10^6$ cells) were lysed. Twelve microliter of platelet lysate, diluted 1:1000, and 12 μL of HTR8/SVneo cell lysate, or beads with β3-integrin were reduced and separated in a 4-12 % SDS polyacrylamide gel (*NP0336 Life Technologies*). Electrophoresed samples were transferred to a PVDF membrane (*LC2005, Life Technologies*) for one hour. Non-specific binding was blocked by *Super blocking buffer (#37525, Thermo scientific),* 0.05 % Tween 20, and 0.2 % goat-IgG (*#31245, Thermo scientific*) for one hour. Antibodies were diluted in Super blocking buffer 0,05 % Tween 20. Primary antibody anti-β3 integrin (1:2000, *Clone EPR2417Y, Epitomics/Abcam*), was added over night at 4˚C. After washing, horseradish peroxidase-conjugated goat anti-rabbit IgG *(1:1000, #32460, Thermo scientific*) was added for 1 hour followed by 3 washes in PBS 0.05% Tween 20. The β3-integrin protein band was visualized using the luminescent image analyzer ImageQuant LAS 4000.

Flow cytometry: amount of antigen on dynabeads

Antibody-coupled beads ($2x10^6$) with bound antigen were blocked with Super Block buffer with 0.05 % Tween 20 and 0.2 % goat IgG for 15 minutes in room temperature. After removal of buffer the beads were incubated with anti-β3 antibody (EPR2417Y, Abcam) for 15 minutes in a tilter, and 10 minutes without movement. The buffer was again removed and the beads

were washed once in Super Block buffer before incubation with secondary antibody goat anti-rabbit IgG-FITC in Super Block buffer 0.05 % Tween 20 for 15 minutes in a tilter, and 10 minutes without a tilter at the dark in room temperature. The beads were washed and resuspended in 400 µL PBS for flow cytometry.

Electron microscopy: Extracellular vesicles isolated from plasma

Extracellular vesicle (EV) suspension was fixed with 8 % formaldehyde in 200 mM Hepes overnight. Samples were then centrifuged, supernatant removed and the EVs pelleted in 12% gelatine. After infiltration in 2.3 M sucrose, small cubes of samples were mounted on aluminium specimen pins and frozen by immersion in liquid nitrogen. Ultrathin sections were cut on a Leica EMUC6 Ultramicrotome with a Ultracut S with a Leica FCS cryochamber using a diamond knife (Diatome Ltd., Nidau, Switzerland). Sections were retrieved from the knife surface with drops of methyl cellulose mixed with 2.3 M sucrose, thawed and mounted on Formvar coated specimen grids. The specimens were examined in an Jeol 1010 Transmission Electron Microscope (Tokyo, Japan) with a Morada digital camera (Olympus, Germany) [13-15].

Flow cytometry: Characterization of extracellular vesicles

Isolated EVs were resuspended in 100 µL 0.2 µm fPBS, and incubated with directly labeled antibodies against surface markers on the EVs for 20-30 minutes, washed once in 1 mL fPBS, and resuspended in fPBS for analysis in flow cytometry. The anti-integrin β3 antibodies used were AP-3 (12 ng/µl) and 26.4 (12 ng/µl), in-house conjugated with AF-488 (Alexa Fluor Antibody Labeling Kits, Life Technologies, MAN0006868), to detect β3 integrin positive and HPA-1a positive vesicles. Anti-CD41 antibody (0.05 µg/ml, clone HIP8, Invitrogen 12-0419-42, 0.05 µg/mL isotype control cat # 12-4714-71 Bioscience) and anti-CD14 antibody (5 µL in 100 µL, Invitrogen, MHCD 1427) were used as markers for non-placental material. Both fPBS, unstained controls, and single stained controls were used in each experiment. In addition, a serial dilution of vesicles to show the detection of single particles, buffer with added antibodies to show potential aggregates, and a detergent analysis with triton 1 % to show membrane-enclosed vesicles was added as controls.

A FACS Canto II flow cytometer (BD) was used to analyze vesicles. The flowrate was set to "low" (10 μL/min), and the samples were diluted to 40-100 events/sec before analysis. FCS threshold and SSC threshold were set to 200, and vesicles were collected in SSC-A/FSC-A scatter plot (figure 3). Log scale was used, and data was analyzed using biexponential axes. Sorting of HPA-1a positive and HPA-1a negative vesicles, was performed with FACS Aria III with instrument settings by CS&T, 70-micron nozzle, yield precision mask, and sort rate 40 events/s. Post sort showed 86.9 % true positive events and 89.4 % true negative events. Flow cytometric data were analyzed using Flow Jo (BD).

Activation of T cells

Cell cultures and TNFα assay

Monocytes ($5x10^4$) were cocultured with platelets ($1x10^6$), beads ($1x10^6$ beads) with different amounts of antigen, or different amounts of extracellular vesicles in 96-well round bottom plates (total volume 200 μL) over night. After co-culture, the plate were centrifuged at 823xg for 5 minutes, 100 μL medium were removed, and $5x10^4$ CFSE-stained [9] T cells from the HPA-1a-specific T cell clone D8T108 or D7T4 were added per well.

To detect TNFα at the cell surface of stimulated T cells, as a measure of activation, a TACE inhibitor was included to block proteolytic release of surface TNFα [16]. Cell surface TNF was detected on the surface of CFSE-stained T cells by an anti-TNFα antibody (PE Mouse Anti-Human TNF, BD biosciences).

ELISpot: Activation of HPA-1a-specific T cells

The INFy-secretion ELISpot assay was performed as described by Ahlen et al (2009) [9]. Synthetic L33 and P33 12-mer peptides (β3 intergrin derived peptides spanning the HPA-1 defining SNP were used as controls [9].

Dynabeads ($4.5x10^6$) were incubated with EVs isolated from 1.5 mL plasma, resuspended in 1 mL PBS, for 4.5 hours, in a tilter at 4° C. After removal of buffer and washing, the beads were resuspended in T cell medium for further experiments. $1x10^6$ beads were incubated with $6x10^4$ monocytes overnight, before monocytes were further co-cultured with D7T4, $3x10^4$

monocytes per well together with $3x10^3$ T cells, in parallel. EVs from an HPA-1a positive random donor, also bound to beads, was used as a positive control.

EVs were sorted from HPA-1aa and HPA-1bb plasma of healthy donors. A total of 120.000 HPA-1a-positive vesicles and 200.000 HPA-1a negative vesicles were sorted, given to monocytes ($6x10^4$ HPA-1a positive EVs per well in parallel), for further analysis in ELISpot according to the protocol described above. In addition, extracellular vesicles from an HPA-1bb woman pregnant with an HPA-1ab fetus, and from an HPA-1bb woman pregnant with an HPA-1bb fetus were isolated by centrifugation of 4 ml plasma per woman.

Proliferation assay: Proliferation of HPA-1a-specific T cells

T cell clones were washed twice in PBS, resuspended in PBSA (0.1 %) and CFSE (200 ng/mL, Invitrogen, C34554) incubated at 37 ^{0}C for 10 minutes, cooled on ice, and resuspended in T cell culture medium (TCM). The cells were further washed twice in PBSA and once in TCM, before transferred to round-bottom wells of a 96-well culture plate (Nunc). Labeled T cells ($5x10^4$) were combined with $6x10^4$ monocytes, and run in parallel. The monocytes were already co-cultured with beads with bound antigen, directly with extracellular vesicles, as described above, or directly pulsed with synthetic L33 (5 μM) or P33 (5 μM) peptide as controls. T cell clones were kept in culture for 7 days before analysis in flow cytometry. An anti-CD14 antibody was used to identify monocytes, and the gate was set on CD14 negative events to analyze T cells only. CFSE fluorescence intensity was measured relative to the non-stimulated T cells, as a measure of proliferation.

Results

HPA-1a positive extracellular vesicles from plasma activate HPA-1a-specific T cells

Extracellular vesicles from plasma were successfully isolated by centrifugation, as shown by flow cytometry and electron microscopy (figure 1A and B). Serial dilution of HPA-1a positive vesicles stained by 26.4-AF488 shows that single events are analyzed (figure 1C). The majority of isolated vesicles were platelet particles and stained positive for CD41 (data not shown) and CD61 (figure 2A). The in-house monoclonal anti-HPA-1a antibody, mAb

26.4, is a highly allo-specific antibody with the potential to differ between maternal and fetal EVs in HPA-1 incompatible pregnancies (figure 2A). HPA-1a positive and HPA-1a negative vesicles were sorted from vesicles isolated from HPA-1a positive plasma and HPA-1a negative plasma respectively, either by staining with 26.4-AF488 and sorting in FACS (figure 2A), or by binding to anti-HPA-1a coated dynabeads. Vesicles bound to beads or vesicles alone were given to monocytes for processing and presentation, and monocytes were further co-incubated with the HPA-1a-specific T cell clone D7T4 (figure 2B, illustration). Activation of D7T4 was measured by secretion of IFNγ in ELISpot assay. Both HPA-1a positive EVs alone and vesicles isolated from HPA-1a positive plasma and bound to anti- HPA-1a-coupled dynabeads activated the HPA-1a-specific T cells (figure 2C).

HPA-1a positive extracellular vesicles of placental origin can be detected in plasma of HPA-1bb women carrying HPA-1a positive fetuses

A very small portion of isolated vesicles from plasma of HPA-1bb women carrying HPA-1ab fetuses were HPA-1a antigen positive (26.4 positive) (figure 3A), indicating that these are vesicles of fetal origin. In comparison, EVs from HPA-1a plasma of a non-pregnant donor contained a big population of HPA-1a positive vesicles, while vesicles from plasma of an HPA-1bb woman carrying an HPA-1bb fetus contained almost no HPA-1a positive vesicles (figure 3A). Staining of vesicles with anti-HPA-1a antibody (26.4) also gave the opportunity to positively sort these vesicles by FACS, or by binding to antibody-coupled dynabeads, for further experiments. About 40 % of the HPA-1a-positive vesicles in an HPA-1bb woman carrying an HPA-1ab fetus were also negative for platelet marker CD41, and monocyte marker CD14, indicating that they are actually derived from placental tissue (figure 3B), while 20 % of the CD14 negative HPA-1a-positive vesicles were CD41 positive, indicating that they are platelet derived. Also, turned around, some isolated CD14- and CD41-negative vesicles were 26.4 positive (figure 3B). In summary, some 26. 4 positive EVs detected in HPA-1bb plasma of a woman pregnant with an HPA-1ab fetus is CD41- and CD14- negative, indicating that these are EVs of fetal placental origin. Antibodies against placental markers as PLAP and HLA-G were also attempted used as positive markers for extracellular trophoblast, however, with much difficulties concerning unspecific binding (data not shown). By examining EVs using multicolour flow cytometry there is a risk of antibody precipitates appearing as EVs in scatter plot, as is also demonstrated in figure 3B. Some of these events

are also included in the true EV gate. However, none of the precipitate events are detected as 26.4 positive.

Fetal HPA-1a positive extracellular vesicles from plasma of HPA-1a negative woman failed to activate HPA-1a specific T cells

HPA-1ab fetal vesicles from plasma of an HPA-1bb woman were isolated either by FACS sorting, or by using 26.4 antibody-coupled dynabeads. However, HPA-1ab fetal vesicles from HPA-1bb maternal plasma did not activate the D7T4 HPA-1a-specific T cell clone, neither alone (data not shown) nor bound to beads (figure 4). The number of HPA-1a positive sorted events, however, was low compared to the number of vesicles isolated from HPA-1a positive plasma that was able to activate the specific T cell clone. The T cell clone, D7T4, was activated by antigen from about $6x10^{4}$ HPA-1a positive extracellular vesicles from HPA1a positive plasma in ELISpot (figure 2C), and was also activated both to secrete IFNγ in ELISpot and to proliferate by antigen from about $5x10^{5}$ beads with bound HPA-1a positive EVs from HPA-1a positive plasma.

β3 integrin is expressed by the first trimester trophoblast cell line HTR8/SVneo

Since HPA-1a positive fetal EVs, assumed to be of trophoblast origin, from HPA-1bb maternal plasma did not activate HPA-1a specific T cells, although possibly due to too low amount of antigen, we attempted to investigate whether HPA-1a antigen from trophoblasts activate T cells in the same way as HPA-1a antigen from platelets. To answer this, HPA-1a antigen was isolated from platelets and trophoblast cell lines. The first trimester extravillous trophoblast cell line, HTR8/SVneo, express β3 integrin (figure 5A), and β3 integrin from both trophoblast cell- and platelet lysates could be immunoprecipitated on anti-β3 antibody-coupled dynabeads (figure 5). The amount of β3 integrin immunoprecipitated on anti-β3 antibody-coupled dynabeads was determined by flow cytometry using a FITC-conjugated anti-β3 antibody that bound another epitope of the integrin than the antibody used for immunoprecipitation, and was found to be at a relatively higher level from platelet lysate compared to lysate from the trophoblast cell line (figure 5B).

HPA-1a antigen from extravillous trophoblast cells activate HPA-1a specific T cells

The HPA-1a specific T cell clone, D8T108, was activated by antigen from trophoblast cells, shown by the expression of TNFα, when processed and presented by *HLA-DRB3*01:01* positive monocytes (figure 6A). The T cell clone was tested for the ability to be activated both by HPA-1a antigen from platelets and extravillous trophoblast cells (HTR8/SVneo and TCL-1). HPA-1a antigen from platelets or trophoblast cells were immunoprecipitated on anti-β3 integrin antibody-coupled beads and given to monocytes. The monocytes were further incubated with the HPA-1a specific T cells, and the activation of T cells was measured by flow cytometry, by the amount of TNFα expressed by activated T cells (figure 6A).

T cells are activated by HPA-1a antigen in a concentration dependent manner, by antigen from both trophoblast cells and platelets

Activation of T cells followed the same pattern in response to the amount of antigen regardless of if the source of antigen was platelets or trophoblast cells (Figure 6B). Two different HPA-1a-specific T cell clones, clone D7T4 and D8T102, were activated with isolated HPA-1a antigen from trophoblast and platelets following uptake and presentation by *DRB3*01:01* positive monocytes. Activation of T cells was determined by the antigen amount from beads (figure 6B). Different amounts of antigen were incubated with antibody-coupled beads, and the amount of antigen bound to beads was measured by flow cytometry, as described above. The same amount of antigen from platelets and HTR8/SVneo cells resulted in the same grade of activation of T cells. Both the percentage of activated T cells secreting TNFα and the amount of secreted TNFα (median PE-intensity of stimulated T cells) were antigen concentration dependent, and independent on the source of antigen, in clone D7T4. For the other clone, clone D8T102, almost all cells secreted TNF upon stimulation by HPA-1a antigen independent of the amount of antigen given from both antigen sources, as long as the antigen concentration was high enough for activation. However, the amount of secreted TNFα fell with falling antigen concentration also for this clone. In conclusion, the activation of HPA-1a specific T cells is equal for HPA-1a antigen from trophoblast and platelets when the antigen is presented in equal amounts from dynabeads.

Discussion

We show here, that extracellular HPA-1a positive vesicles can be isolated from plasma by centrifugation, and that such vesicles also may activate HPA-1a specific T cells when taken up, processed and presented by *DBR3*01:01* positive APCs. Also, HPA-1a positive vesicles, necessarily of foetal origin, and most likely also of placental origin, were detected in - and isolated from plasma of HPA-1bb women pregnant with an HPA-1ab foetus. However, we were not able to show that these vesicles also activate specific T cells, and the possible reasons for this are discussed below. HPA-1a specific T cell clones isolated from HPA-1a immunized women can be activated by HPA-1a antigen both from trophoblast cells and platelets. The activation of T cells is dependent on the amount of antigen. However, it does not matter for the specific T cells if the antigen comes from placental cells, αVβ3, or from platelets, primarily αIIbβ3, as long as the antigen can be taken up, processed and presented by an antigen-presenting cell (APC) to the HPA-1a specific T cell.

The number of foetal platelets entering the maternal circulation during a normal pregnancy, or even at delivery, following transplacental haemorrhage is not known. However, as reviewed by Curtis [17], studies of blood exchange in Haemolytic Disease of the Foetus and Newborn (HDFN) estimates that between 0.5 and 1.0 mL of foetal blood enters the maternal circulation at parturition [17-19]. With a normal platelet count (150-400x10 ^{9}/L) in the foetus at least from the second trimester of gestation [20], a maximum of about 4 x10^{8} foetal platelets will enter the maternal circulation with such bleeding. We do not know if this is enough antigen for a primary immune response to occur. And, since the number of platelets entering the circulation at delivery not for certain carry enough antigen to cause HPA-1a immunization, it is even more uncertain if smaller amounts of platelets entering the maternal circulation, as a cause of smaller haemorrhages, during pregnancy may cause immunization. A fair percentage of HPA-1a immunized women are, however, immunized during their first pregnancy [1, 4, 21], suggesting that other sources than platelets also may be responsible for antigen in HPA-1a immunization.

From the above discussion, it is not definite that HPA-1a alloimmunization is caused by antigen from foetal platelets *during* pregnancy. In addition, anti-HPA-1a antibodies have been

shown both to be directed against β3-integrin alone, against the αIIbβ3 complex, or against the αVβ3 complex [7, 22-24]. The existence of anti-HPA-1a antibodies that preferentially bind to the αVβ3 complex also indicates that a source of HPA-1a antigen is the vitronectin receptor, either alone or in addition to fibrinogen receptor. The vitronectin receptor is expressed on platelets, although the expression is very low [25, 26]. HPA-1a antigen is, however, also expressed as part of the αVβ3 also in the placenta, on both extravillous cytotrophoblasts and on syncytiotrophoblast. And we know that extracellular vesicles of different kinds and different sizes enters the maternal circulation as the placenta grows [27]. Herein, we therefor asked whether HPA-1a antigen from placental cells, extracellular vesicles, and/or other placental debris in the HPA-1a$^-$ maternal circulation is able to cause immunization.

We show that HPA-1a antigen from placental cells (vitronectin receptor, αVβ3) activates HPA-1a specific T cells in the same way as antigen from platelets (fibrinogen receptor, αIIbβ3). The T cells were also activated by HPA-1a antigen on extracellular HPA-1a positive vesicles from HPA-1a positive plasma. However, we were not able to show that foetal EVs with HPA-1a antigen in negative women could activate the same specific T cells. We argue that a possible reason for this is that the amount of antigen isolated in our experiments was not sufficient to activate our T cells. There is no doubt, from our experiments with isolated integrin β3 from trophoblast cells and platelets, that integrin β3 from trophoblast activates HPA-1a specific T cells, when the antigen amount is sufficient. As shown, in flow cytometry, the number of HPA-1a positive EVs in plasma of HPA-1bb mothers with HPA-1ab fetuses is very low. The number of EVs or amount of antigen per bead in all our experiments done with beads and microvesicles are not known, and using an approach to try to control for the amount of antigen in those cases would have given us even less antigen for further use in activation experiments. Neither is the maximum amount of HPA-1a positive vesicles possible to isolate from plasma of HPA-1bb women pregnant with HPA-1ab foetuses known, and will probably also vary both between individuals and between different time points in pregnancy. Regardless, the amount of foetal HPA-1a positive vesicles in maternal HPA-1bb plasma will be very small, compared to the total amount of isolated vesicles, and compared to the number of positive vesicles isolated from HPA-1a positive plasma, which are probably mostly platelet vesicles. In addition, HPA-1a positive platelet vesicles carry more antigen per vesicle than

EVs from placental cells, and the expression of antigen per vesicle will also differ between vesicles from the same parent cell.

The detection of relatively few HPA-1a positive events in HPA-1bb pregnant plasma demanded a highly specific system. As such, both the 26.4 antibody and the HPA-1a specific T cell clones proved to be adequate tools. The 26.4 antibody is highly specific in separating HPA-1a- positive and -negative events. Our isolation protocol, however, was simple, and probably gave us a wide range of vesicles both in range of size and type. Regardless of this, the isolated material was representative for answering our main question. The possible wider range of vesicle types isolated by this protocol, however, may have affected our result regarding the activation of T cells. Different vesicle types may for example affect the uptake in phagocytosing cells and probably also the activation of T cells, in different ways [8, 28].

Plasma from pregnant HPA-1bb women in this study was taken from somewhat different time points in pregnancy. We assume that the samples from later in pregnancy contain more fetal material [29]. However, we do not know how the amount of fetal HPA-1a positive vesicles in maternal circulation will differ along pregnancy. Some of the material was also already isolated and frozen for some time, while some of the material were fresher when analyzed. This may also affect the number of vesicles available for isolation. The main result, the existence of fetal/placental HPA-1ab EVs in maternal HPA-1bb plasma, however, is not affected by the variety in gestation age or age of the plasma sample.

Flow cytometric analysis of EVs in plasma is challenging, and research data differ in respect of isolation protocol, flow cytometer used, gating strategy, and antibodies used. There are many possibilities for especially false positive results by using multicolour staining of microvesicles in flow cytometry. One of these is the accidental detection of immunoprecipitants of antibodies appearing as stained vesicles. Also, in our control of fPBS mixed with all antibodies used for multicolour staining, some events occurred in our vesicle gate in the SSC-A/FSC-A scatter plot. However, none of these events were 26.4 positive, and this did not interfere with our detection of HPA-1a positive vesicles in plasma of an HPA-1bb woman carrying an HPA-1ab foetus. In addition, by swarm detection, there is also a potential

risk to detect two vesicles (events), each single positive for one surface marker, as one vesicle (event) positive for both markers. However, since the HPA-1a positive vesicles we wanted to detect were only positive for the 26.4 antibody used, and negative for the other markers, we also avoided this problem. We have attempted to follow the recommendations in MIFlowCyt-EV for our flow cytometry, at least in our reporting of data [30]. However, the data shown here are from experiments done before the framework for standardized reporting of EVs and flow cytometry was made.

