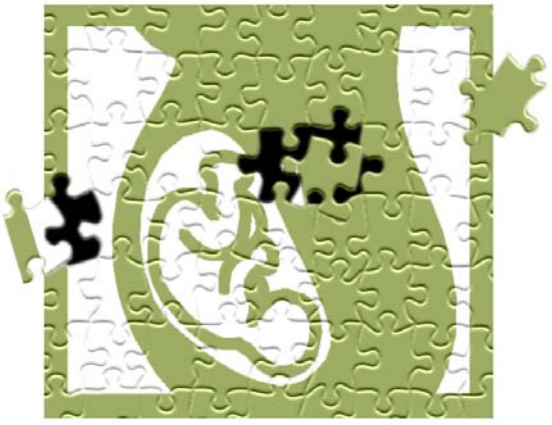
UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF IMMUNOLOGY

Specificity and HLA-restriction of CD4 T Cells Associated with Neonatal Alloimmune Thrombocytopenia



Maria Therese Ahlen

A dissertation for the degree of Philosophiae Doctor

January 2011

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Maria Therese Ahlen, Tromsø, January 2011.

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II. Ahlen MT, Husebekk A, Killie IL, Haney D, Betts MR, Skogen B and Stuge TB. T cell responses associated with neonatal alloimmune thrombocytopenia: HPA-1a-specific T cell clones recognize a "self"-epitope that does not include the allogeneic Leu33 residue. Manuscript.

III. Ahlen MT, Heide G, Husebekk A, Skogen B, Kjeldsen-Kragh J and Stuge TB. HLA-DR-DQ haplotypes in HPA-1a-immunised women: DR3-association is stronger than expected by random distribution. Manuscript.

Abbreviations

NAIT	Neonatal Alloimmune Thrombocytopenia
HLA	Human Leukocyte Antigen
MHC	Major Histocompatibility Complex
FMH	Foeto-maternal haemorrhage
CD	Cluster of differentiation
HPA	Human Platelet Antigen
IFN	Interferon
TNF	Tumor Necrosis Factor
ICS	Intracellular Cytokine Staining
CFSE	Carboxyfluorescein succinimidyl ester
IL	Interleukin
FACS	Fluorescence Activated Cell Sorting
TCR	T cell receptor
APC	Antigen-Presenting Cell
B-LCL	B-lymphoid cell line
AdEtOH	Adamantane Ethanol
MLE	MHC loading enhancer
CEH	Conserved extended haplotypes
AH	Ancestral Haplotype
DC	Dendritic cell
MAIPA	Monoclonal antibody immobilisation of platelet antigen assay

Thesis Summary

The immune system is efficiently protecting us against infections by recognizing foreign structures; however it can occasionally cause complications during pregnancy by eliciting immune responses against foetal blood cells. Blood platelets have surface proteins that exist in different variants in the population. Those that are known to be a target for alloimmune responses are referred to as human platelet antigens (HPA). In cases where the foetus has inherited an HPA-determinant from its father that differs from the mother's own, there is a risk of immunisation. Maternal antibodies are transferred over the placenta to the foetus during pregnancy, where platelet-reactive antibodies can cause depletion of foetal platelets and increase the risk of bleeding – a condition defined as neonatal alloimmune thrombocytopenia (NAIT). There is currently no treatment that can prevent immunisation. The vast majority of NAIT cases are due to incompatibility in the HPA-1 system, defined by a single amino acid difference (Leu33/Pro33) in β3-integrin on platelets. The knowledge of the underlying cellular mechanisms that result in production of maternal platelet-reactive antibodies has been limited. This thesis sheds light on cellular mechanisms, by characterizing HPA-1a-specific T cells isolated from HPA-1a-immunised women who have given birth to a child affected by NAIT. Formal evidence for these cells is important, as antigen-specific T cells are generally orchestrating any given antigen-specific immune response. Furthermore, the characteristics of these HPA-1a-specific T cells were studied; both regarding specific recognition and HLA-restriction.