Still, the question of what induces the immune response against HPA-1a antigen in HPA-1a negative women remains unanswered. In conclusion, however, we now know that HPA-1a antigen from cells from a trophoblast cell line activates HPA-1a specific T cells similarly to HPA-1a antigen from platelets, that HPA-1a antigen on EVs from HPA-1a positive plasma also activates HPA-1a specific T cells, and that HPA-1a positive extracellular vesicles can be detected in plasma of HPA-1a negative women pregnant with HPA-1a positive fetuses. Earlier studies, and the strong association between the *HLA-DRB3*01:01* allele and HPA-1a alloimmunization, supports that activation of HPA-1a specific T cells likely is crucial for the production of HPA-1a specific antibodies. And both trophoblast - and platelet derived HPA-1a antigen are indeed potential sources for HPA-1a specific T cell activation.

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Author contributions

GH, MTA and TBS planned the study. GH did all the experiments, except the electron microcopy. GH wrote the manuscript. MTA and TBS revised the manuscript.

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Legend: Figure 1. Extracellular vesicles were successfully isolated from plasma. (A) Gating of microvesicles isolated from plasma of an HPA-1a positive woman shown in flow cytometry; 1. Extracellular vesicle gate. Scatter plot of isolated EVs. 2. True vesicles disappears after lysis. Scatter plot of EVs after lysis. 3) . True vesicles can be distinguished from buffer background. Scatter plot of PBS filtrated through a 0.2 μm filter (fPBS) (collected for at least 2 minutes). **(B)** Isolated EVs by centrifugation shown in electron microscopy. The first picture shows vesicular extracellular vesicles of variables size and some platelet rests (1) (Bar size 5 μm). Compared to isolated platelets, also viewed in EM (2) (Bar size 5 μm). **(C) Single EV events are detected.** Serial dilution experiment for EVs gated in scatter plot A. Samples diluted 1:1, 1:2, 1:4 and 1:10, stained with the same concentration of 26.4-AF488 antibody. Mean fluorescence intensity (MFI) for AF488 by analysis 5,000 EVs does not change with serial dilution. fPBS= filtrated PBS, MFI= Mean fluorescence intensity.

Legend Figure 2. Sorted HPA-1a positive extracellular vesicles activate HPA-1a specific T cells. HPA-1a positive extracellular vesicles were sorted either by 26.4 antibody-coupled beads, or by sorting using the same antibody in FACS. **(A) Strategy for sorting of HPA-1a positive and HPA-1 negative EVs.** HPA-1a positive and HPA-1a negative EVs were isolated by centrifugation from healthy HPA-1aa and HPA-1bb plasma donors. Showing already gated EV events. Strategy for sorting HPA-1bb EVs: staining with the anti-β3 antibody PM6/13 (HPA-1a negative plasma). Strategy for sorting HPA-1aa EVs: staining with the anti-HPA-1a antibody 26.4 (HPA-1a positive plasma). HPA= Human platelet antigen, APC= Antigen presenting cell, Tc= T cell, EVs= Extracellular vesicles**. (B) IFNy secretion by D7T4 T cells activated by monocytes with antigen from EVs.** Vesicles sorted in A were given to monocytes, which were subsequently cocultured with D7T4 for further analysis in Elispot. Both HPA-1a positive vesicles bound to dynabeads (E), and sorted HPA-1a positive vesicles alone (e) activated the T cell clone D7T4 to secret IFNγ in Elispot. Dynabeads were incubated with EVs isolated from plasma, resuspended in T cell medium and given to monocytes, before monocytes were further cocultured with D7T4, and analyzed for activation in Elispot, as described in methods. A and a: T cell only, B and b: monocytes and T cells, without antigen, C and c: positive control, L33 peptide, monocytes and T cells, D and d: negative control, P33 peptide, monocytes and T cells, E: beads with HPA-1a positive EVs, monocytes and T cells, F: Beads without antigen, monocytes and T cells. e: sorted HPA-1a positive EVs, monocytes and T cells, f: sorted HPA-1a negative EVs, monocytes and T cells. Numbers = number of spots/T cells secreting IFNγ in each well. EVs= Extracellular vesicles, HPA= Human platelet antigen, mnct= monocytes, Tc= T cells.

Legend: Figure 3. HPA-1a positive extracellular vesicles can be detected in HPA-1a negative plasma of women pregnant with an HPA-1a positive fetus. (A) Conjugated 26.4 and AP3 antibodies were used for staining of HPA-1a positive and β3 integrin positive extracellular vesicles (EVs) in flow cytometry, respectively. EVs were isolated from plasma of a random HPA-1a positive donor, an HPA-1bb woman pregnant with an HPA-1ab fetus, pregnancy week 26, and an HPA-1bb women pregnant with an HPA-1bb fetus, pregnancy week between 22-26. The AP-3 antibody binds β3 integrin independent of HPA-1a status, while 26.4 antibody binds specific only to HPA-1a positive β3-integrin. HPA-1a positive EVs

are detected in plasma of HPA-1 positive donors (AP-3 and 26.4 positive), and in HPA-1bb women pregnant with an HPA-1ab fetus (small percentage of 26.4 positive), but not in an HPA-1bb woman carrying an HPA-1bb fetus (AP3 positive, 26.4 negative). The experiment was repeated with similar results (Supplementary figure 1). Only one of the experiments is shown. EVs= extracellular vesicles, HPA= Human platelet antigen. **(B)** HPA-1a positive EVs isolated from plasma of an HPA-1bb woman pregnant with an HPA-1ab fetus were further characterized. 1. EVs were gated in scatter plot as shown. EV scatter plot is compared also to fPBS with all antibodies used in the experiment, to show possible immunoprecipitates. 2. Single staining is shown for 26.4-AF488, CD41-PE, and CD14-APC-AF750. The 26.4 positive events detected are further also gated on CD41-PE and CD14-APC-AF750, and about 40 % of all 26.4 positive events were CD41 and CD14 negative. 3. Also, for EVs double negative for CD41 and CD14, HPA-1a positive events were detected. No events in the EV gate of "fPBS+antibodies" are CD41 and CD14 negative and HPA-1a positive. fPBS= filtrated PBS, EVs= Extracellular vesicles.

Legend Figure 4. Fetal HPA-1a positive extracellular vesicles do not activate HPA-1a specific T cells. EVs from an HPA-1bb woman pregnant with an HPA-1ab fetus, and from an HPA-1bb woman pregnant with an HPA-1bb fetus were isolated from plasma. **(A) IFNγ secretion by D7T4 T cells activated by monocytes with antigen from EVs**. Dynabeads were first incubated with isolated EVs, and further given to monocytes. Monocytes were subsequently cocultured with HPA-1a specific T cells, clone D7T4, in parallel, and analyzed for activation of T cells in Elispot. EVs from an HPA-1a positive random donor, also bound to beads, was used as a positive control. IFN= Interferon, mnct= monocytes, Tc= T cells, HPA= Human platelet antigen. **(B) Proliferation of D7T4 T cells activated by monocytes with antigen from EVs.** The same approach was used for analysis of the same clone in a proliferation assay. D7T4 was activated by the positive peptide control, L33, and by beads with EVs from HPA-1a positive plasma. The same T cells were not activated by EVs from an HPA-1bb woman pregnant with an HPA-1ab fetus. A: T cells only, B: Monocytes and T cells, C: Monocytes pulsed with L33-peptide (positive control), and T cells, D: Monocytes pulsed with P33-peptide (negative control), and T cells, E: Monocytes incubated with beads with EVs from HPA-1a plasma, and T cells, F: Monocytes incubated with beads with EVs from HPA-1bb plasma of a woman pregnant with an HPA-1bb fetus, and T cells, G: Monocytes

incubated with beads with EVs from HPA-1bb plasma of a woman pregnant with an HPA-1ab fetus, and T cells, H: Monocytes incubated with beads only, and T cells. Mnct= monocytes, Tc= T cells, HPA= Human platelet antigen, EVs= Extracellular vesicles, CFSE= Carboxyfluorescein succinimidyl ester.

Legend: Figure 5. Integrin β3 is expressed by HTR8/SVneo cells and platelets and is immunoprecipitated on anti-β3-coated dynabeads. (**A) The presence of β3-integrin shown by western blots,** and β3 integrin was detected in lysates from both platelets and HTR8/SVneo (lane 3 and 5, from two different blots). Integrin from both platelet and trophoblast cell lysates could also be immunoprecipitated on SZ21-antibody-coupled dynabeads (lane 7 and 8). Recombinant αVβ3 protein was used as a positive control for β3 integrin (lane 2, 4, and 6). **(B)** Anti-β3 coupled dynabeads, about $6{,}67x10^6$ beads, were incubated with platelet - and HTR8/SVneo lysates. After removal of the lysate supernatant, the amount of β3-integrin bound to beads was detected in flow cytometry, by further staining of about $2x10^6$ beads. Different combinations of anti-β3 antibodies coupled to dynabeads and anti-β3 detection antibodies were used in flow cytometry. Shown here is detection by the combination of clone SZ21 (Beckman Coulter) coupled to beads and detection in flow cytometry by using an anti-β3 antibody binding the C-terminal part of the integrin (rabbit monoclonal EPR2417Y, Abcam), followed by a FITC-conjugated goat anti-rabbit secondary antibody (goat pAb to Rb IgG (FITC), Abcam, ab6717). FITC intensity for a defined number of beads were used for comparison of antigen amount bound to beads.

Legend: Figure 6. HPA-1a specific T cells were activated by HPA-1a antigen both from platelets and trophoblast cells. (A) *HLA-DRB3*01:01* positive monocytes were incubated over-night with platelets or beads ($1x10^6$) with captured β3 integrin from platelet - or trophoblast lysates. Antigen-pulsed monocytes were further cocultured with clone D8T108 together with PE-conjugated anti-TNF antibody and TAPI-0. The cells were finally analyzed in flow cytometry. Cells were gated on living T lymphocytes, and further on CFSE positive D8T108 T cells. T cells were CFSE-stained to distinguish from APCs. Activation D8T108 was measured by TNFα-expression. "No antigen" refers to T cells only. Numbers indicate %

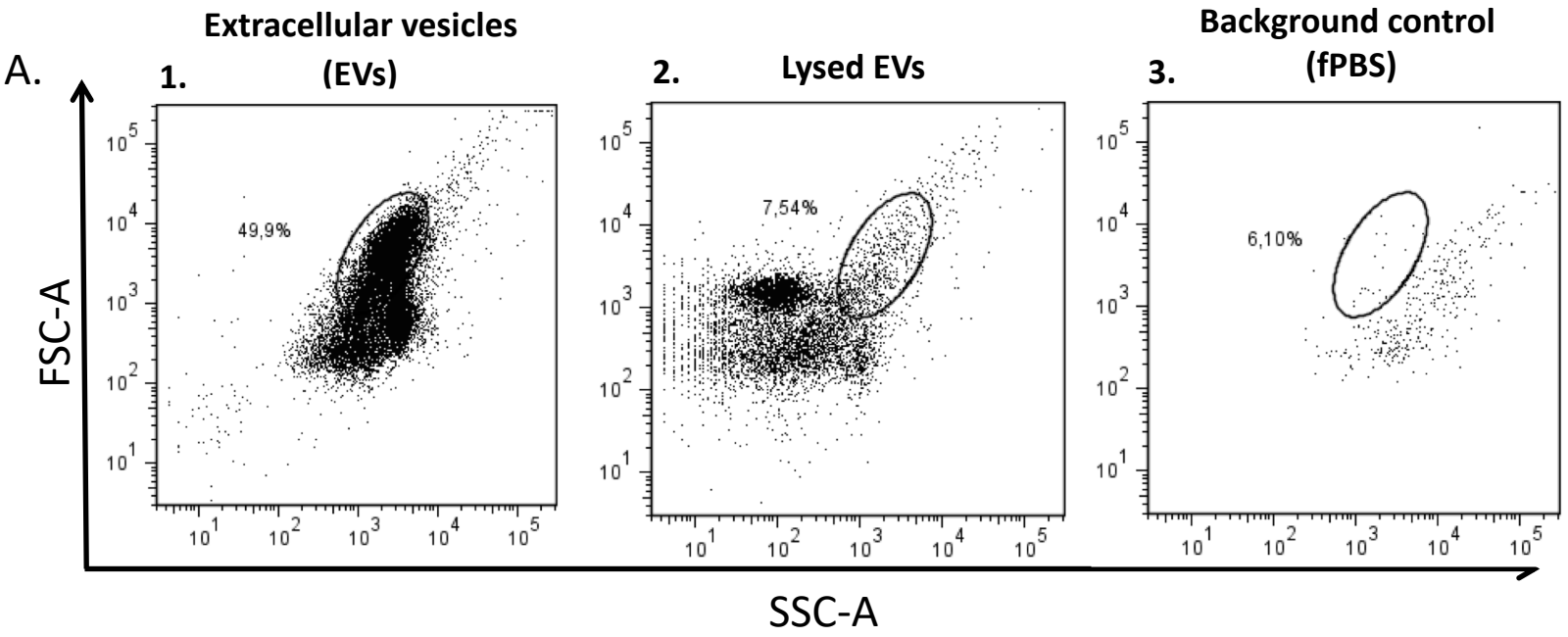
of gated events in upper right quadrant. The clone D8T108 is activated by HPA-1a positive platelets, by beads with β3 integrin from platelets, and by beads with β3 integrin from trophoblast (HTR8/SVneo and TCL-1 lysates). CFSE= Carboxyfluorescein succinimidyl ester, HPA= Human platelet antigen, TNF= Tumor necrosis factor. **(B)** Beads with different amounts of bound HPA-1a antigen were incubated with HPA-1a negative *HLA-DRB3*01:01* positive monocytes. Monocytes were further cultured with HPA-1a specific T cells, and the activation of T cells was measured both by % of cells secreting TNFα and the median PE-intensity of stimulated T cells, in flow cytometry. "Antigen amount" on the x-axis refers to mean FITC intensity of $1x10^4$ dynabeads with bound antigen in flow cytometry (also described in figure 1). "Relative antigen amount" refers to mean FITC intensity of a given number of dynabeads with bound antigen relative to mean FITC intensity for beads only. Activation of T cells are shown on the y-axis both as percentage of TNF positive cells, and by median PE intensity/TNF intensity. TNF= Tumor necrosis factor.

Legend: Supplementary figure 1. A. Conjugated 26.4 and AP3 antibodies were used for staining of HPA-1a positive and β3 integrin positive extracellular vesicles (EVs) in flow cytometry, respectively. EVs were isolated from plasma of a random HPA-1a positive donor, an HPA-1bb woman pregnant with an HPA-1ab fetus. The AP-3 antibody binds β3 integrin independent of HPA-1a status, while 26.4 antibody binds specific only to HPA-1a positive β3-integrin. HPA-1a positive EVs are detected in plasma of HPA-1 positive donors (AP-3 and 26.4 positive), and in HPA-1bb women pregnant with an HPA-1ab fetus (small percentage of 26.4 positive). **B.** Conjugated 26.4 antibody was used for staining of HPA-1a positive extracellular vesicles (EVs) in flow cytometry. EVs were isolated from plasma of a random HPA-1a positive donor, and an HPA-1bb woman pregnant with an HPA-1ab fetus. HPA-1a positive EVs are detected in plasma of HPA-1 positive donors and in HPA-1bb women pregnant with an HPA-1ab fetus (small percentage of 26.4 positive). **C.** Conjugated 26.4 antibody was used for staining of HPA-1a positive extracellular vesicles (EVs) in flow cytometry. EVs were isolated from plasma of an HPA-1bb woman pregnant with an HPA-1ab fetus, and an HPA-1bb woman pregnant with an HPA-1bb fetus. HPA-1a positive EVs are detected in plasma of HPA-1bb women pregnant with an HPA-1ab fetus (small percentage of 26.4 positive), but not in plasma of the HPA-1bb woman pregnant with an HPA-1bb fetus. **D**. Conjugated 26.4 and AP3 antibodies were used for staining of HPA-1a positive and β3 integrin positive extracellular vesicles (EVs) in flow cytometry, respectively. EVs were

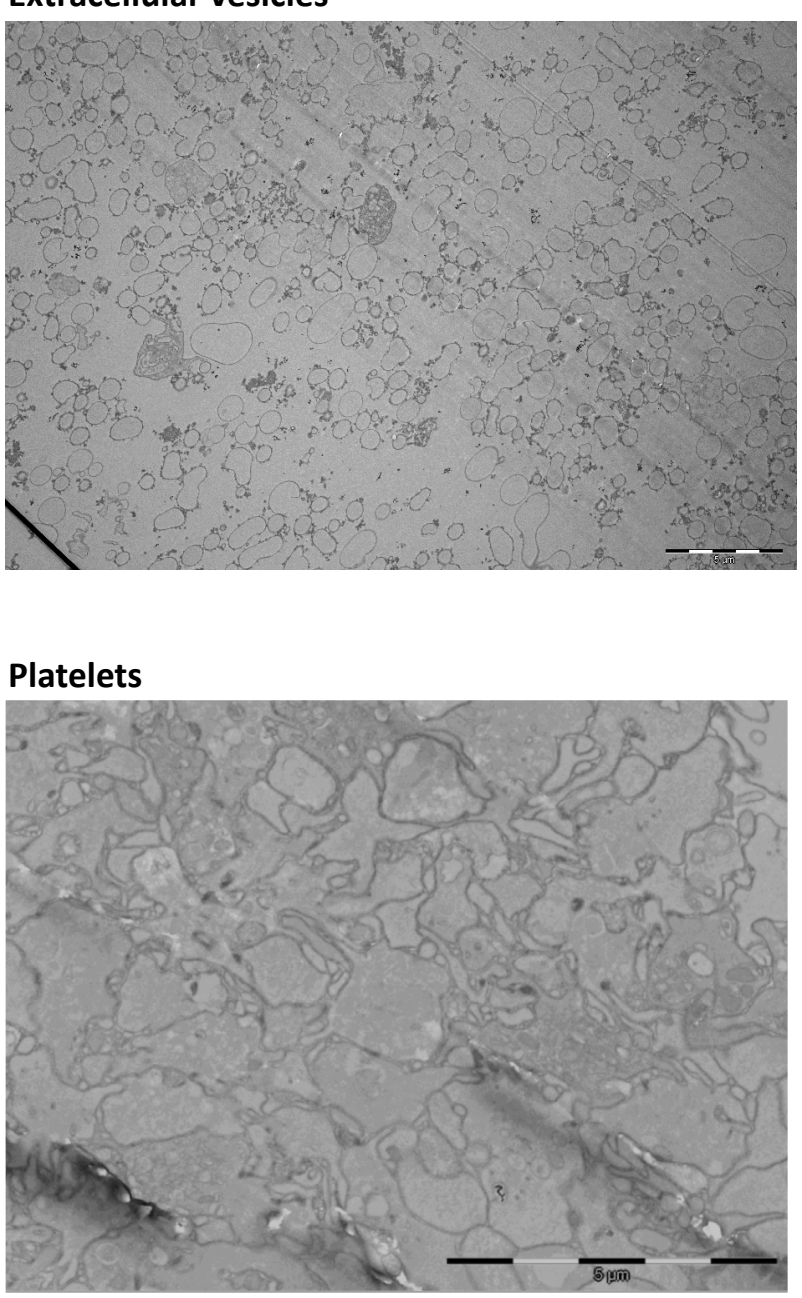
isolated from plasma of a random HPA-1a positive donor, an HPA-1bb woman pregnant with an HPA-1ab fetus, and an HPA-1bb women pregnant with an HPA-1bb fetus. HPA-1a positive EVs are detected in plasma of HPA-1 positive donors (AP-3 and 26.4 positive), and in HPA-1bb women pregnant with an HPA-1ab fetus (small percentage of 26.4 positive), but not in an HPA-1bb woman carrying an HPA-1bb fetus (AP3 positive, 26.4 negative). HPA= Human platelet antigen.

Figure 1

A.


Extracellular vesicles (EVs)
1.
2.
Lysed EVs
3.
Background control (fPBS)
49,9%
7,54%
6,10%
FSC-A
SSC-A

B.


Extracellular vesicles
Platelets
5µm

C.

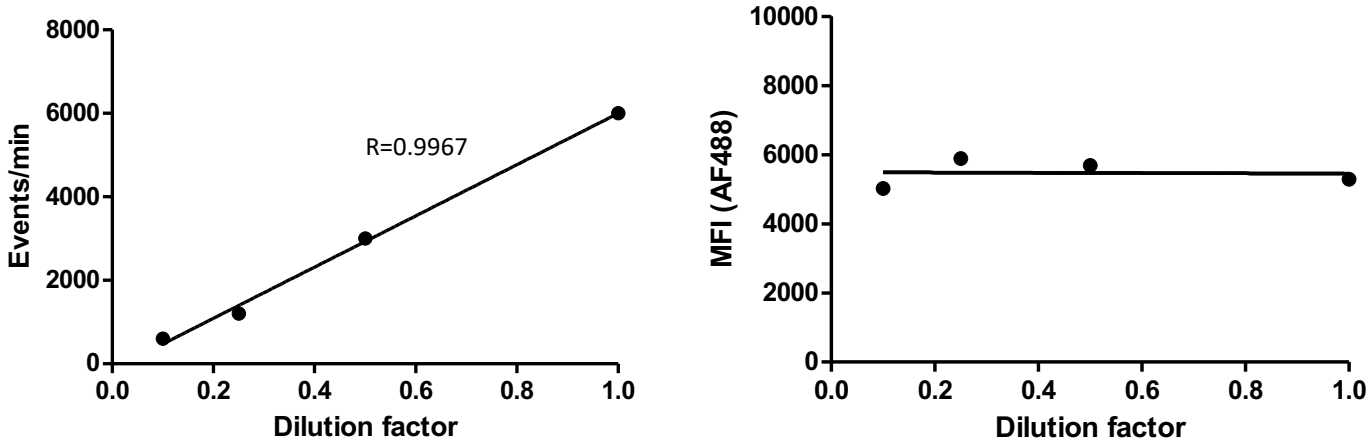
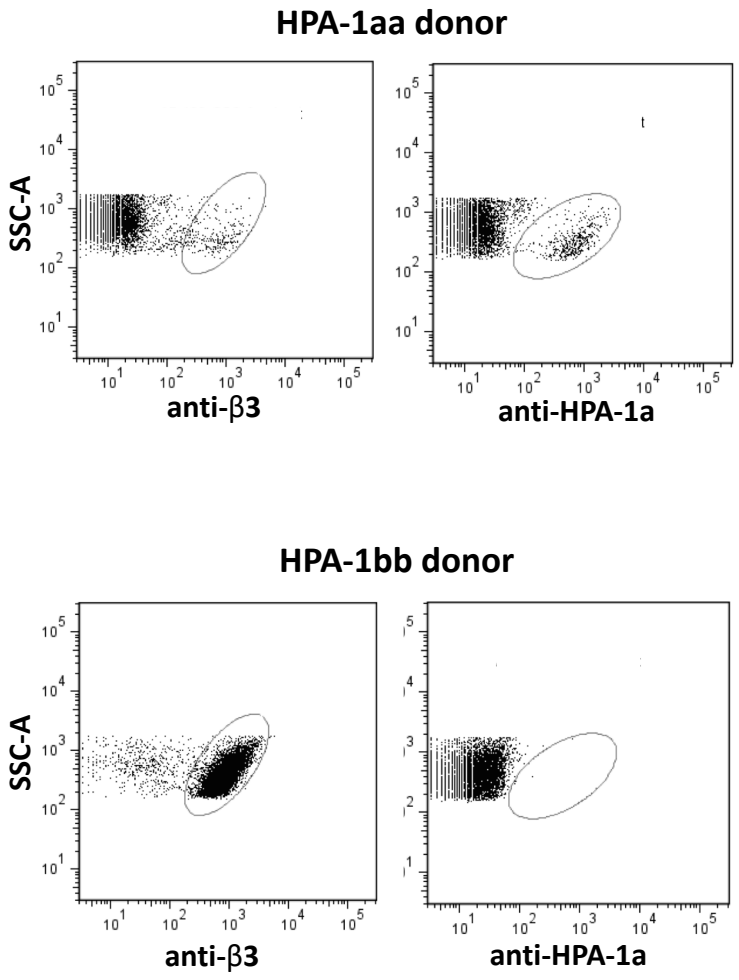
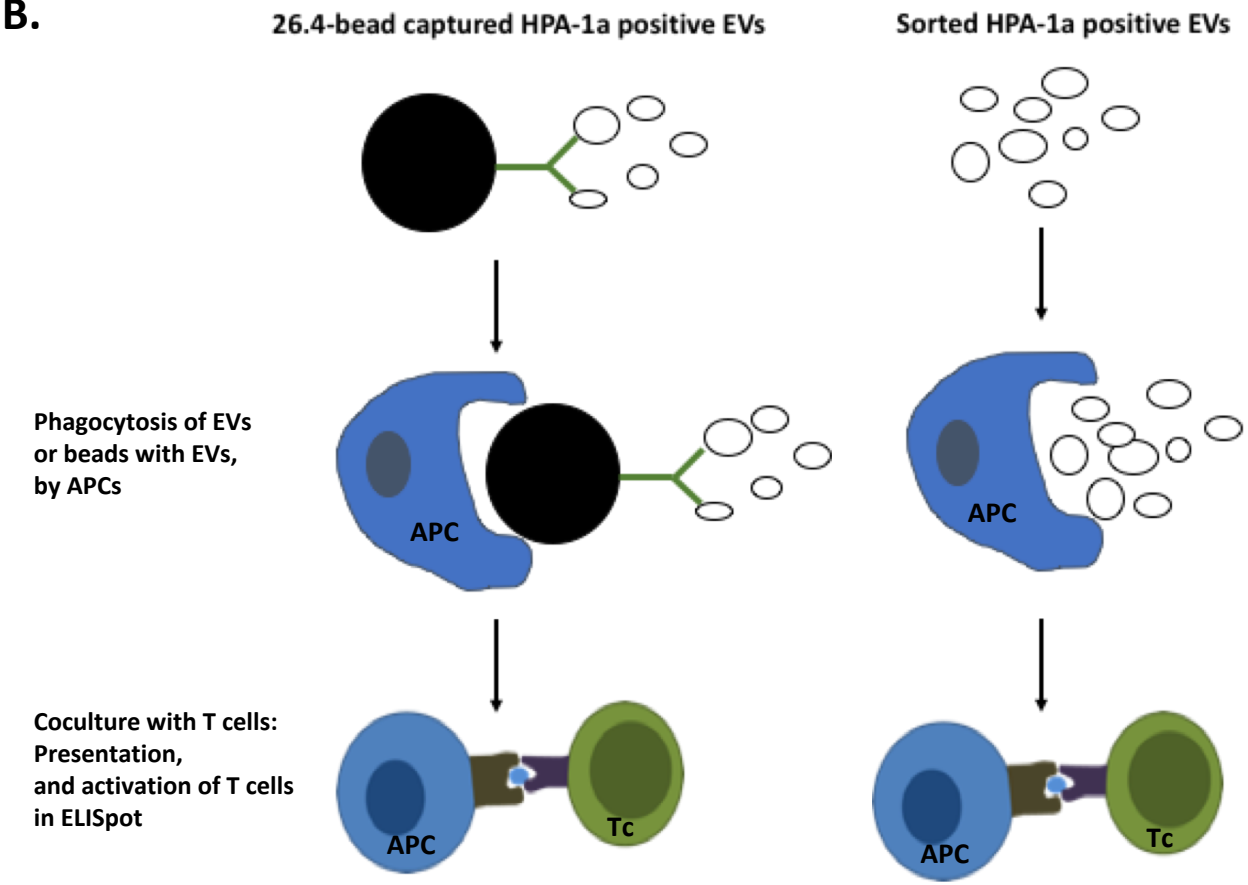

Events/min
R=0.9967
Dilution factor
MFI (AF488)
Dilution factor

Figure 2

A.


HPA-1aa donor
SSC-A
anti-β3
anti-HPA-1a
HPA-1bb donor
SSC-A
anti-β3
anti-HPA-1a

B.


26.4-bead captured HPA-1a positive EVs
Sorted HPA-1a positive EVs
Phagocytosis of EVs or beads with EVs, by APCs
APC
APC
Coculture with T cells: Presentation, and activation of T cells in ELISpot
APC
Tc
APC
Tc

C.

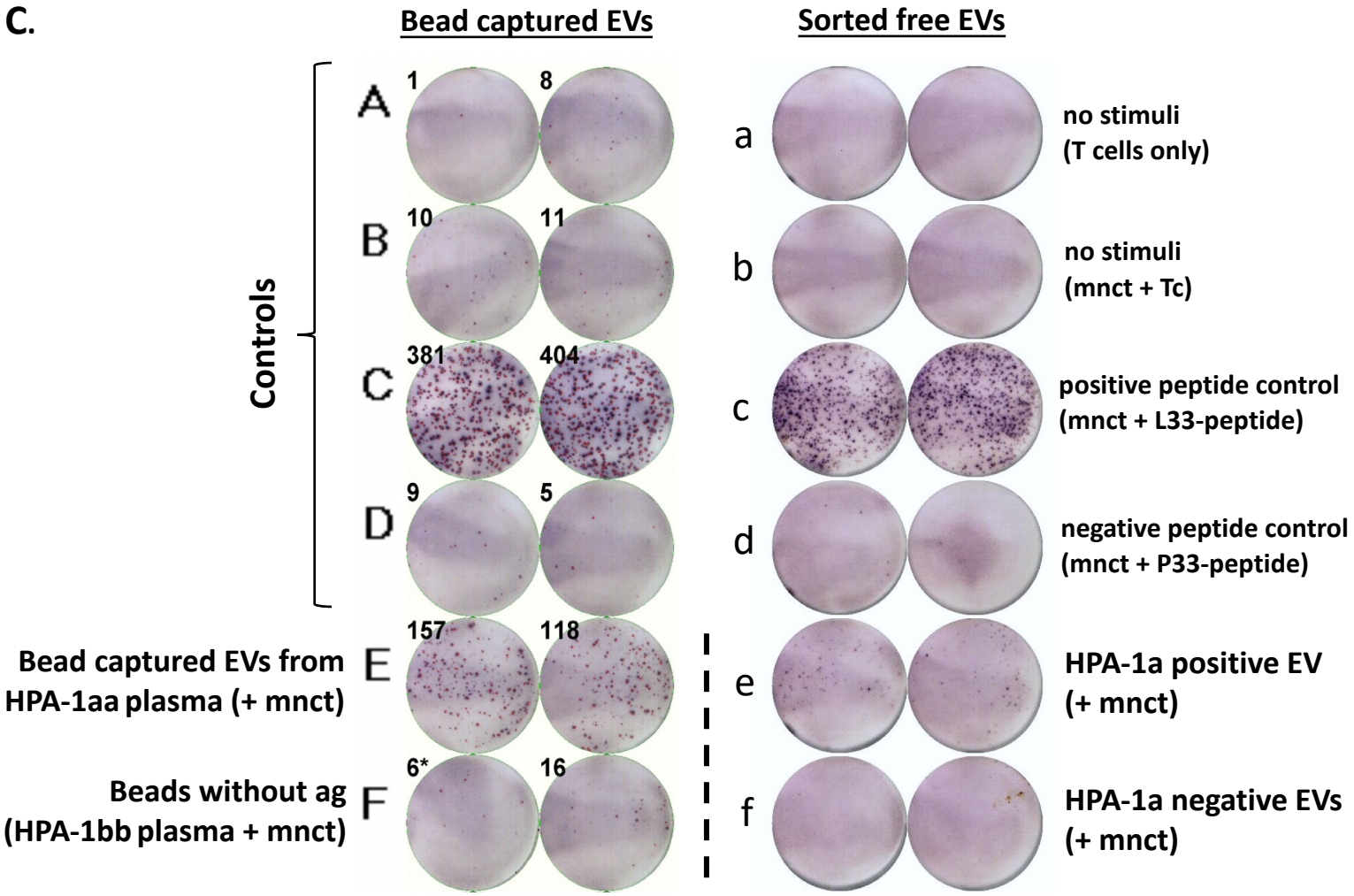
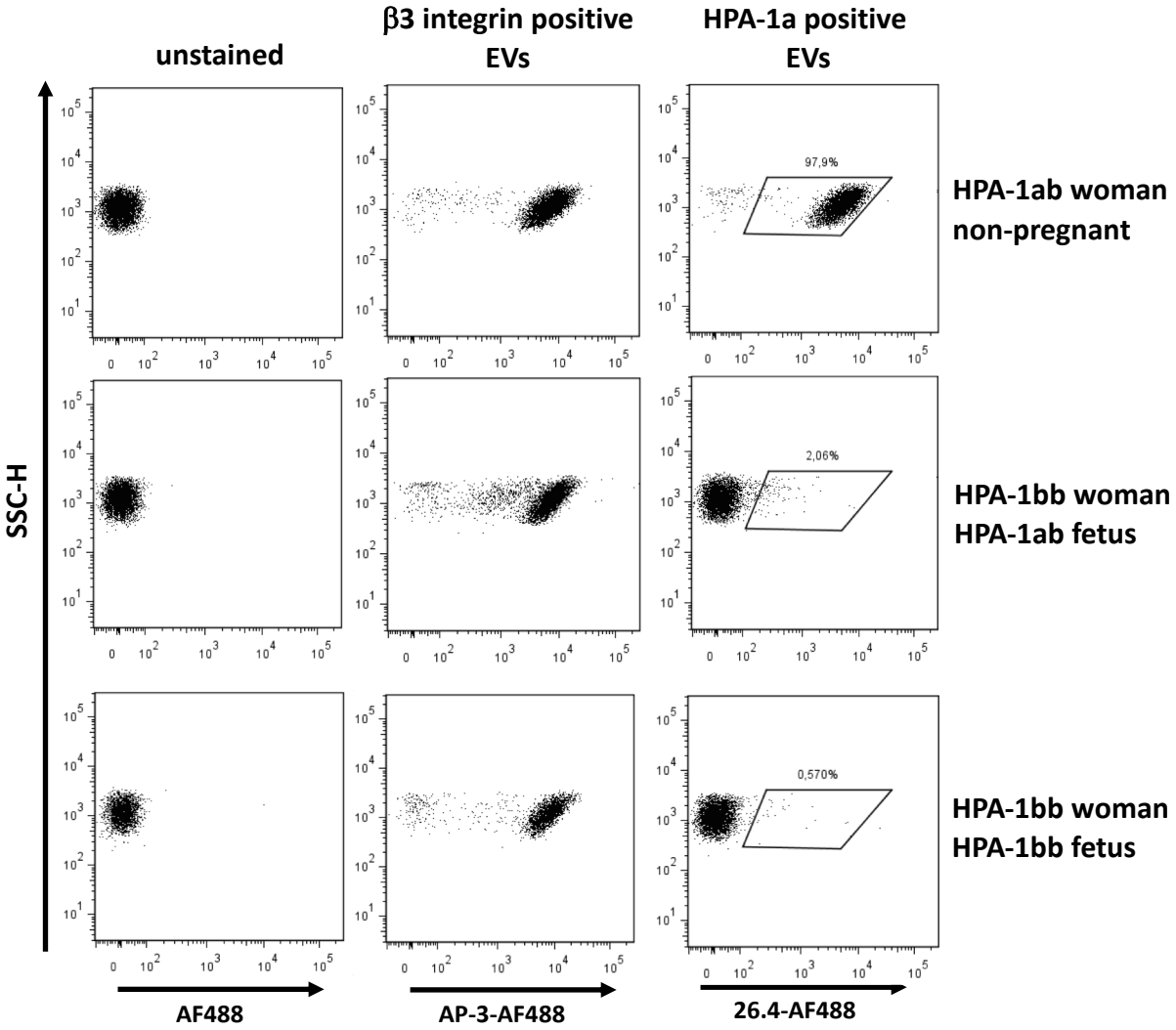
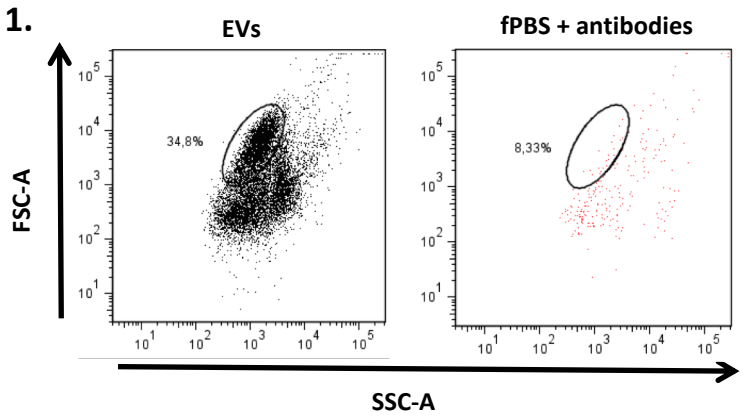

Bead captured EVs
Sorted free EVs
Controls
A
1
8
a
no stimuli
(T cells only)
B
10
11
b
no stimuli
(mnct + Tc)
C
381
404
c
positive peptide control
(mnct + L33-peptide)
D
9
5
d
negative peptide control
(mnct + P33-peptide)
Bead captured EVs from HPA-1aa plasma (+ mnct)
E
157
118
e
HPA-1a positive EV
(+ mnct)
Beads without ag (HPA-1bb plasma + mnct)
F
6*
16
f
HPA-1a negative EVs
(+ mnct)

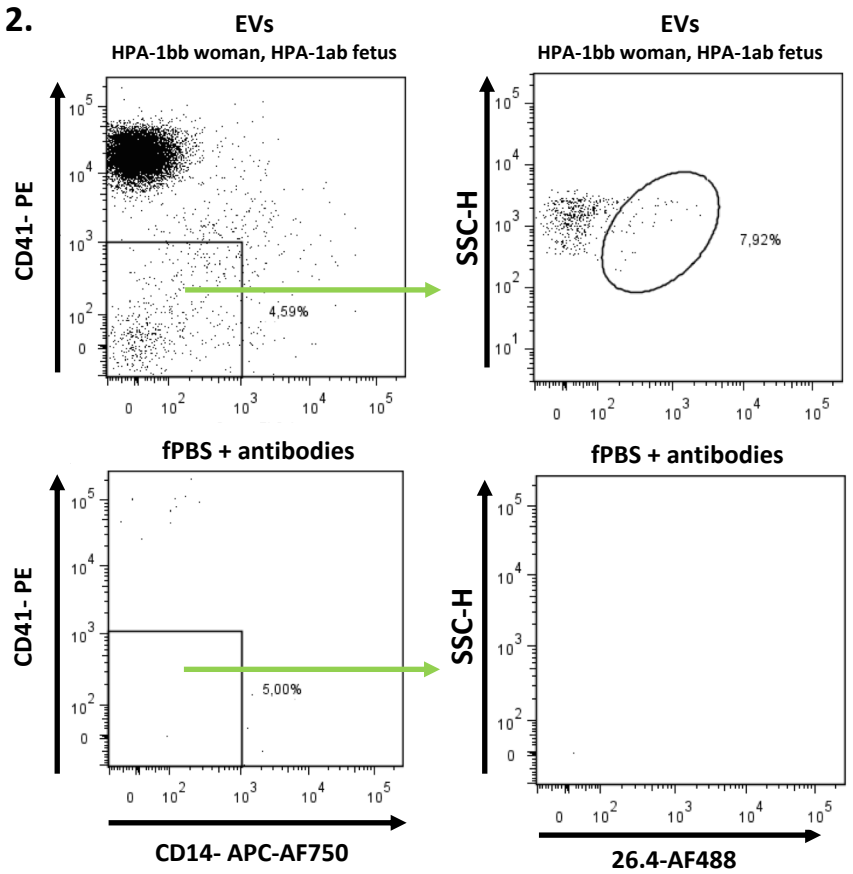
Figure 3

A.


unstained
β3 integrin positive EVs
HPA-1a positive EVs
97,9%
2,06%
0,570%
SSC-H
AF488
AP-3-AF488
26.4-AF488
HPA-1ab woman non-pregnant
HPA-1bb woman HPA-1ab fetus
HPA-1bb woman HPA-1bb fetus

B.


1.
EVs
fPBS + antibodies
34,8%
8,33%
FSC-A
SSC-A


2.
EVs
HPA-1bb woman, HPA-1ab fetus
EVs
HPA-1bb woman, HPA-1ab fetus
CD41- PE
4,59%
SSC-H
7,92%
fPBS + antibodies
fPBS + antibodies
CD41- PE
5,00%
SSC-H
CD14- APC-AF750
26.4-AF488

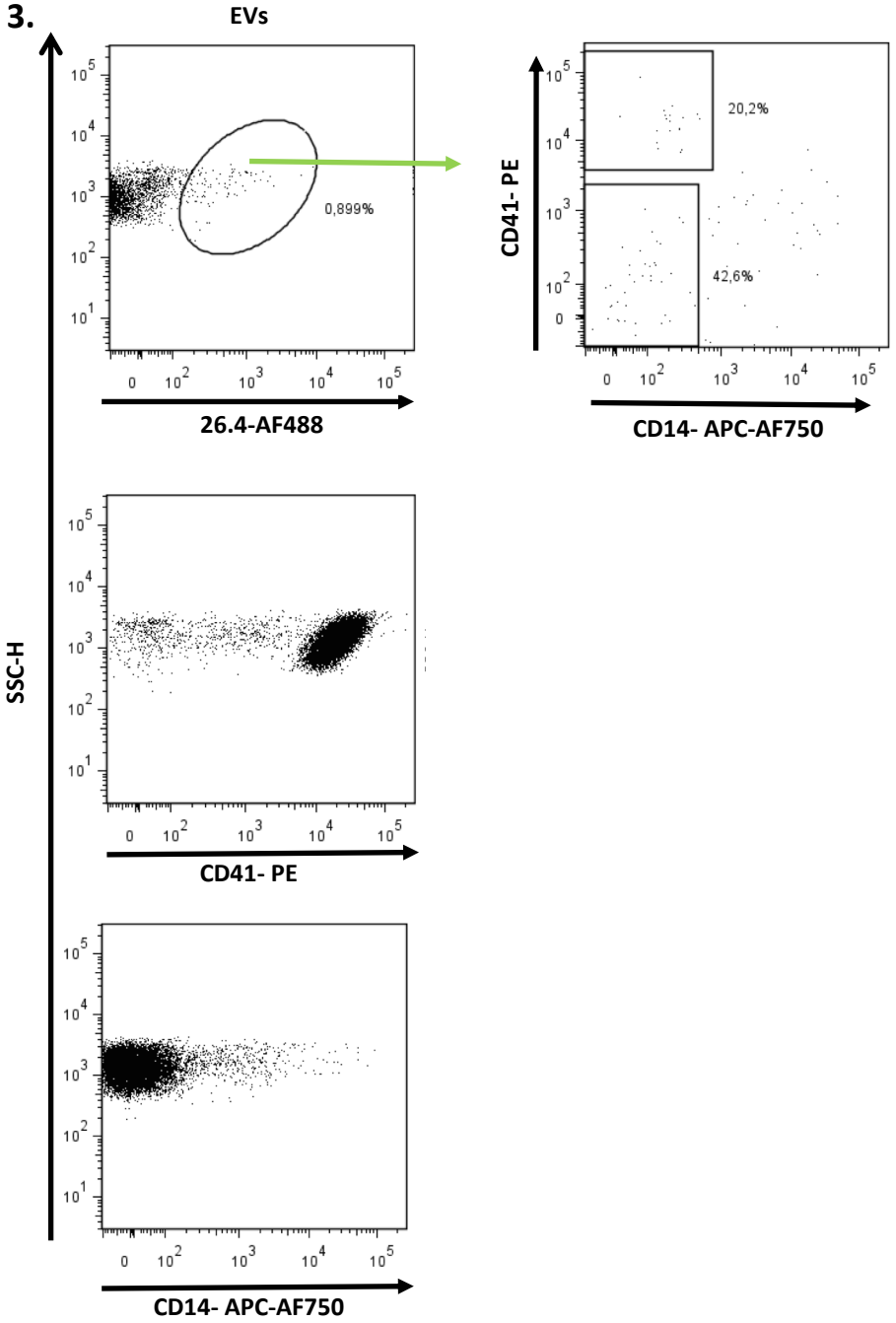
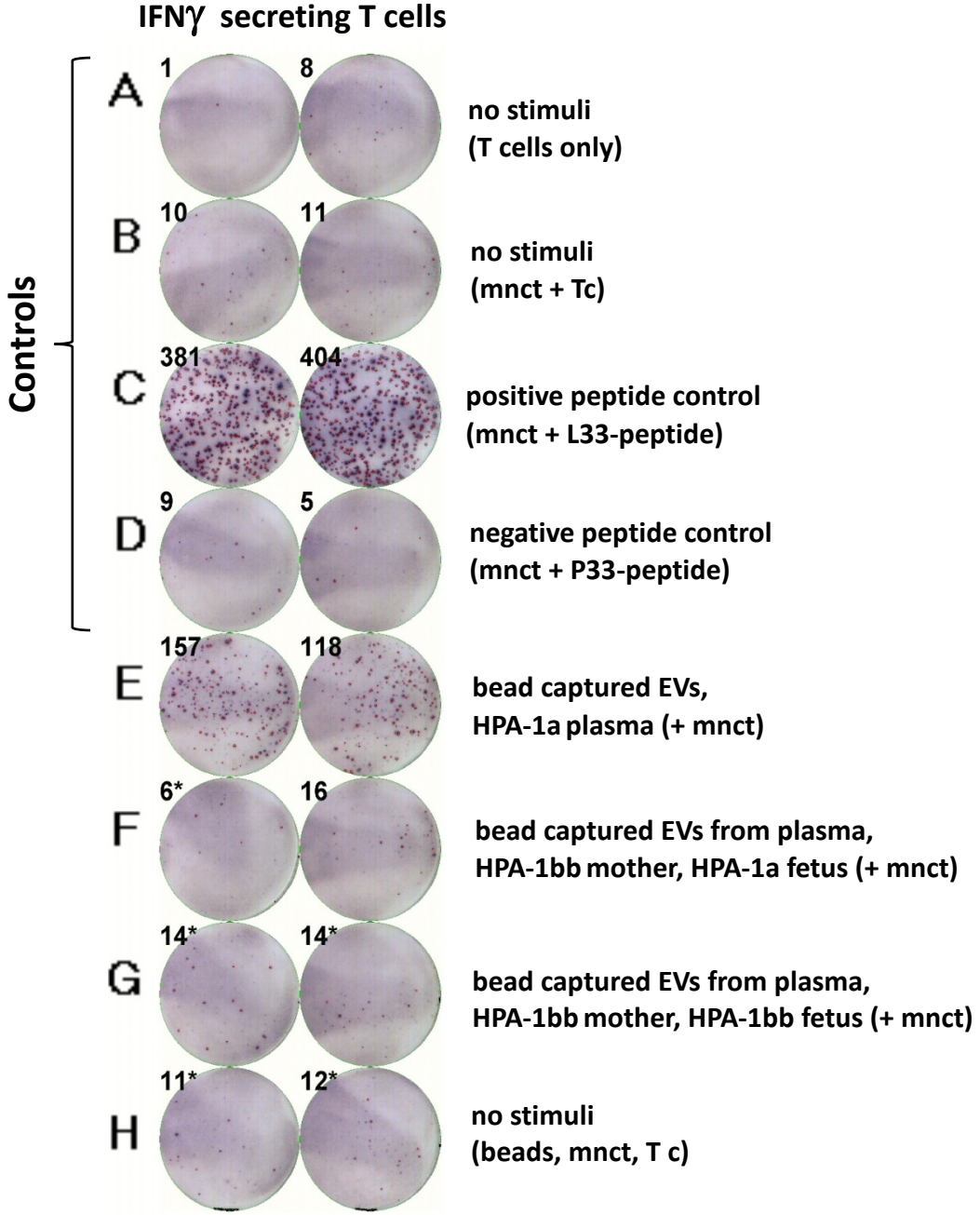

3.
EVs
0,899%
26.4-AF488
20,2%
42,6%
CD41- PE
CD14- APC-AF750
SSC-H
CD41- PE
SSC-H
CD14- APC-AF750

Figure 4

A.


IFNγ secreting T cells
Controls
A
1
8
no stimuli
(T cells only)
B
10
11
no stimuli
(mnct + Tc)
C
381
404
positive peptide control
(mnct + L33-peptide)
D
9
5
negative peptide control
(mnct + P33-peptide)
E
157
118
bead captured EVs,
HPA-1a plasma (+ mnct)
F
6*
16
bead captured EVs from plasma,
HPA-1bb mother, HPA-1a fetus (+ mnct)
G
14*
14*
bead captured EVs from plasma,
HPA-1bb mother, HPA-1bb fetus (+ mnct)
H
11*
12*
no stimuli
(beads, mnct, T c)

B.

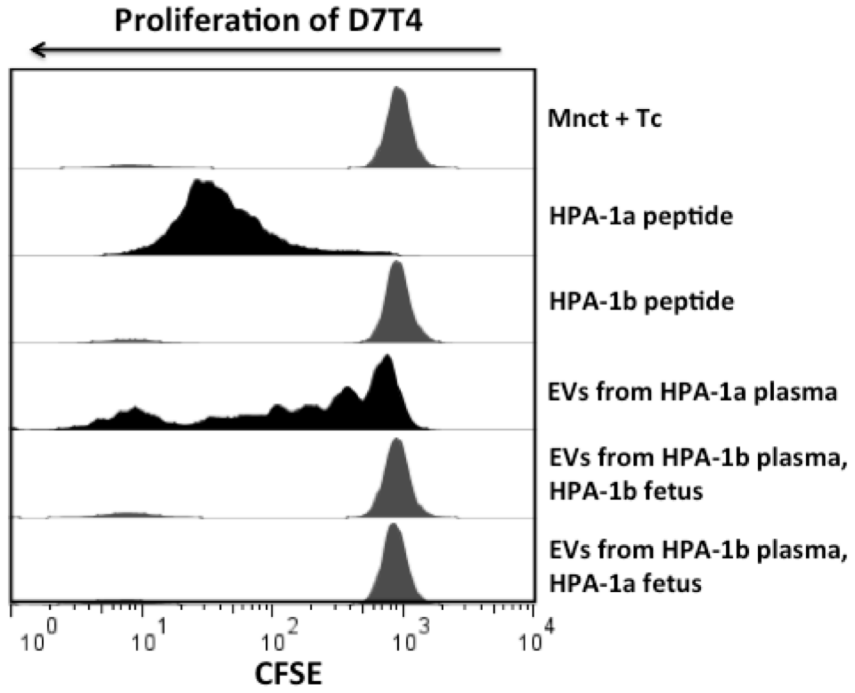
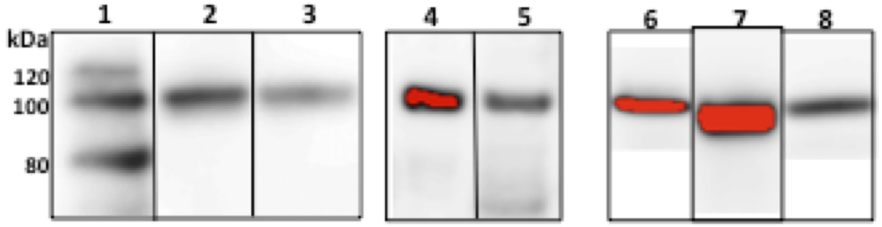

Proliferation of D7T4
Mnct + Tc
HPA-1a peptide
HPA-1b peptide
EVs from HPA-1a plasma
EVs from HPA-1b plasma,
HPA-1b fetus
EVs from HPA-1b plasma,
HPA-1a fetus
10^0
10^1
10^2
10^3
10^4
CFSE

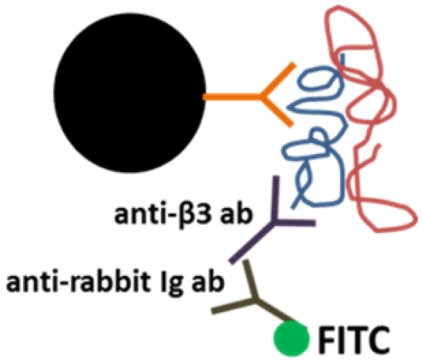
Figure 5

A.



1. Protein marker
2. Recombinant αVβ3
3. Platelet lysate
4. Recombinant αVβ3
5. HTR8/SVneo lysate
6. Recombinant αVβ3
7. Beads + platelet lysate
8. Beads + HTR8/SVneo lysate

B.



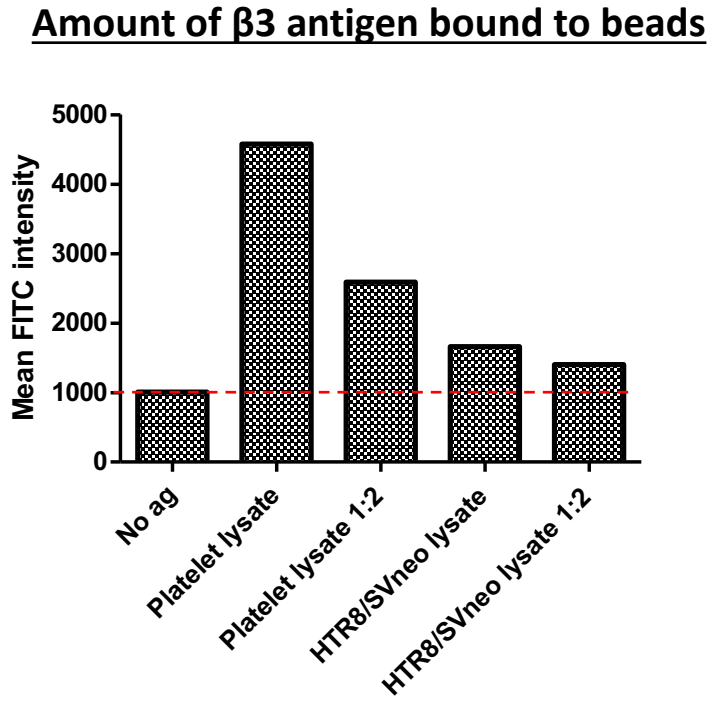
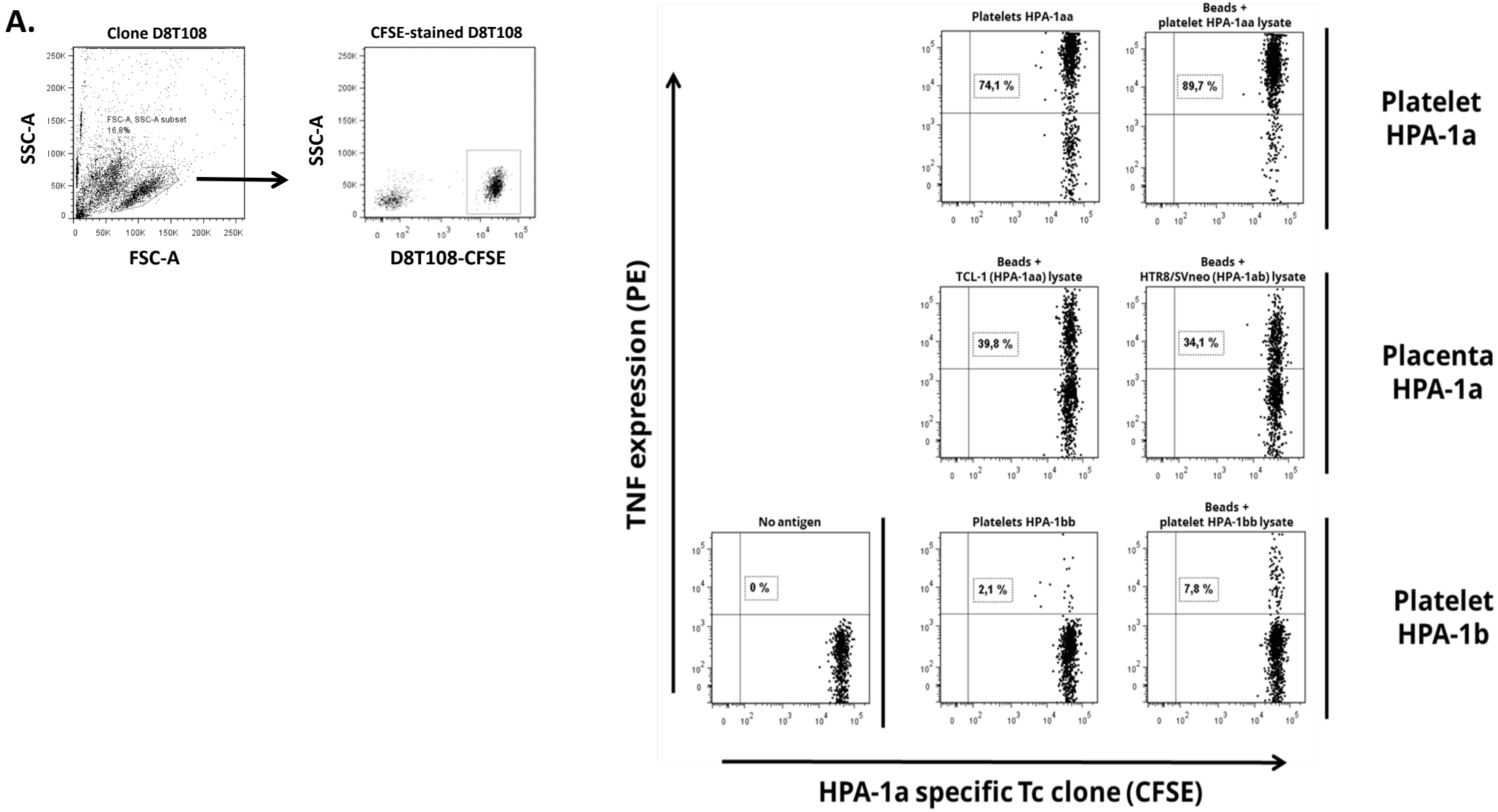
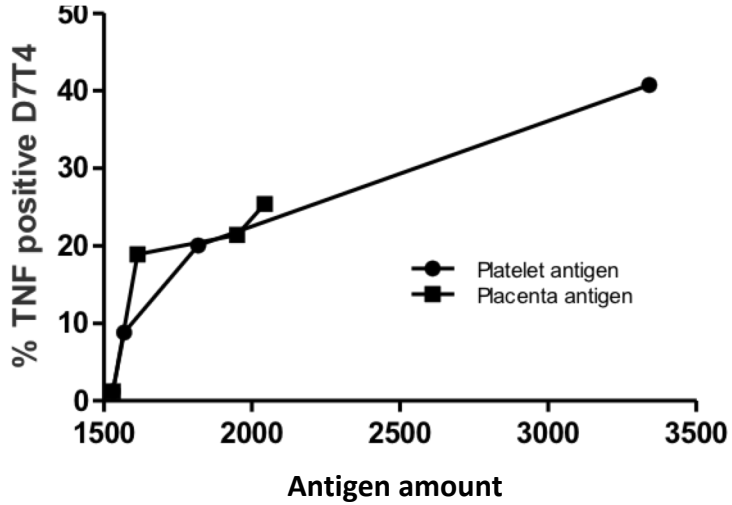


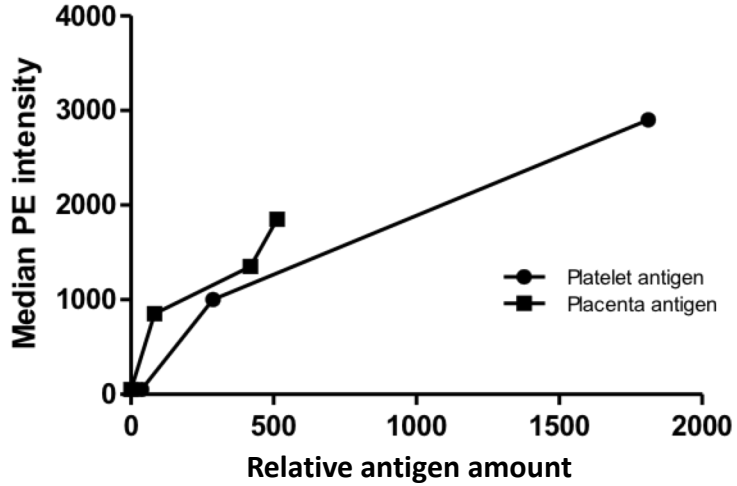
Figure 6


A.
Clone D8T108
CFSE-stained D8T108
SSC-A
FSC-A
FSC-A, SSC-A subset
16,8%
D8T108-CFSE
TNF expression (PE)
HPA-1a specific Tc clone (CFSE)
Platelets HPA-1aa
Beads + platelet HPA-1aa lysate
74,1 %
89,7 %
Platelet HPA-1a
Beads + TCL-1 (HPA-1aa) lysate
Beads + HTR8/SVneo (HPA-1ab) lysate
39,8 %
34,1 %
Placenta HPA-1a
No antigen
Platelets HPA-1bb
Beads + platelet HPA-1bb lysate
0 %
2,1 %
7,8 %
Platelet HPA-1b

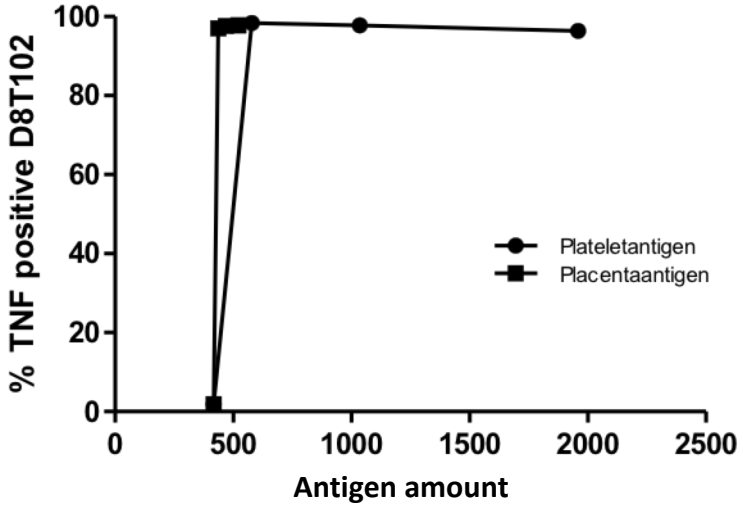
B.

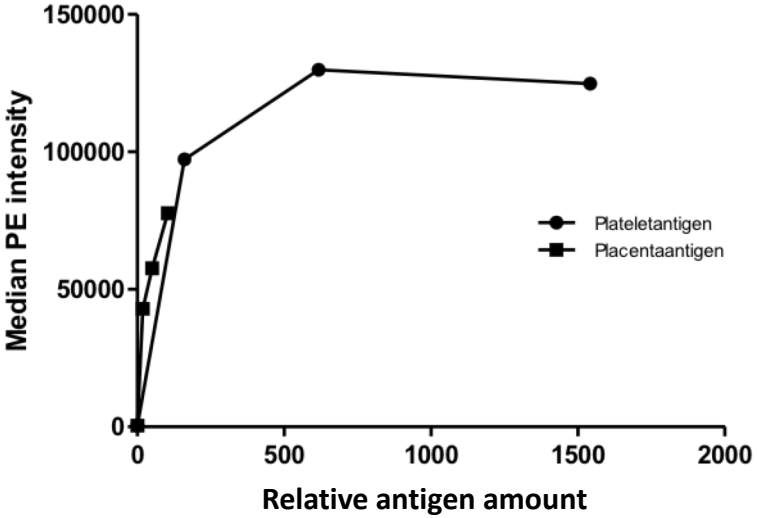
Activation of D7T4 T cells by integrin β3 from trophoblast and platelets





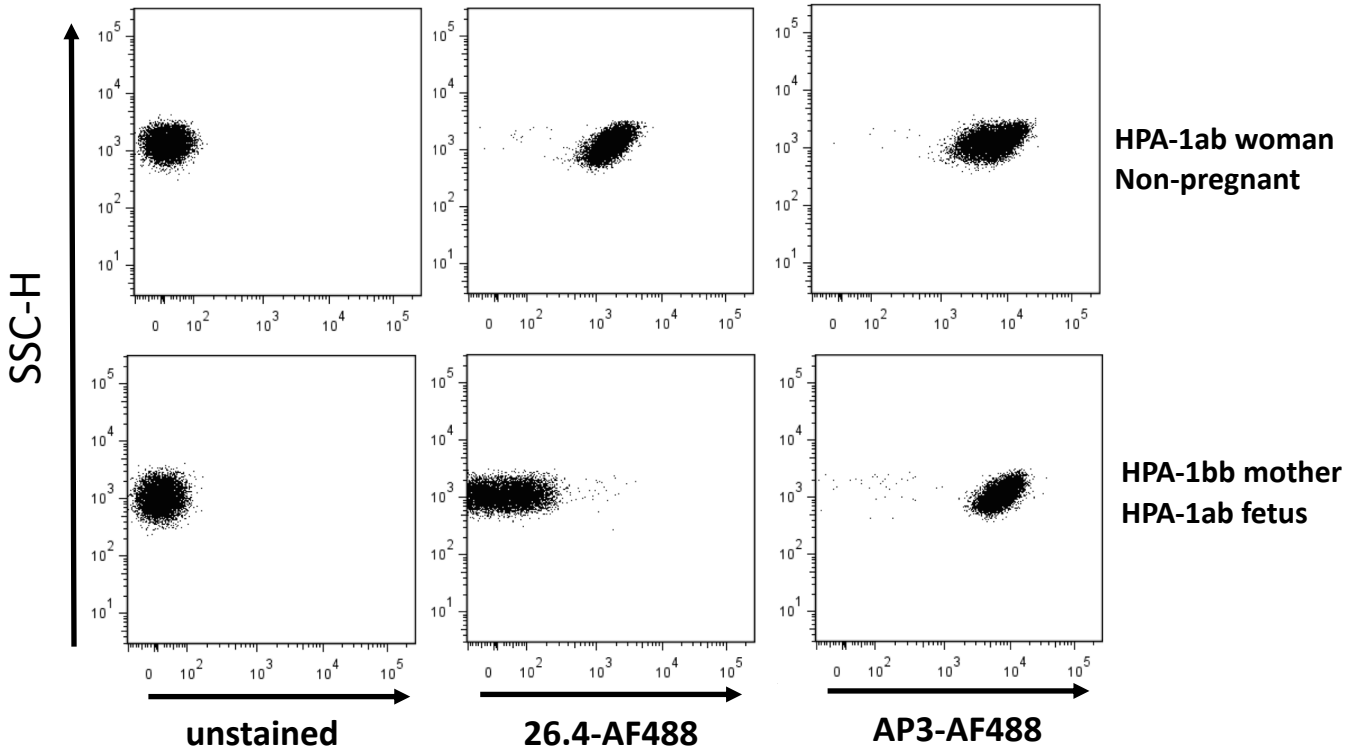
Activation of D8T108 T cells by integrin β3 from trophoblast and platelets



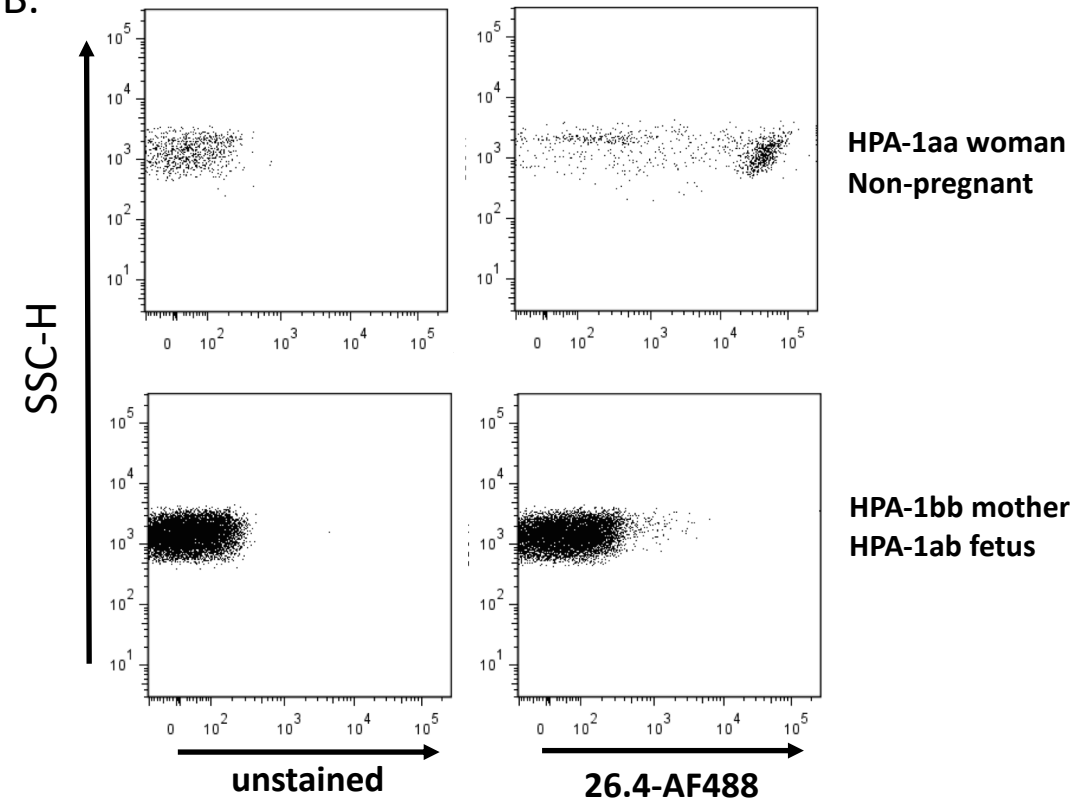


Supplementary figure 1

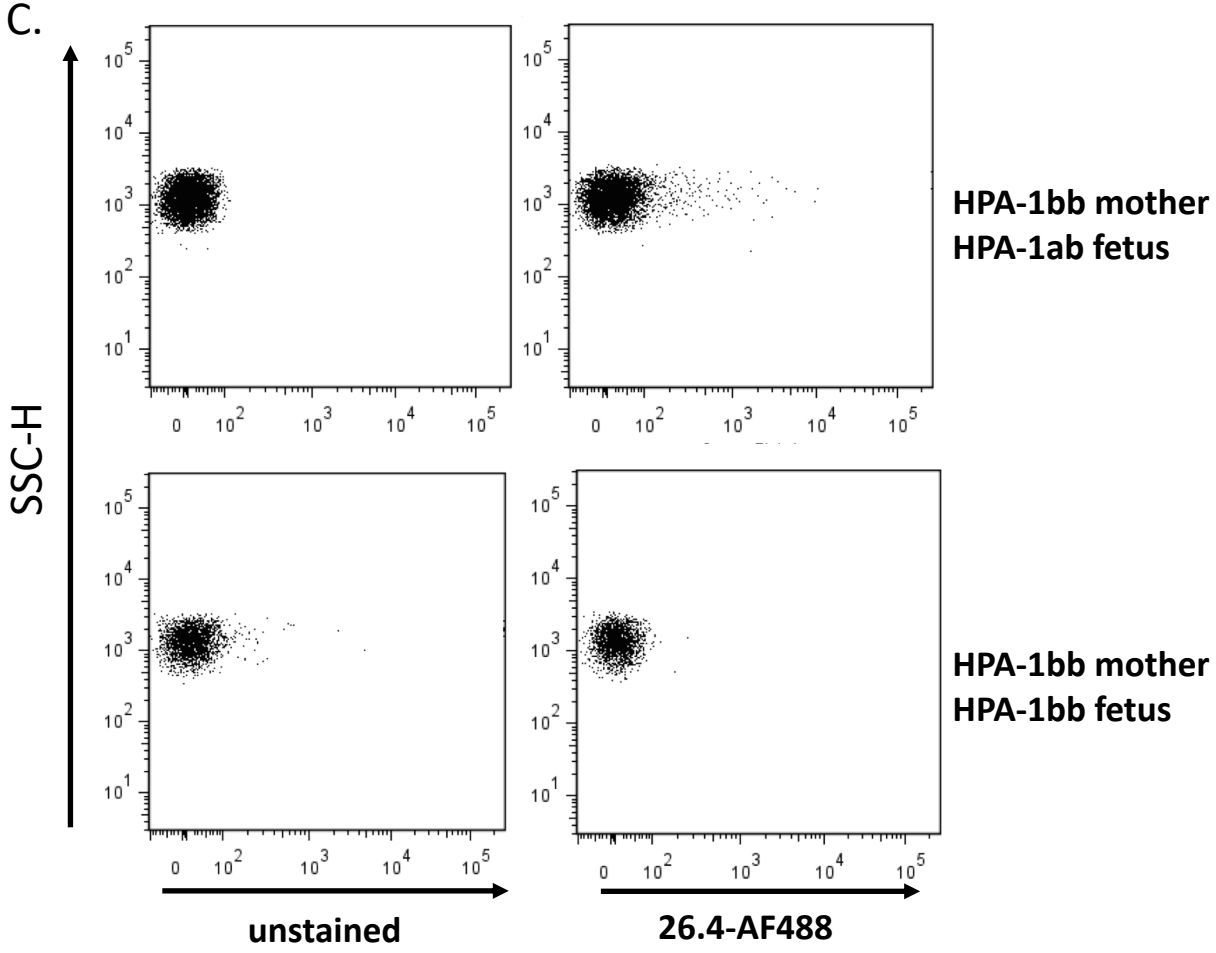
A.



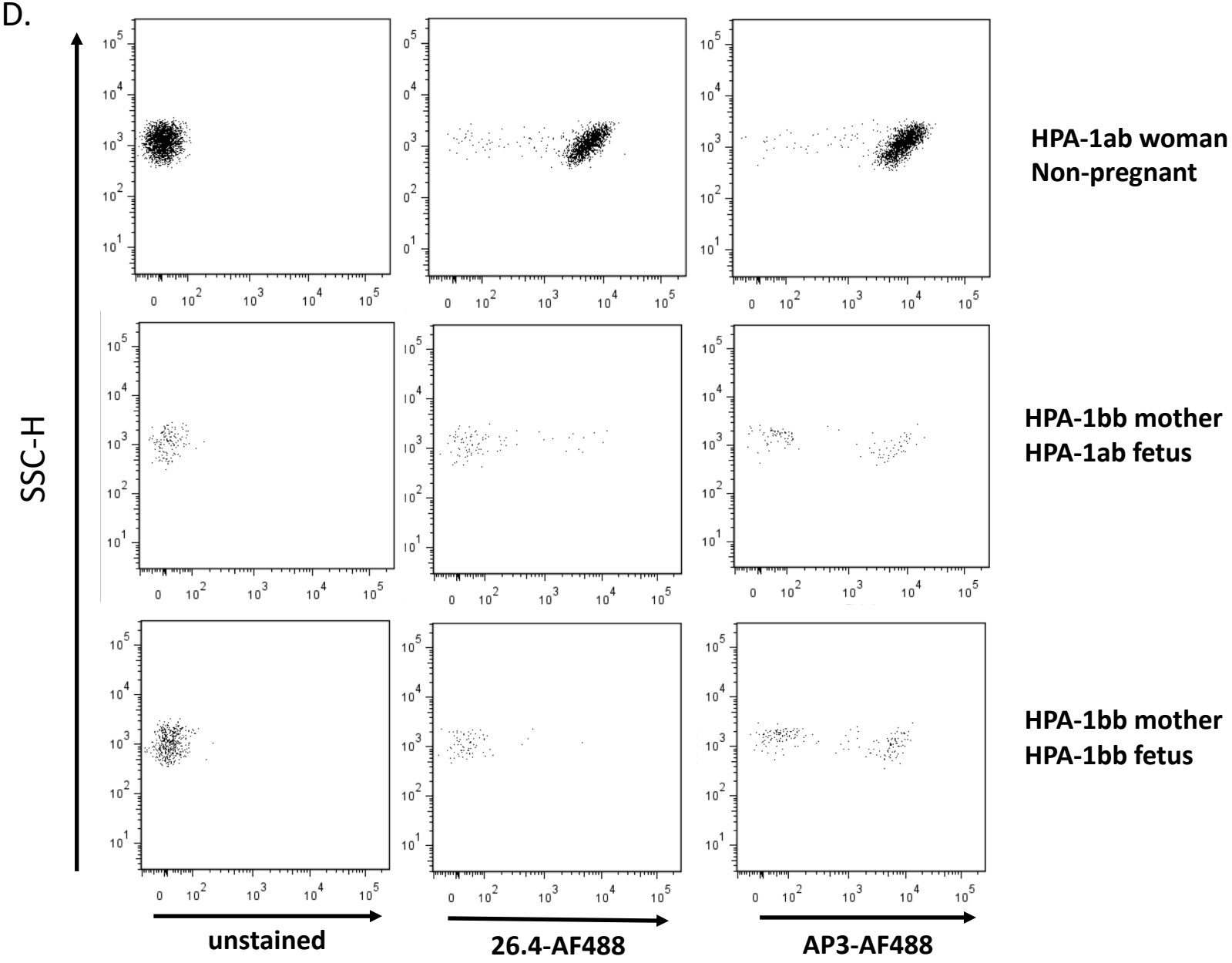
B.



C.


SSC-H
unstained
26.4-AF488
HPA-1bb mother
HPA-1ab fetus
HPA-1bb mother
HPA-1bb fetus

D.


SSC-H
unstained
26.4-AF488
AP3-AF488
HPA-1ab woman
Non-pregnant
HPA-1bb mother
HPA-1ab fetus
HPA-1bb mother
HPA-1bb fetus

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The prevalence of HPA-1a alloimmunization and the potential risk of FNAIT depend on both the *DRB3*01:01* allele and associated DR-DQ haplotypes

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Norges Forskningsråd; Helse Nord RHF

Abstract

Alloimmunization against human platelet antigen (HPA)-1a during pregnancy can cause foetal/neonatal alloimmune thrombocytopenia (FNAIT) and severe bleeding in the foetus or newborn and likely depends on several factors. HPA-1a alloimmunization is associated with *DRB3*01:01,* which is associated with several DR-DQ haplotypes. However, it is not known to what extent these haplotypes contribute to the prevalence of HPA-1a alloimmunization. HPA-1a–alloimmunized women, identified in a prospective study, and random donors were typed for selected *DRB3*, *DRB4, DRB1*, *DQA1* and *DQB1* alleles to determine allele and DR-DQ haplotype frequencies. *DRB3*01:01* was carried by 94% HPA-1a–immunized women compared to 27% in the general population. In the first population, the DR3-DQ2 haplotype was overrepresented ($P < .003$). The prevalence of HPA-1a alloimmunization was estimated to be about twice as frequent with DR3-DQ2 compared to DR13-DQ6, together accounting for about 90% of *DRB3*01:01*–positive individuals. Further, we examined *DQB1*02* and *DRB4*01:01* alleles for their reported association with HPA-1a alloimmunization, in the context of DR-DQ haplotypes. Since ~ 80% of *DQB1*02* alleles are linked to the DR3-DQ2 haplotype, the association might be coincidental. However, the *DQB1*02:02*–associated DR7-DQ2 haplotype was also overrepresented in alloimmunized women, suggesting a role for this allele or haplotype in HPA-1a alloimmunization. As *DRB4*01:01* is predominantly associated with the DR7-DQ2 haplotype in HPA-1a–alloimmunized individuals, the reported association with FNAIT may be coincidental. Typing for DR-DQ haplotypes revealed important genetic associations with HPA-1a alloimmunization not evident from typing individual alleles, and the presence of different DRB3-associated DR-DQ haplotypes showed different prevalence of HPA-1a alloimmunization.

Maria Therese Ahlen and Gøril Heide contributed equally to this study.

1 | INTRODUCTION

FNAIT is a rare condition that can cause severe complications such as intracranial haemorrhage in the foetus or newborn due to the transfer of platelet-depleting alloantibodies from the mother to the foetus during pregnancy. The most common cause of FNAIT is maternal alloantibodies directed against HPA-1a on foetal platelets. This fetomaternal incompatibility is defined by a leucine/proline polymorphism at residue 33 in integrin β3.[1] About 2% of Caucasian women are homozygous for the HPA-1 variant with proline (HPA-1bb). Alloimmunization occurs in about 10% of these women,[2] and about one third of these will give birth to a child with FNAIT. The most severe complication is intracranial haemorrhage (ICH), which occurs in approximately 1 in 10,000 unselected pregnancies.[2] Several factors may in theory affect the natural history of FNAIT, as reviewed by Sachs and Santoso (2017),[3] including the influence of the maternal HLA class II genotype. The MHC class II allele *HLA-DRB3*01:01* is strongly associated with HPA-1a alloimmunization; more than 90% of immunized women carry this MHC allele,[4-6] which also shows a dose-dependent association to the severity of HPA-1a immunization.[7] This strong genetic association suggests that HPA-1a immunization is dependent on T cells restricted by the MHC class II molecule encoded by the *HLA-DRA/DRB3*01:01* alleles. There is support for this notion: HPA-1a– but not HPA-1b–derived peptides bind this molecule, and the allogeneic residue Leu33 serves to anchor the peptide.[8,9] Furthermore, HPA-1a–specific $CD4^+$ T cell clones have been isolated from alloimmunized women,[10-12] and these T cell clones are restricted by DRA/DRB3*01:01.[10] However, it is possible that there exist additional genetic factors that predispose for immunization. In that respect, both *DQB1*02* and *DRB4*01* have been shown to be associated with FNAIT.[13,14] However, these alleles and other genetic elements that may influence HPA-1a alloimmunization do not segregate entirely independently of each other. Rather, they are in linkage disequilibrium with other genes in conserved haplotypes. In this respect, the *DRB3*01:01* allele is known to be in linkage disequilibrium with several different DR-DQ haplotypes, and one of these contains a *DQB1*02* allele.[15,16]

In the present study, we aimed to examine the impact of *DRB3*01:01*–associated DR-DQ haplotypes on HPA-1a alloimmunization, and to determine the haplotype associations and relative importance in HPA-1a alloimmunization for *HLA-DQB1*02:01/*02:02* and *HLA-DRB4*01:01* alleles.

2 | METHODS

The study was approved by the Regional Committee for Medical Research Ethics, North Norway (approval no. P REK NORD 66/2005, 2009/1585 and 2012/1917). Blood samples were drawn from patients and healthy volunteers after written informed consent.

2.1 | Study groups

In total, 167 HPA-1a–immunized women were included in the study comprising two different populations: one group of HPA-1a–negative women who developed anti-HPA-1a IgG antibodies in connection with pregnancies, included from the Norwegian prospective FNAIT screening study (1995-2004) (prospective screening group, N = 123)[4] (More than 2/3 of these immunized women were included from the southern part of Norway), and one group of women referred to the Norwegian National Unit for Platelet Immunology (NNUPI) after giving birth to a child with severe thrombocytopenia and clinical signs of bleeding, also producing anti-HPA-1a IgG antibodies (retrospective group, N = 44). The criteria for inclusion of both subgroups were detectible levels of anti-HPA-1a antibodies.

The control group was made of 781 healthy random blood donors at the University Hospital of North Norway, referred to as the general population. No information regarding ethnicity was available for neither the random donors nor the immunized women. The Sami population is known to differ from the Norwegian population in several HLA types.[17] However, most individuals were expected to be Caucasians in both groups as all donors were recruited from the Norwegian population, and not from specific Sami areas of Norway. The frequency of *DQB1*02* in our control population also fits the frequency of *DQB1*02* in a group of about 8000 Norwegian donors at the bone marrow registry (32,6%), lending support to the notion that the control population in this study reflects the general population in Norway.

2.2 | DNA isolation

Cryopreserved genomic DNA was available from about 40% of the HPA-1a–alloimmunized women. In addition, DNA was isolated from blood samples from the rest of alloimmunized women and all randomly selected donors, using Blood Mini Kit (QIAGEN) and eluted in sterile H_2O. Short-time storage of DNA was carried out at −20ºC whereas −70ºC was used for long-time storage.

2.3 | Genotyping

Primer sequences, characteristics and cycling programs are listed in supplementary data (Tables S1 and S2). All primers were synthesized by Eurogentec (Belgium). All PCR were

performed in a total volume of 25μL, using 0.5U HotStarTaq (Qiagen) per reaction, and run on a GeneAmp9700 (Applied Biosystems). *HGH* was used as an internal DNA amplification control.[18] Genomic DNA from IHWG cell lines was used as controls for amplification, specificity and enzyme digestion: STEINLIN, DUCAF, EMJ, LZL, CEPH-1413, HAG, BER, WDV and EK (Table S3).

Restriction cutting of PCR products was performed with endonucleases (all from New England Biolabs) according to references (Table S2).

The risk of false-positive detection within each genotyping assay, for example amplification of infrequent alleles by group-specific primers, could be evaluated by conferring dbMHC (the MHC database) with updated sequences.

2.3.1 | Designation of HLA-DR-DQ haplotypes

HLA-DR-DQ haplotypes for each donor were designated based on known DR-DQ haplotypes in the Norwegian population,[15,17,19] based on typing of selected DR- and DQ alleles. To answer our main questions in this study, alleles known to be associated with *DRB3*01:01* and *DQB1*02* were specifically typed for (Table 1).

2.3.2 | Detection of *DRB3*01:01P*

Detection of *DRB3*01:01P* (http://hla.alleles.org/alleles/p_groups.html) was performed for all immunized women and random donors by PCR using sequence-specific primers for *DRB3*01:01/*03:01*,[20] combined with restriction fragment length polymorphism (RFLP) by the enzyme *Kpn*I, cutting amplified *DRB3*01:01,* but not *DRB3*03:01*. All *DRB3*01:01*–positive random donors (213) and immunized women (157) were included for further detection of HLA-DR and –DQ alleles.

Because the above-mentioned method of *DRB3*01:01* detection is both time- and resource demanding, we also developed a new *DRB3*01:01* typing assay. This assay was performed for almost all individuals as duplex assay for *DRB3*01:01* and *GAPDH* in a TaqMan RQ-PCR assay on genomic DNA: 10 to 100 ng DNA was used in 25 μL reactions, in parallel reactions, with TaqMan Fast Universal PCR Master Mix, run on Fast mode (95°C for 20 seconds followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds) on ABI Prism7900HT (Applied Biosystems).

Oligos for HLA-DRB3: HLA-DRB3 primers (1000nM) 5'- TCTTGGAGCTGCGTAAGTCTGA-3', 5'-TGTTCCAG GACTCGGCGA-3' and a specific HLA-DRB3*01:01 probe (150 nM) 5'-6-FAM-TCTPTCCAGGZACCG-BHQ-1-3' (P= G-LNA, Z=T-LNA nucleotides). For GAPDH as reference, primers (500nM) 5'-CCCCACACACATGCACT TACC-3', 5'-CCTAGTCCCAGGGCTTTGATT-3' and a probe (100nM) 5'-VIC-AAAGAGCTAGGAAGGACAGGC AACTTGGC-BHQ-1-3'. Samples from fully HLA typed cell lines were used as controls for method development: 2 copies of DRB3*01:01 (STEINLIN), 1 copy of DRB3*01:01 (D4BL4 in-house), as well as *DRB3*01:01*–negative, *DRB3*02:02/DRB3*03:01*–positive samples (DUCAF/EMJ).

2.3.3 | Detection of DQB1*02 and DQA1*05

Group-specific primers for *DQB1*02* and *DQA1*05* were multiplexed.[21,22] The primers for *DQA1*05* and *DQB1*02* theoretically amplify all alleles in the two different groups. To discriminate between the allele *DQB1*02:02* and other *DQB1*02 alleles,* typing for *DQB1*02* [23] was combined with digestion by the restriction endonuclease *Msc*I, cutting only the amplified product of *DQB1*02:02* in exon 3 of the DQB1 gene.

2.3.4 | Detection of DRB1*03:01, (*11:01/*11:02), *13:01, *13:02, *13:03, (*14:01)

Group-specific primers for two groups of alleles were used.[24] Primers for Group-1 amplified *DRB1*11:01*, **13:02*, and **13:03.* The restriction endonucleases, *Fok*I and *Sfa*NI, were

TABLE 1 Common HLA alleles and HLA DR-DQ haplotypes in the Norwegian population, specifically typed for in this study. Adapted from Spurkland et al, Harbo et a and Lande et al,[15,17,19] with modifications in DRB1*13[31]

Haplotype	*DRB1*	*DQA1*	*DQB1*	Additional DRB association
DR3-DQ2	****03:01***	****05:01***	****02:01***	***DRB3*01:01***
DR13(01)-DQ6	****13:01***	**01:03*	**06:03*	***DBR3*01:01***
DR13(03)-DQ3	****13:03***	**05:01*	**03:01*	***DRB3*01:01***
DR7-DQ2	****07:01***	**02:01*	****02:02***	***DRB4*01***
DR7-DQ3	****07:01***	**02:01*	**03:03*	***DRB4*01***

All alleles are written in italic. Alleles specifically typed for in this study are in bold.

used to distinguish between these three *DRB1* alleles. Primers for Group-2 amplified *DRB1*03:01*, **11:02*, **13:01*, and **14:01*. The restriction endonucleases *Fok*I, *Sfa*NI and *Kpn*I were used to distinguish between these alleles. For details, see Table S2.

2.3.5 | Detection of *DRB1*07*

Detection of *DRB1*07* was done by group-specific PCR for *DRB1*07*.[25]

2.3.6 | Detection of *DRB4*01:01*

Two sets of primers and restriction endonucleases, for exon 2 and exon 3, were used. The first group-specific primers for *DRB4*01* in exon 2 amplify all *DRB4*01* alleles except *DRB4*01:05*.[26] Samples positive in the first PCR were further typed for exon 3, with primers specific for *DRB4*01:01, -*01:02, -*01:03, -*01:06, -*02:01,* and *-*03:01*.[27] The restriction endonuclease *Eae*I cuts *DRB4*01:01* and *DRB4*01:06*. Hence, samples with amplified products in both reactions and only restricted by the *Eae*I enzyme could be *DRB4*01:01* and/or *DRB4*01:06* positive.

2.3.7 | Confirmation of haplotypes and additional haplotype detection

To support the assigned *DRB3*01:01*–associated haplotype, for each immunized women and each individual in the control group, based on the results of PCR-RFLP genotyping and to uncover the additional haplotype, low-resolution typing for *DRB1* was performed by the Norwegian Bone Marrow Registry in Oslo, Norway; donors were typed for HLA-DRB1 by oligonucleotide probing defining HLA-DR1, DR2, DR3, DR4, DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR14 and DR103 specificities. Due to too low amounts of DNA, some donors could not be low-resolution typed or typed for *DRB4*01:01*. However, all individuals included for analysis of the *DRB3*01:01*–associated haplotype were still sufficient typed to define the associated haplotype. Donors not typed for *DRB4*01:01* were excluded from the statistical analysis of *DRB4*01:01* or its associations. 'N' is given for each statistical analysis.

2.4 | Estimating the prevalence of HPA-1a alloimmunization with different *DRB3*01:01*–associated haplotypes

The prevalence of alloimmunization was estimated by combining data from the Norwegian FNAIT screening study with data from the general population (control population in the current study). The number of HPA-1a–negative women in the screening study who carried the various *DRB3*01:01*–associated DR-DQ haplotypes was estimated by applying the population frequencies of these haplotypes determined for the general population (control population in the current study). Patient samples of HPA-1a–alloimmunized women from the screening study were also examined for these haplotypes. The prevalence of HPA-1a alloimmunization was calculated as the per cent of HPA-1a–negative women who eventually became alloimmunized, for each DR-DQ haplotype. Not all HPA-1a–immunized women were included for HLA typing due to lack of DNA. The results from our typing of a proportion of immunized women were therefore adjusted to apply to the whole population of immunized women. Estimates are shown in Figure 1. Statistical analyses could not be used for estimated prevalence numbers. In support of the accuracy of these estimates, the binomial test for measured frequencies of these haplotypes showed that the HPA-1a–immunized individuals were significantly different from the general population.

2.5 | Genotyping of TNF-308, LTA252 and AGER-429 SNPs

TNF −308G>A (rs1800629), LTA 252A>G (rs909253) and AGER-429T>C (rs1800625) SNPs were determined with allelic discrimination assays. Primers and probes are listed in Table S4.

2.6 | Statistics

To compare allele and haplotype frequencies between two groups, the chi-square test was used. Differences between groups were considered significant if $P < .05$. As the number of a priori hypotheses was small and related to specific haplotypes, correction for multi-significance was not applied as recommended by Perneger TV.[28]

3 | RESULTS

3.1 | The DR3-DQ2 haplotype occurs at a higher frequency among HPA-1a–alloimmunized women than would be expected by random distribution

To identify the most common DR-DQ haplotypes in linkage disequilibrium with the HLA-DRB3*01:01 allele, we first identified *DRB3*01:01* positives among

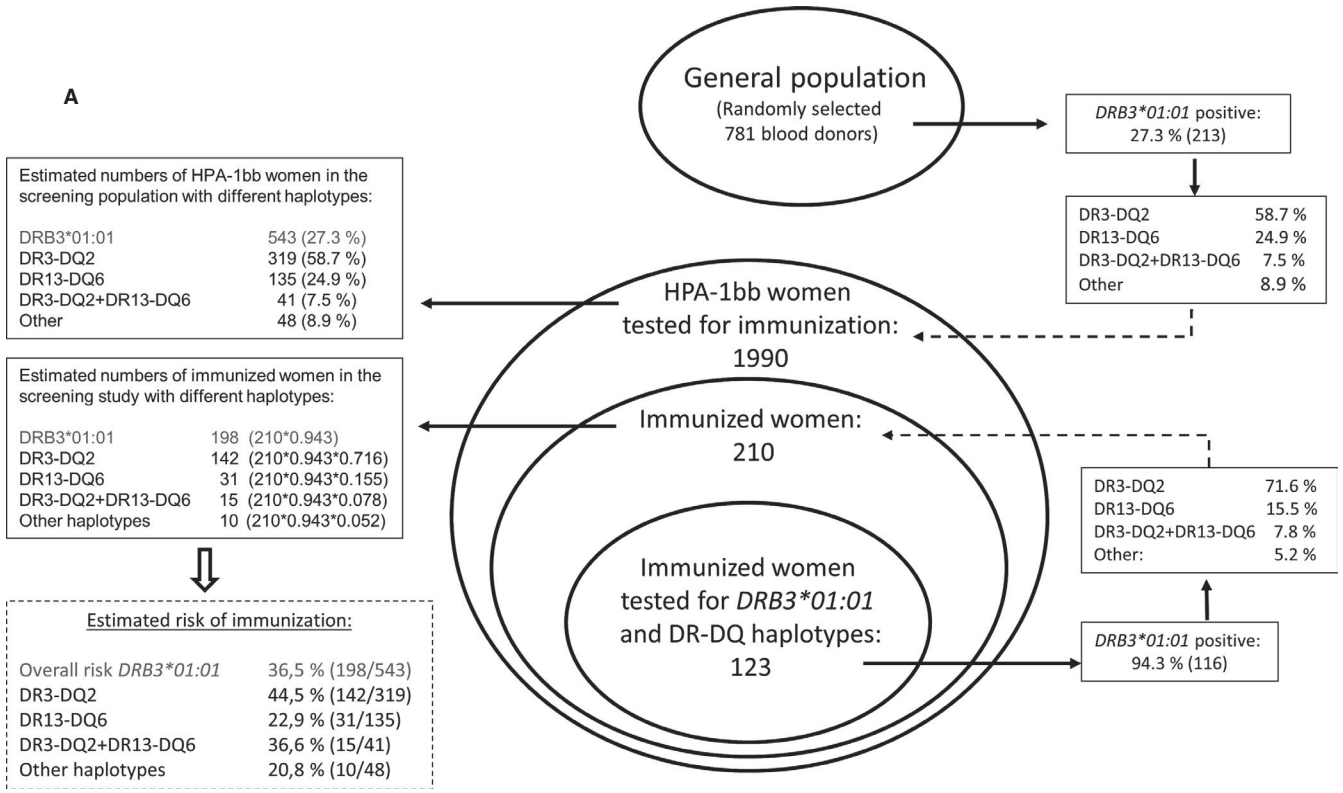

FIGURE 1 The estimated prevalence for HPA-1a alloimmunization is twice as high for women carrying DR3-DQ2 compared to DR13-DQ6. (A) The prevalence of HPA-1a alloimmunization for groups of women with different *DRB3*01:01*–associated DR-DQ haplotypes in a prospectively selected population was examined by combining data from the Norwegian screening study [4] and DR-DQ haplotype data generated in the current study. (B) Each bar represents individuals enrolled in the Norwegian screening study projected to carry one or two *DRB3*01:01*–associated DR-DQ haplotypes. The width of each bar represents the proportion of individuals with the indicated DR-DQ haplotype, projected from measured frequency of individuals carrying the different *DRB3*01:01*–associated DR-DQ haplotypes in the general population. The height of each bar represents the estimated prevalence of HPA-1a alloimmunization and is based on the actual number of HPA-1a–alloimmunized individuals determined to carry the specific haplotype. The overall prevalence of HPA-1a alloimmunization in *DRB3*01:01*–positive individuals is 36.5%. The prevalence of immunization with DR3-DQ2 (44.5%) is about twice as high as for DR13-DQ6 (22.9%). The accuracy of these estimates is supported from binomial test of statistical significance for measured frequencies of these haplotypes in the HPA-1a–immunized individuals against the general population: Frequency of HPA-1a–alloimmunized women with DR3-DQ2 (71,8%, n = 116) against the general population (58.7%): $Z = 2.67$, $P = .0038$. Frequency of HPA-1a–alloimmunized women with DR13-DQ6 (15.5%, n = 116) against the general population (24.9%). $Z = 2.35$, $P = .009$

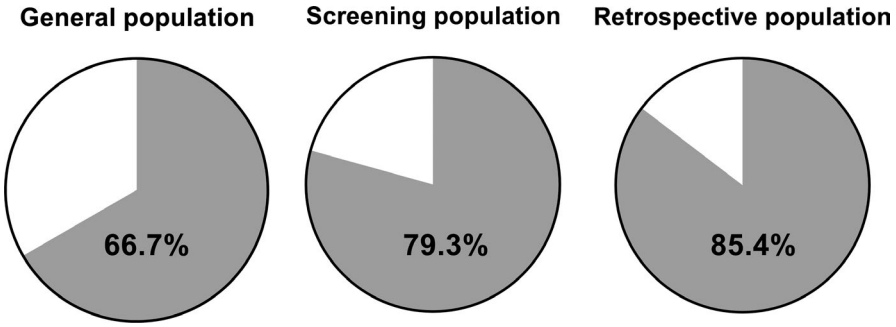

FIGURE 2 The frequency of the DR3-DQ2 haplotype in the general population and in HPA-1a–immunized women. Both in a population of HPA-1a–alloimmunized *DRB3*01:01*–positive women identified in a prospective screening study,[4] 'Screening population', and in a population consisting of women referred from the clinic following identification of suspected FNAIT cases, 'Retrospective population', the per cent individuals carrying the DR3-DQ2 haplotype were higher than in a general population of *DRB3*01:01*–positive individuals (screening: difference12.6%, $P = .016$. Retrospective: difference 18.7%, $P = .017$). The 'Retrospective population' is likely enriched in women who gave birth to severely thrombocytopenic neonates compared to the 'Screening population', in which roughly a third of affected neonates were severely thrombocytopenic

HPA-1a–immunized individuals (n = 167) (both prospectively and retrospectively selected) and in the general population (n = 781). The same populations were also typed for *HLA-DQB1*02* and *HLA-DRB4*01* alleles since these have also been reported to be associated with HPA-1a immunization.

As expected, in total, 94% (157) of the HPA-1a–immunized women carried the *DRB3*01:01* allele, compared to only 27.3% (213) of the general population ($P < .0001$; Table 2); notably near similar frequency of *DRB3*01:01* was found in the general population in other countries.[29]

Almost all immunized women and random donors were also typed by the TaqMan assay for detection of *DRB3*01:01,* and the results were in concordance with the old assay for typing of *DRB3*01:01*.

In total, 82.0% of the HPA-1a–immunized women carried a *DQB1*02* allele, compared to only 31,0% in a random selection of the general population ($P < .0001$) (Table 2).

Only 10.5% of HPA-1a–immunized women carried the *DRB4*01:01* allele, not significantly different from 6.9% in a random selection of the general population ($P = .23$) (Table 2).

Next, in a detection regime to identify *DRB3*01:01*–associated DR-DQ haplotypes, *DRB3*01:01*–positive individuals were further genotyped for selected alleles (as outlined in 'Methods'). *DRB3*01:01* is known to be associated with DRB1*03, DRB1*11, DRB1*12, DRB1*13 and DRB1*14 alleles.[29] However, some of the combinations are very rare in Caucasians.[29] DR alleles known to be associated with *DRB3*01:01* were successfully identified in 98.6% of random donors, and all but one of the immunized women. For the remaining random donors and immunized woman, we did not have enough DNA to perform low-resolution typing for DRB1; the typing results were therefore uncertain.

A comparison of the *DRB3*01:01*–associated DR-DQ haplotypes (Table 3) showed that there were two dominating haplotypes, DR3-DQ2 and DR13-DQ6, together accounting for > 90% of the immunized women. Furthermore, individuals with DR3-DQ2 were overrepresented among HPA-1a–immunized women (80.9%), compared to the general population (66.7%; $P < .003$). In the remaining few per cent of immunized women, *DRB3*01:01* was associated with less frequent haplotypes.

3.2 | DR3-DQ2 is overrepresented in both the retrospective and the screening groups

The HPA-1a–alloimmunized women in the study comprised of women identified in connection with a prospective screening study described previously [4] and women referred from the

TABLE 2 Frequency of individuals carrying *DRB3*01:01*, *DQB1*02* and *DRB4*01:01* alleles in HPA-1a–alloimmunized women and in the general Norwegian population

Allele	HPA-1a–alloimmunized women	General population	
*DRB3*01:01*	94.0[a] (N = 167)	27.3 (N = 781)	$P < .0001$
*DQB1*02*	82.0 (N = 167)	31.0 (N = 200)	$P < .0001$
*DRB4*01:01*	10.5 (N = 153)	6.9 (N = 204)	$P = .23$

Note: Number of individuals typed for the indicated allele in each population.

[a]Per cent of individuals in each population that typed positive for the indicated allele.

TABLE 3 Frequencies of the two most common *DRB3*01:01*–associated DR-DQ haplotypes in HPA1a–alloimmunized women and in the general population of Northern Norway

Haplotype	*DRB3*01:01*–positive HPA-1a–immunized women(n = 157)	*DRB3*01:01*–positive General population(n = 213)	
*DRB1*03:01-DQA1*05:01-DQB1*02:01* (DR3-DQ2)	80.9 (127)	66.7 (142)	$P < .003$
*DRB1*13:01-DQA1*01:03-DQB1*06:03* (DR13-DQ6)	20.4 (32)	32.9 (70)	$P < .008$

clinic following a pregnancy where FNAIT was diagnosed. Both groups showed overrepresentation of DR3-DQ2, 79.3% in the prospective group and 85.4% in the retrospective group (Figure 2), compared to 66.7% in the general population (diff 12,6, $P = .016$; diff 18,7, $P = .017$). Population frequencies of *DRB3*01:01*–associated DR-DQ haplotypes in each of these groups are shown in Figure S1.

3.3 | More women become HPA-1a alloimmunized when DRB3*01:01 is associated with the DR3-DQ2 haplotype

Since the proportion of those who have the DR3-DQ2 haplotype is higher in the immunized population in this study compared to the control population, we wanted to determine the prevalence of HPA-1a alloimmunization among *DRB3*01:01*–positive individuals having this or other DR-DQ haplotypes, that is the proportion of women with different *DRB3*01:01*–associated DR-DQ haplotypes who have become alloimmunized after one or several incompatible pregnancies. In lack of a control group of non-immunized HPA-1a–negative women, which would have been our preferred control group, the prevalence of immunization among women with different haplotypes had to be estimated. For this estimation, we used data from the Norwegian FNAIT screening study, in which more than 100.000 women were screened for HPA-1a negativity and HPA-1a alloimmunization in connection with pregnancy. Since DR-DQ haplotypes were not determined for subjects in the screening study, we estimated the proportion of the women carrying the different *DRB3*01:01*–associated DR-DQ haplotypes by applying the frequency of each of the *DRB3*01:01*–associated DR-DQ haplotypes determined for the control population in the current study. The prevalence of alloimmunization was then calculated from the number of HPA-1a–alloimmunized women with a particular *DRB3*01:01*–associated DR-DQ haplotype (typed in the current study) and the estimated number of HPA-1a–negative women in the screening study who carried the same haplotype (Figure 1A). The prevalence of alloimmunized HPA-1a–negative, *DRB3*01:01*–positive, DR3-DQ2–positive women was 44.5%, that for DR13-DQ6 haplotype is only 22.9% (Figure 1A,B). Hence, in HPA-1a–negative *DRB3*01:01*–positive women, we estimate that about twice as many will become HPA-1a–alloimmunized during or after one or multiple HPA-1a–incompatible pregnancies when the *DRB3*01:01* allele is associated with the DR3-DQ2 haplotype, compared to those with DR13-DQ6. The same approach was used to calculate the prevalence of alloimmunized HPA-1a–negative, *DRB3*01:01*–positive women: 36.5%.

3.4 | The DR7-DQ2 haplotype and DRB4*01:01 are overrepresented and DR15-DQ6 is underrepresented in DRB3*01:01–positive HPA-1a–alloimmunized women

Both haplotypes were successfully determined, by low-resolution DRB1 typing combined with typing for specific alleles, in 197 of 213 (92%) individuals in the *DRB3*01:01*–positive general population group and in 139 of 157 (88%) of HPA-1a–immunized *DRB3*01:01*–positive women.

Although all individuals in this study carry the *DRB3*01:01* allele on one or both chromosomes, *HLA-DQB1*02* is also strongly associated with HPA-1a alloimmunization.[13] *HLA-DQB1*02* is present in two different DR-DQ haplotypes. The *HLA-DQB1*02:01* allele is in linkage disequilibrium with *DRB3*01:01* in the DR3-DQ2 haplotype, while *HLA-DQB1*02:02* is present in the DR7-DQ2 haplotype, which segregates independent of *DRB3*01:01*. Furthermore, a negative association with HPA-1a alloimmunization has been reported for *HLA-DRB1*15:01*.[30] This allele is present in DR15-DQ6, which also segregates independent of *HLA-DRB3*01:01*. To determine the relative influence of these two DR-DQ haplotypes on HPA-1a alloimmunization, we independently typed for DR7-DQ2 and DR15-DQ6 on the second, non–*DRB3*01:01*-associated chromosome in each individual.

The DR7-DQ2 haplotype was seen more frequently among the HPA-1a–immunized women (11.7%) compared to the general population (5.4%; $P = .03$) (Figure S2A). This suggests that there is a weak association of the DR7-DQ2 haplotype in *DRB3*01:01*–positive HPA-1a–immunized women. However, there were too few individuals included in the study to conclude upon a potential dose effect of DQ2 on immunization. Also, DR15-DQ6 is underrepresented in HPA-1a–immunized women ($P = .01$) (Figure S2B).

To also determine whether the *DRB4*01:01* allele is associated with HPA-1a alloimmunization, we examined its presence in *DRB3*01:01*–positive immunized women and

random blood donors. When comparing these two groups, the frequency of *DRB4*01:01* is higher in immunized women, 9.2% (Table S5), than in the random population (2.9%; $P = .01$). *DRB4*01* is known to be associated with DRB1*04, DRB1*07 and DRB1*09.[29]

All individuals carrying the *DRB4*01:01* allele, both in the group of immunized women and in the general population, also carried either the DR7-DQ2 (all *DRB4*01:01*–positive individuals except one) or the DR7-DQ3 haplotype (one immunized individual) (data not shown).

3.5 | An inflammation-associated single nucleotide polymorphism occurs at a higher frequency in alloimmunized compared to general population DR3-DQ2–positive individuals

Since DR3-DQ2 was found to be more associated with HPA-1a immunization, we wanted to examine whether this haplotype is associated with known factors that may increase the chance of immunization. Therefore, most individuals with a detected DR3-DQ2 haplotype were also typed for the Conserved Extended Haplotype (CEH) markers *TNF-308A/ LTA252G/ AGER-429C* by in-house allele discrimination assays (117 immunized women and 145 random donors were tested). Ninety per cent and 86% carried these markers, among immunized women and general population, respectively (data not shown). In addition, we observed that 29.4% of the immunized women with the DR3-DQ2 haplotype in the retrospective group were homozygous for the *TNF-308A* marker, a more frequent occurrence compared to both the prospective group (10.9%; $P < .025$) and the control group (7.0%; $P < .0008$).

4 | DISCUSSION

The association of *HLA-DRB3*01:01* with HPA-1a alloimmunization is well documented. In the present study, we show that also DR-DQ haplotype association significantly influences the prevalence of HPA-1a alloimmunization; women carrying DR3-DQ2 are estimated to be about twice as likely to become HPA-1a alloimmunized compared to those carrying DR13-DQ6, even though both of these haplotypes are associated with the *DRB3*01:01* allele. One third of HPA-1a–negative, *DRB3*01:01*–positive pregnant women with an HPA-1a–positive child were immunized; the estimated prevalence is, however, 44.5% if the *DRB3*01:01* allele is linked to the DR3-DQ2 haplotype.

The frequency of different HLA-DR-DQ haplotypes in the Norwegian population is known from typing by the Norwegian Bone Marrow Registry.[15,17,19] We have focused on the most common haplotypes. The *DRB3*01:01* allele is associated with several of these HLA-DR-DQ haplotypes,[15,29,31,32] and DR3-DQ2 and DR13-DQ6 represent the major two of these.[29] The increased risk of alloimmunization with DR3-DQ2 suggests that other genetic elements that

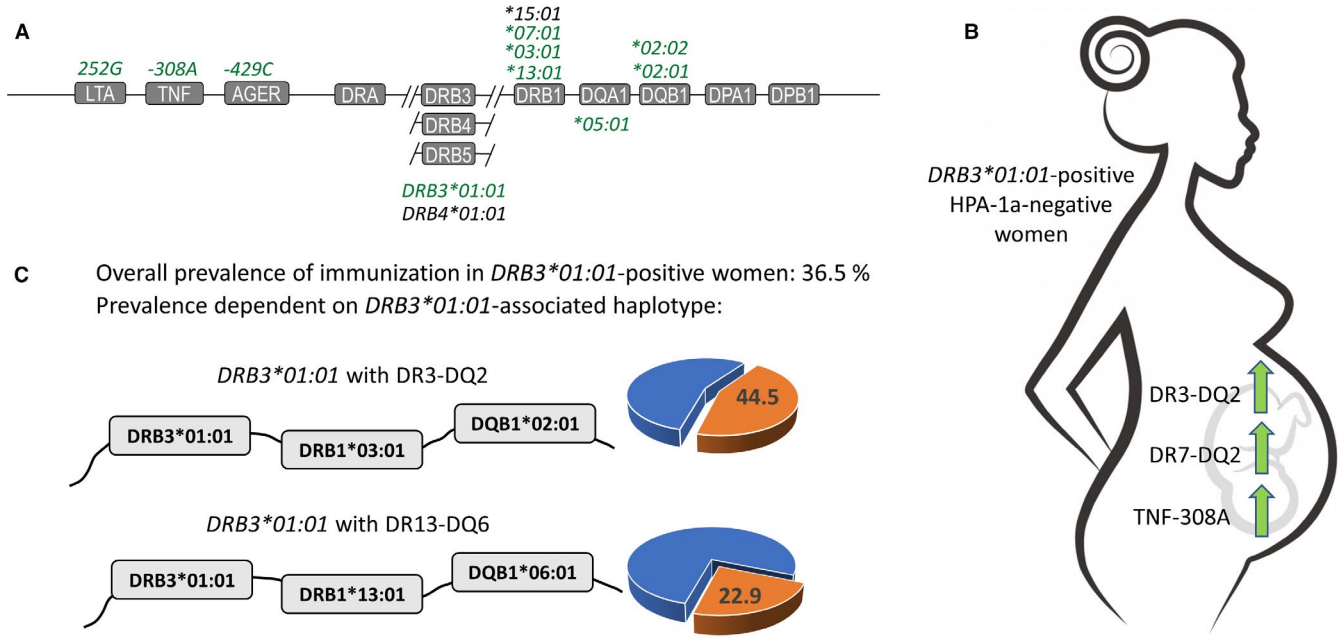

FIGURE 3 HLA-DR-DQ haplotype association with HPA-1a alloimmunization. (A) Alleles and SNPs that were specifically examined in this study in relation to HPA-1a alloimmunization. Those positively associated with HPA-1a alloimmunization are marked in green. (B) Haplotypes and SNPs positively associated with HPA-1a alloimmunization in HPA-1a–negative *DRB3*01:01*–positive women. C. Prevalence of HPA-1a alloimmunization in HPA-1a–negative women carrying *DRB3*01:01* with DR3-DQ2 or with DR13-DQ6

somehow increase the chance of HPA-1a alloimmunization are associated with this haplotype.

In addition to *DRB3*01:01*, the *DQB1*02* alleles are also strongly associated with HPA-1a alloimmunization.[13,30] As shown herein, this association can largely be accounted for by its genetic linkage to the DR3-DQ2 haplotype; in the present study, 80.9% of *DRB3*01:01*–positive women carried the DR3-DQ2 haplotype. This linkage was even stronger (85.4%) when only retrospective cases were considered, which is similar to that analysed in a previous study.[13] Therefore, the reported DQ2 association with alloimmunization may be coincidental. Alternatively, it is possible that *DQB1*02* may represent a genetic element in the DR3-DQ2 haplotype that contributes to increased risk of alloimmunization with HPA-1a. In support of the latter, DQ2 is associated with alloimmunization also by a second allele not linked to DR3-DQ2. This second allele, *DQB1*02:02*, is present in the DR7-DQ2 haplotype,[33] which is the only other DR-DQ haplotype including DQ2 besides DR3-DQ2.[15]

The *DQB1*02:02* allele and, thus, the DR7-DQ2 haplotype are present at a higher frequency in HPA-1a–alloimmunized *DRB3*01:01*–positive women compared to in *DRB3*01:01*–positive individuals in the general population. Unlike the *DRB3*01:01* allele, which has been functionally associated with alloimmunization, there is no evidence for a similar function associated with DQ2 alleles. Still, the possibility that such cells exist in HPA-1a–immunized women cannot be ruled out.

Taken together, there is still no direct evidence for a functional role of *DQB1*02* in HPA-1a alloimmunization. Whether it is coincidental that both haplotypes found to be associated with HPA-1a immunization and FNAIT contain a *DQB1*02* allele remains to be determined.

The *DRB4*01:01* allele was also suggested by Loewenthal et al[14] to be important for HPA-1a immunization, by acting in synergy with the *DRB3*01:01* allele to cause more serious immunization in women positive for both these alleles.[14] The opposite was found, however, by L'abbé et al,[13] where *DRB4*01* seemed to be more frequent in random donors than in immunized women. Wienzek-Lischka et al,[34] Delbos et al [35] and Sainio et al[36] subsequently examined the role of this allele and did not find it to be overrepresented in HPA-1a–immunized women. Wienzek-Lischka et al also found that the combination of *DRB3*01:01* and *DRB4*01:01/03* did not enhance immunization compared to only carrying the *DRB3*01:01* allele alone.[34] Although earlier reports[14,30,34] did not distinguish between *DRB4*01:01* and *DRB4*01:03*, because there is no difference in exon 2 of these alleles and they phenotypically are the same, we chose to only focus on *DRB4*01:01*. We were more curious about the different DRB1 allele associations of *DRB4*01:01* than the potential function of the molecule it codes for.

In this study, the *DRB4*01:01* allele was relatively rare, but more frequent in already *DRB3*01:01*–positive immunized women compared to the general population. For all women, except one, carrying this allele, it was associated with the DR7-DQ2 haplotype. Taken together, this points to a role for the DR7-DQ2 haplotype, and thereby may be for *DQB1*02:02*, rather than for the *DRB4*01:01 allele*, in HPA-1a immunization.

A major question addressed in this study is whether particular DR-DQ haplotypes are associated with increased prevalence of HPA-1a alloimmunization. This was determined by using prospective data from the Norwegian FNAIT screening study [4] and DR-DQ haplotype frequencies determined for the control population (random Norwegian blood donors that have the *DRB3*01:01* allele) in the current study. The measured allele frequency of *DRB3*01:01* in the Norwegian population in the current study was 14.3% (781 random donors with a total of 1562 alleles, 224 positively typed DRB3*01:01 alleles), close to the allele frequency (14.9%) measured in a large European Caucasian population in the United States,[29] lending support to the validity of our control population. We show that the prevalence of HPA-1a alloimmunization in the group of *DRB3*01:01*–positive women carrying DR3-DQ2 is twice as high as for women carrying the DR13-DQ6 haplotype. In support of these findings, both Sainio et al[36] and Wienzek-Lischka et al[34] found that both *DR3-DRB3*01:01* and *DR13-DRB3*01:01* are overrepresented in immunized women compared to controls and that *DR3-DRB3*01:01* is found more often than *DR13-DRB3*01:01*. Both these studies are based on retrospective data, showing a *DRB3*01:01* frequency of 100% and 98%, respectively. Benefiting from prospective data, however, we show a lower frequency of this allele. Most important, by comparing already *DRB3*01:01*–positive individuals we show here that *DRB1*13:01* is overrepresented only by association with *DRB3*01:01* and that the prevalence of immunization in the group of *DRB3*01:01*–positive women carrying DR13-DQ6 actually is lower than the prevalence of immunization in other *DRB3*01:01*–positive women.

The DR3-DQ2 haplotype has been studied for years, due to its association with a number of immune-mediated diseases, and especially autoimmune disorders.[37-40] In Caucasians, the DR3-DQ2 haplotype is commonly a part of the conserved extended haplotype AH 8.1, (HLA-A1 - B8 - DRB3*01:01 - DRB1*03:01 - DQB1*02:01). In addition to the antigen-presenting role of the separate HLA class II variants encoded within the haplotype, the non-MHC coding alleles embedded have also been studied, with *C4A, TNF, LTA* and *AGER* as candidate markers.[39,41] There are several reports of constitutively higher systemic levels of TNFα in healthy individuals with AH 8.1; however, evidence for direct influence of the 8.1 AH marker (TNF-308A) on TNFα levels is ambiguous.[42] Our finding that almost all DR3-DQ2–positive women also

carry this 8.1 AH marker and that the frequency of homozygous TNF-308A is higher in immunized women suggests that it may contribute to increase the likelihood of HPA-1a immunization.

Associations between HLA alleles and diseases have been known for about 50 years. *DRB3*01:01* has also been shown to be associated with several diseases, in addition to HPA-1a immunization; for example, *DRB3*01:01* is increased in patients with Grave's disease in Jamaicans[43] and has been associated with sarcoidosis.[44] Whether the association between *DRB3*01:01* and several different autoimmune diseases is coincidental because of the close linkage to other alleles in the AH8.1 haplotype is, however, uncertain. In addition to this, other HLA alleles and haplotypes have also been associated with different diseases. One such well-known association is the linkage between *DQB1*02* and celiac disease, where the disease also is shown to be more frequent with a double dose of DQB1*02.[45]

In contrast to the positively associated alleles, the *DRB1*15:01* allele has previously been reported as negatively associated with FNAIT, suggesting a regulatory or suppressive role of this allele.[30] This negative association was also found in the current study, although we here suggest that the negative association can be accounted for by the presence of this allele in an underrepresented haplotype: DR15-DQ6. It is possible that the negative association with FNAIT could be due to other elements present in this haplotype and not necessarily the *DRB1*15:01* allele itself.

As the methods for HLA typing that were applied were published some years ago and do not reflect the current standard of HLA typing, it cannot completely be ruled out that new HLA alleles could have been amplified by the primers used for HLA typing. Alignments of all DRB3 alleles known to this date, with primers in the current study for detection of *DRB3*01:01,* show that the primer pairs would also have amplified, among others, DRB3*02:06, *02:08, *02:21, *02:44, and *02:56. Common for all these alleles, however, is that they have only been found once by one lab and only one of them is confirmed. In addition, the prevalence of these alleles in different ethnic populations is not known. Thus, if one or two new HLA alleles had erroneously been amplified, it is unlikely that this would have influenced our main results.

The main results of this study are summarized in Figure 3. In summary, this study represents one of few studies in which MHC allele associations with HPA-1a immunization have taken into consideration the strong linkage disequilibrium that exist between specific MHC alleles. More importantly, and based on data from a prospective screening study, our results indicate that the haplotype which is associated with the *DRB3*01:01* allele has a considerable impact on the chance of HPA-1a alloimmunization, likely due to yet not identified associated genetic elements. The haplotypes and alleles identified herein point to genetic elements that will be investigated for greater insight into the immune response that results in FNAIT. Potentially, these may guide a more accurate identification of pregnancies most at risk of FNAIT, in a clinical setting.

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CONFLICT OF INTEREST

AH, BS and J.K-K. are three of the founders and owners of Prophylix AS, which has been developing a hyperimmune anti-HPA-1a IgG for the prevention of foetal and neonatal alloimmune thrombocytopenia. J.K-K. is a consultant for Rallybio IPA, LLC, which recently acquired the assets of Prophylix AS.

AUTHORS' CONTRIBUTIONS

Contribution**:** MTA and TBS planned the study. MTA and GH conducted the study. MTA, GH and TBS wrote the manuscript. AH, JKK and BS reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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The DR7-DQ2 Haplotype in a Native Norwegian Population

To the Editor

Non-random association of HLA-DR and DQ alleles in haplotypes has become important in dissecting the genetic influence in different diseases. Recently, it was shown that *DRB1*07* and *DQB1*02:01* represent the most common DR7-DQ2 association in one defined Asian immigrant population in Norway [1], while *DRB1*07* and *DQB1*02:02* are more common in European Americans [2]. The association between these alleles in native Norwegians is not known. Because DR7-DQ2 associations have direct relevance to our research related to foetal and neonatal alloimmune thrombocytopenia, we set out to address this question.

In the Norwegian population, 24 different HLA-DR-DQ haplotypes are considered common, with frequencies $\geq 1\%$ in the population [3, 4]. Spurkland *et al.* [3] reported in 1992, that the DR7-DQ2 haplotype consists of *DRB1*07:01* and *DQB1*02:01.* Only later was the distinction made between the *DQB1*02:01* allele and a second *DQB1*02* allele, *DQB1*02:02* [5] (assigned in 1994; IMGT/HLA database). Thus, because the original paper on HLA class II haplotypes in Norwegians only typed for polymorphisms in exon 2 [3] and the polymorphism distinguishing *DQB1*02:01* and *DQB1*02:02* lies in exon 3, the reported DR7-DQ2 haplotype in Norway could have included both *DRB1*07:01-DQB1*02:01* and *DRB1*07:01-DQB1*02:02*.

In 2003, Klitz *et al.* [2] reported haplotype frequencies of the MHC class II loci DRB1, DQA1 and DQB1 (high-resolution typing) in European Americans. In this population, the *DRB1*07:01* allele was in linkage disequilibrium with *DQB1*02:02* (11.1%), *DQB1*03:03* (3.7%) and *DQB1*02:01* (1.1%). We therefore expected this to be the case also in the Norwegian population, and this was our assumption until the recent report on DR-DQ haplotypes in Pakistani immigrants in Norway [1], where the *DRB1*07:01:01-DQB1*02:01:01* is clearly more frequent (9.9%) than the *DRB1*07:01:01-DQB1*02:02* haplotype (1.1%). The finding by Rønningen *et al.* [1] prompted us to determine the nature of this association in a native Norwegian population.

To assess the occurrence of the *DRB1*07-DQB1*02:01:01* haplotype, 202 random native Norwegian blood donors were typed for *DRB1*07*, *DQB1*02* and *DRB1*03* by group-specific primers [6–8]. All *DQB1*02-*, *DRB1*03-* and/or *DRB1*07*-positive individuals ($n = 61$) were further analysed by sequence-based typing of DQB1 for exons 2 and 3 [9]. Haplotypes were assigned to individuals according to the identified alleles. In 21 of the DR3- or DR7-positive individuals, additional typing for DRB1 allowed identification of both haplotypes (data not shown), providing confidence to the assignment. The DR and DQ alleles in the Norwegian population are reported to have a good fit to the Hardy–Weinberg proportion model [10].

We found that *DRB1*07* was seen in combination with *DQB1*02:02* and *DQB1*03:03:02*, and in one individual, in a rare combination with either *DQB1*04:02:01* or *-*05:01:01* (Table 1). In contrast, *DQB1*02:01:01* was only found in combination with *DRB1*03.* Two donors were positive for both *DQB1*02:01:01, DQB1*02:02, DRB1*07* and *DRB1*03;* which corresponds to haplotypes *DRB1*03-DQB1*02:01:01* and *DRB1*07-DQB1*02:02*. Frequencies of the different haplotypes in our material are in agreement with the haplotype frequencies given in the sample of European Americans in 2003 [2].

In conclusion, we have determined that in a native Norwegian population, the DR7-DQ2 haplotype consists mainly or only of *DRB1*07-DQB1*02:02*, and not *DRB1*07-DQB1*02:01:01.*

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Author contributions

GH, TBS and MTA designed and planned the study. GH and MTA planned and performed the laboratory

Table 1 Occurrence of DR3- and DR7-DQ haplotypes in a random native Norwegian population.

DRB1-DQB1 haplotypes	Number of individuals ($n = 202$)	%
*DRB1*03-DQB1*02:01:01*	31	15.3
*DRB1*03-DQB1*06:02*	0	
*DRB1*07-DQB1*02:01:01*	0	
*DRB1*07-DQB1*02:02*	19	9.4
*DRB1*07-DQB1*03:03:02*	8	4.0
*DRB1*07-DQB1*04:02:01/05:01:01*	1	0.5
*DRB1*07-DQB1*02:02* and *DRB1*03-DQB1*02:01:01*	2	1.0
Non-DR3 and -DR7 haplotypes	141	69.8

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experiments and analysed data. GH drafted the manuscript. All authors discussed data and edited the manuscript.

Conflict of interest disclosure

The authors report no conflict of interest in connection with this work.

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RESEARCH Open Access

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

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Abstract

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a bleeding disorder caused by maternal antibodies against paternal human platelet antigens (HPAs) on fetal platelets. Antibodies against HPA-1a are accountable for the majority of FNAIT cases. We have previously shown that high levels of maternal anti-HPA-1a antibodies are associated with clinically significant reduced birth weight in newborn boys. Chronic inflammatory placental lesions are associated with increased risk of reduced birth weight and have previously been reported in connection with FNAIT pregnancies. The HPA-1a epitope is located on integrin β3 that is associated with integrin αIIb (the fibrinogen receptor) on platelets and megakaryocytes. Integrin β3 is also associated with integrin αV forming the αVβ3 integrin heterodimer, the vitronectin receptor, which is expressed on various cell types, including trophoblast cells. It is therefore thinkable that maternal anti-HPA-1a antibodies present during early pregnancy may affect placenta function through binding to the HPA-1a antigen epitope on invasive trophoblasts. The aim of the study was to examine whether interaction of a human anti-HPA-1a monoclonal antibody (mAb) with HPA-1a on trophoblast cells affect adhesion, migration and invasion of extravillous trophoblast cells.

Methods: An in vitro model with human anti-HPA-1a mAb, clone 26.4, and the first trimester extravillous trophoblast cell line HTR8/SVneo was employed. The xCELLigence system was utilized to assess the possible effect of anti-HPA-1a mAb on adhesion and migration of HTR8/SVneo cells. Specially designed chambers precoated with Matrigel were used to assess the effect on the invasive capacity of cells.

Results: We found that human anti-HPA-1a mAb 26.4 partially inhibits adhesion and migratory capacity of HTR8/SVneo cells.

Conclusions: Our findings suggest that anti-HPA-1a antibodies may affect trophoblast functions crucial for normal placental development. Future studies including primary throphoblast cells and polyclonal anti-HPA-1a antibodies are needed to confirm these results.

Keywords: Alloimmunization, HPA-1a, Anti-HPA-1a antibodies, Trophoblast cells, Placental development, αVβ3, Vitronectin receptor, Fetal and neonatal alloimmune thrombocytopenia

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Background

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal antibodies against alloantigens on fetal platelets. It is a rare, but potentially life threatening disorder with intracranial hemorrhage (ICH) as the most severe complication. Severe gastrointestinal and pulmonary hemorrhages have also been reported [1]. Antibodies against human platelet antigen (HPA)-1a are accountable for nearly 85% of FNAIT cases [2]. The frequency of FNAIT due to anti-HPA-1a antibodies is around one per 1100 live births [2, 3]. We have previously found that high levels of maternal anti-HPA-1a antibodies are associated with clinically significant reduced birth weight in newborn boys [4]. A similar observation was made in an international multicenter study of FNAIT-associated ICH, showing that 23% of neonates with ICH were small for gestational age [5]. Chronic inflammatory placental lesions like chronic villitis and intervillositis have been reported in association with FNAIT cases [6] and such placental lesions are known to be associated with increased risk of fetal growth restriction.

Integrin β3, carrying the HPA-1 antigen epitope, is expressed on platelets and megakaryocytes as part of αIIbβ3 integrin heterodimer, the fibrinogen receptor. Integrin β3 is also associated with αV integrin forming integrin heterodimer αVβ3, also known as vitronectin receptor. The vitronectin receptor is expressed on various cell types, including trophoblast cells [7–9].

During early pregnancy, a population of trophoblast cells differentiates into highly invasive extravillous trophoblasts (EVT). EVT invade the decidualized endometrium reaching the inner third of the myometrium, and migrate along the spiral arteries remodeling them into large diameter low resistance vessels [10]. EVT migration and invasion into the uterus continues until mid-gestation and is regulated by various factors of both maternal and embryonic origin [11]. Impaired trophoblast invasion and insufficient remodeling of placental spiral arteries are common histopathological findings in placentas from pregnancies complicated by preeclampsia and low birth weight [12, 13].

During migration and invasion, EVT cells undergo integrin switch and upregulate expression of adhesion molecules on cell surface, including the αVβ3 [8, 14]. The important role of αVβ3 in mediating migration and invasion of primary cytotrophoblasts (CTB) was demonstrated in vitro [8, 15]. It has therefore been speculated that anti-HPA-1a antibodies may affect placental development [4]. Anti-HPA-1a antibodies can bind HPA-1a on αVβ3 expressed on trophoblast cells [9, 16], and we hypothesize that this binding may affect EVT invasion, spiral artery remodeling, and in turn lead to reduced placental function.

The objective of this study was to test whether anti-HPA-1a antibodies affect adhesion, migration and invasive capacity of EVT cells. For functional experiments we used an experimental in vitro model with human recombinant anti-HPA-1a monoclonal antibody (mAb), clone 26.4 [16], and a first trimester human EVT-derived cell line, HTR8/SVneo [17].

Methods

Cell culture

Human first trimester extravillous trophoblast-derived cell line, HTR8/SVneo, was kindly provided by Charles Graham (Department of Anatomy and Cell Biology at Queen's University, Kingston, ON, Canada). The cell line was generated by immortalization of primary villous explant culture from first trimester human placenta (8–10 WG) with SV40 virus [17]. HTR8/SVneo is a hypotriploid cell line (3n-) [18]. Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% FBS (Lonza, Basel, Switzerland), 100 U/ml penicillin, 100 U/ml streptomycin (Lonza) and maintained at 37 °C, in a 5% CO_2 humidified atmosphere. The cells were grown to 70–80% confluency and passaged 24 h prior to experiments. The cells were detached by incubation with 2 mM EDTA in PBS for 5 min at 37 °C.

Antibodies

A recently developed human recombinant anti-HPA-1a IgG1 mAb (clone 26.4) [16] was used to explore the effect on invasive trophoblast cells. Murine anti-human αVβ3 mAb, clone LM609 (Millipore, Billerica, MA) was used as positive control for cell functional studies. Sodium azide from LM609 sample was removed by buffer exchange with PBS using PD SpinTrap G-25 (GE Healthcare, Little Chalfont, UK). Integrin β3 was detected using murine mAb, clone SZ21, HPA-1-reactive [19] (Dako, Glostrup, Denmark) and rabbit mAb, clone EPR2417Y (Abcam, Cambridge, UK). Alexa Fluor 488-conjugated goat anti-mouse and goat anti-human antibodies (Invitrogen, Carlsbad, CA) were used as secondary antibodies in flow cytometry experiments. Human myeloma plasma IgG1 (Sigma) and murine IgG1 (Beckman Coulter, Brea, CA) were used as isotype controls. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Scientific, Waltham, MA) was used as a detection antibody in the western blot experiment.

Western blot

Platelets from an HPA-1aa-genotyped donor (16×10^8 cells) and HTR8/SVneo cells (20×10^6 cells) were lysed using 3 ml RIPA buffer (Sigma) in the presence of protease inhibitor (cOmplete Tablets Mini EDTA-free, Roche Diagnostics, Basel, Switzerland). Twelve microliters of

platelet lysate diluted 1:1000 and 12 μl of HTR8/SVneo cell lysate were reduced and separated in a 4–12% SDS polyacrylamide gel (Life Technologies, Carlsbad, CA). Electrophoresed samples were transferred to a PVDF membrane (Life Technologies). Nonspecific binding sites were blocked by Super blocking buffer (Thermo Scientific) containing 0.05% Tween 20 and 0.2% goat IgG (Thermo Scientific) for 1 h. Primary and secondary antibodies were diluted in Super blocking buffer containing 0,05% Tween 20. The PVDF membrane was incubated overnight at 4 °C with rabbit anti-β3 antibody diluted 1:2000 (clone EPR2417Y). After a washing step, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG diluted 1:1000 for 1 h at RT followed by a washing step with PBS 0.05% Tween 20. The membrane was covered by 3 ml of Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and left for 5 min in the dark at RT. Integrin β3 was visualized using the luminescent image analyzer ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK). Integrin αVβ3 purified from human placenta was used as a positive control (Millipore, Billerica, MA). The expected β3 subunit band is of approximately 90–110 kDa.

Flow cytometry

To stain cell surface membrane integrins, the HTR8/SVneo cells were harvested, washed and re-suspended in PBS 0.2% bovine serum albumin, and incubated 10 min at RT with unconjugated mouse anti-human β3 (clone SZ21) or human anti-HPA-1a (clone 26.4) mAbs. Mouse and human IgG1 were used as isotype controls. After a washing step, cells were stained with Alexa Fluor 488-conjugated goat anti-mouse and goat anti-human antibodies respectively, and analyzed by flow cytometry (Canto, Becton Dickinson, Franklin Lakes, NJ). The acquired data was analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

HPA-1 genotyping

The DNA and RNA from HTR8/SVneo cells and donor samples were isolated and used for HPA-1 genotyping by TaqMan 5′ nuclease assay as described previously [20, 21].

Y-chromosome DNA test

The DNA isolated from HTR8/SVneo cells was used for Y-chromosome DNA test by TaqMan 5′ nuclease assay. The primers used for the assay were described previously [22] and FAM-labelled probe was designed in house.

Monitoring cell adhesion and migration

Cell adhesion and migration were monitored in real time using the xCELLigence system (Roche Applied Science, Penzberg, Germany) [23]. For determining the rate of cell adherence, E-plate 16 assemblies were coated with human vitronectin (Promega, Madison, WI) by incubating 1 μg/ml solution in 100 μl volume for 1 h at 37 °C. The wells were washed twice with PBS before 50 μl complete medium was added and the background measurements recorded. The cells were seeded at 20,000 cells/well in a 40 μl volume. From a solution of 200 μg antibodies/ml PBS, 10 μl were added to each well (human IgG1 as negative control, 26.4 and LM609). Each plate was then assembled on the RTCA DP analyzer, and data were gathered at 5-min intervals for 7 h at 37 °C, in a 5% CO_2 humidified atmosphere.

Cell migration was monitored using specially designed CIM-plate 16 with 8-μm pores. The sensor side (bottom side) of each well of the upper chamber was coated with human vitronectin by incubating 30 μl of the 1 μg/ml solution for 30 min at RT. The lower chambers were filled with medium containing 10% FBS, used as chemoattractant. The upper chambers were filled with serum-free medium (50 μl/well), and the plate was incubated at 37 °C in 5% CO_2 for 1 h. After recording background measurements, the cells were seeded into the upper chamber at 40,000 cells in 40 μl per well and 10 μl of 200 μg/ml antibodies in PBS were added. The plate was then incubated for 30 min at RT, assembled on the RTCA DP analyzer and data collected every 15 min for 24 h at 37 °C, in a 5% CO_2 humidified atmosphere. The obtained data were analyzed using the RTCA 1.2 software supplied with the instrument.

Invasion assay

Cell invasion was evaluated using BD BioCoat Matrigel Invasion Chambers (BD Biosciences). The Chambers (24 well Plate 8 Micron with Control inserts) were prepared following the manufacturer's instructions. The RPMI 1640 medium with 5% FBS was used as chemoattractant. HTR8/SVneo cells were seeded into each insert at 40,000 cells/well in a 180 μl volume in serum free medium and 20 μl of 200 μg/ml antibodies in PBS were added (total antibody concentration of 20 μg/ml). The plate was incubated for 48 h at 37 °C, in a 5% CO_2 humidified atmosphere. After incubation, the non-invading cells were scrubbed from the upper part of the inserts by a cotton swab.

The invaded cells were measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The MTT (Sigma) at 5 mg/ml in RPMI 1640 medium without phenol red, was diluted 1:10 and 350 μl of the dilution was added to each clean well. The inserts were transferred to MTT solution and incubated for 2 h at 37 °C, in a 5% CO_2 humidified atmosphere. Next, the inserts were transferred into clean wells with 220 μl of 0.04 M HCl in pure isopropanol and incubated for 5 min

at RT. The inserts were removed and the solution transferred to centrifuge tubes and centrifuged for 2 min at 16,000 x g. Of the solution, 100 μl was transferred into a 96-well microtiter plate and absorption at 560 nm was measured by an ELISA- reader (Multiskan Ex, Thermo Scientific).

Statistical Analysis

A one-way analysis of variance (ANOVA) in SPSS software (SPSS Inc., Chicago, IL, USA) was used to analyze adhesion, migration and invasion experimental data. A *P*-value of < 0.05 was considered significant. Sigma Plot 13 software (San Jose, CA) was used to present the data.

Results

A human anti-HPA-1a mAb 26.4 binds HPA-1a epitope on HTR8/SVneo cells

Integrin β3 expression by HTR8/SVneo cells was assessed with Western blot and flow cytometry techniques. Both techniques demonstrated expression of integrin β3 by HTR8/SVneo cells (Fig. 1a and b). The cells expressed αV, but were negative for αIIb (data not shown), indicating that HTR8/SVneo cells express β3 integrin only in association with αV integrin. Next, HTR8/SVneo cells were genotyped HPA-1ab. Finally, flow cytometry analysis demonstrated that human anti-HPA-1a mAb bound to intact HTR8/SVneo cells (Fig. 1a).

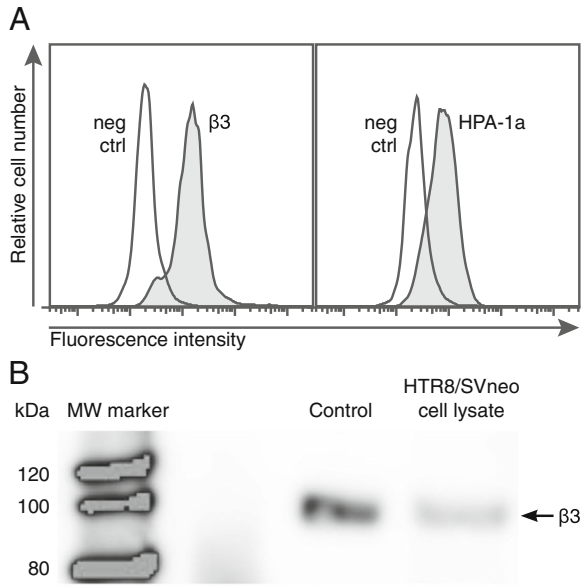

Fig. 1 Expression of integrin β3 and HPA-1a on HTR8/SVneo cells. **a** Flow cytometric analysis of cells incubated with murine anti-human β3 HPA-1-reactive mAb SZ21 and human HPA-1a-specific mAb 26.4, or isotype control. **b** Detection of integrin β3 in lysed HTR8/SVneo cells by Western blot. Integrin αVβ3 isolated from human placenta used as control. Integrin β3 detected as a band of about 100 kDa in the control and cell lysate. Western blot image was spliced to rearrange the order of samples within one experiment. Dashed lines indicate where the images were joined. Figure A and B are representative for at least three independent experiments

A human anti-HPA-1a mAb 26.4 partially inhibits adhesion and migratory capacity of HTR8/SVneo cells

The effect of a mAb 26.4 on trophoblast cell adhesion and migration was explored using the xCELLigence system. mAb 26.4 was used at a concentration of 20 μg/ml, which corresponds to about 400 IU/ml of anti-HPA-1a antibody activity as measured by quantitative mAb immobilization of platelet antigens (MAIPA) assay [24]. mAb 26.4 significantly inhibited adhesion and migration of HTR8/SVneo cells to vitronectin-coated membranes by 15–20% (Fig. 2a and b) and 18–23% (Fig. 2c and d), respectively. Anti-αVβ3 murine mAb (clone LM609) similarly inhibited adhesion and migration of HTR8/SVneo cells (data not shown).

The effect of mAb 26.4 on invasive capacity of first trimester trophoblast cells was studied utilizing Matrigel pre-coated invasion chambers. The mAb inhibited invasive capacity of cells in three out of four independent experiments by 9, 15 and 25% (Fig. 3). The inhibition was not statistically significant ($p = 0.13$). Anti-αVβ3 murine mAb (clone LM609) did not affect invasive capacity of HTR8/SVneo cells (data not shown).

HTR8/SVneo cells originate from a female conceptus

To identify whether HTR8/SVneo cells originate from a female or male conceptus, the Y-chromosome DNA test has been performed. HTR8/SVneo cells were tested negative for Y-chromosome DNA, indicating that the cells originate from a female conceptus.

Discussion

In the present study, we have demonstrated that a human HPA-1a-specific mAb inhibit adhesion and migratory capacity of EVT cells in an in vitro model.

To study the possible effect of anti-HPA-1a antibodies on EVT we utilized transformed first trimester EVT-derived cells (HTR8/SVneo cell line), which were reported to share phenotypic and functional characteristics of EVT cells [17, 25]. We have shown that HTR8/SVneo cells express HPA-1a epitope as part of αVβ3 integrin complex, and importantly, that anti-HPA-1a antibodies interact with HPA-1a on these cells. In this study, we used a human anti-HPA-1a mAb, clone26.4, generated from a B cell derived from a woman HPA-1a immunized in connection with pregnancy, who had two children affected by FNAIT. mAb 26.4 was expressed recombinantly and found to be highly specific for HPA-1a, bound strongly to HPA-1a epitopes on αIIbβ3 from platelets as well as αVβ3 from trophoblasts [16]. Thus, the HTR8/SVneo cell line with mAb 26.4 could be a

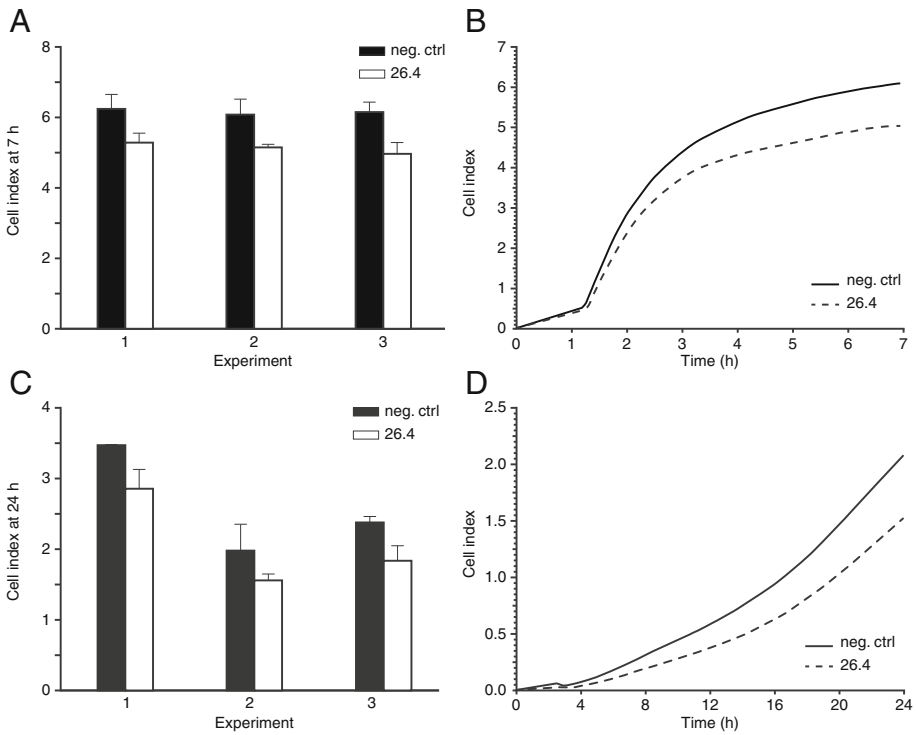

Fig. 2 Effect of anti-HPA-1a antibodies on EVT adhesion and migration. HTR8/SVneo cells were plated on E-plate 16 (for adhesion experiments) and CIM-plate 16 (for migration experiments) in the presence of mAb 26.4 or human IgG1 as a negative control. Cell adhesion was monitored using xCELLigence system over 7 h, and cell migration over 24 h. The samples were run in triplicates and experiments repeated three times each. Each column represent the range in cell index of one sample run in triplicate. Results presented in the graphs (distinguished by experiment due to high variations in cell index between the experiments) are from three independent experiments: **a** adhesion; **c** migration. Plots visualize cell spreading and attachment in real time: **b** adhesion; **d** migration. Plots are representative for three independent experiments

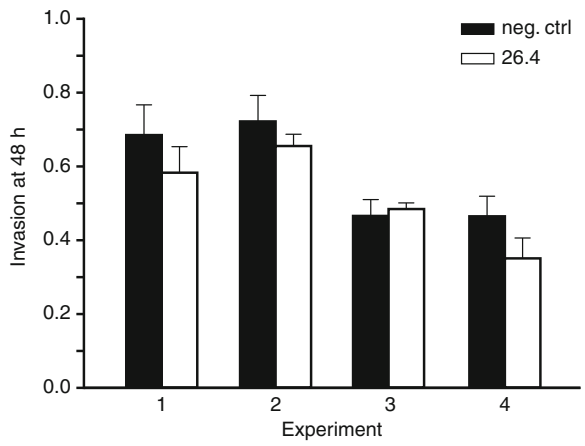

Fig. 3 Effect of anti-HPA-1a antibodies on invasive capacity of EVT. HTR8/SVneo cells were cultured in Matrigel coated chambers in the presence of mAb 26.4 or human IgG1 as a negative control for 48 h. The samples were run in quadruplicates and experiment repeated four times. Each column represent the range in cell index of one sample run in quadruplicate. Cell invasion was evaluated by MTT-assay. The invasion data is expressed as the ratio of invasion through the Matrigel Matrix and membrane relative to the migration through the control membrane

useful model to study possible effect of anti-HPA-1a antibodies on EVT.

The idea that alloantibodies reactive with fetal integrins expressed on trophoblast cells can impair placental function is not new. A histological study of placentas from FNAIT-affected pregnancies described chronic villitis in pregnancies not treated with IVIG [26]. And in a recent histopatological study, FNAIT was associated with chronic chorioamnionitis, basal chronic villitis and intervillositis [6]. In addition, a case of FNAIT associated with massive chronic intervillositis has also been described [27]. Chronic villitis and intervillositis are placental lesions known to be associated with poor fetal growth [28, 29]. Further, 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 early stages of pregnancy [30, 31].

Vitronectin receptor, carrying HPA-1 antigen epitope, is crucial for cell-matrix and cell-cell interactions, modulating growth, survival, motility and differentiation of angiogenic endothelial cells (EC), osteoclasts, tumor cells and other cell types [32]. Blocking αVβ3 was shown to disrupt the invasive and proliferative program of sprouting

EC, and suppress angiogenesis [33–35] impede tumor progression [36], and hinder osteoclast adhesion and migration [32]. The important role of αVβ3 in mediating EVT cells invasion [8] and adhesion to ECs [37, 38] was shown in vitro.

Further, the capacity of anti-HPA-1a antibodies to affect αVβ3-expressing EC in vitro has been reported [35, 39, 40]. Anti-HPA-1a maternal sera affected spreading and monolayer integrity of human umbilical cord endothelial cells (HUVEC) [39] and inhibited HUVEC proliferation and formation of capillary-like networks [35]. The latter findings suggest that anti-HPA-1a antibodies can cause systemic vascular damage, impair angiogenesis, and subsequently can be an independent cause of FNAIT-associated ICH. Further, in a recent study, Santoso S. with co-workers have shown that only anti-HPA-1a antibodies binding selectively to the αVβ3 complex interfere with angiogenesis [40].

Mechanisms of inhibitory effects of anti-HPA-1a antibodies are still incompletely understood. It has been shown that anti-HPA-1a antibodies can impair angiogenic and increase proapoptotic signaling in HUVECs [35]. It has also been hypothesized that anti-HPA-1a IgG antibodies block the ligand RGD binding site on αVβ3 and αIIbβ3 by indirect competition (i.e., steric hindrance) [41].

The HPA-1a epitope is formed by only one amino acid change, L33P, in integrin β3, and, therefore, all anti-HPA-1a antibody epitopes overlap reacting with the L33 residue. Yet, anti-HPA-1a antibodies are reported to be heterogeneous in their footprint on integrin β3 [42] and binding affinity [43–45]. In fact, recently it was found that antibodies of this specificity can be even more complex; αVβ3-, αIIbβ3-specific, or bind antigen independently of the complex [40]. Thus, the effect of a single mAb specific for HPA-1a, as used in this study, may not be representative for different polyclonal antibody profiles among immunized women. Still, our finding that an anti-HPA-1a mAb could affect functions of HTR8/SVneo cells is interesting, indicating that anti-HPA-1a antibodies may affect functions of extravillous trophoblast cells in vivo.

Only male neonates had significantly reduced birth weight in pregnancies with high levels of maternal anti-HPA-1a antibodies in a retrospective observational study [4]. Male sex of the fetus is a well known risk factor for adverse pregnancy outcome [46]. Evidence is emerging on the influence of fetal sex on placental development and function [47]. The placenta displays sexually dismorphic differences in gene expression and responds to maternal factors in a sex-dependent manner [48]. The magnitude of the effects of anti-HPA-1a antibodies on trophoblast cells may depend on the sex of the placenta. In this study we used a cell line HTR8/SVneo which we found to originate from a female placenta. In the follow up studies, it is therefore important to compare the effects of anti-HPA-1a antibodies on trophoblast cells originating from male and female placentas.

Conclusions

We have demonstrated that a human anti-HPA-1a mAb impaired adhesion and migratory capacity of EVT-derived cell line in vitro. We speculate that anti-HPA-1a antibodies may hinder placental development, and consequently, may be involved in early pregnancy loss as well as poor placental function. Further studies with primary trophoblast cells and maternal anti-HPA-1a sera, together with a histopathological study of placentas from pregnancies affected by FNAIT are important to support our finding.

Abbreviations

CTB: Cytotrophoblast; EC: Endothelial cells; EVT: Extravillous trophoblast; FNAIT: Fetal and neonatal alloimmune thrombocytopenia; HPA-1a: Human platelet antigen-1a; ICH: Intracranial hemorrhage; WG: Weeks gestation

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

MK planned the overall study protocol and together with ME and GH designed and performed the experiments, analyzed data and wrote the manuscript. HT analyzed data and wrote the manuscript. YZ supervised and performed immunocytochemistry experiments (not included in the paper). NHN produced the recombinant version of the mAb 26.4. IM supervised the xCELLigence experiments and analyzed data. AH, BS and MK conceived the project and together with TS supervised the study. All authors contributed to analyses and interpretation of data, critically revised the manuscript and approved the final version.

Competing interests

NHN, AH, BS and MK have financial relationship with the Prophylix Pharma AS. Prophylix Parma AS did not finance or influence the study. The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics Approval and Consent to Participate

Not applicable.

Publisher's Note

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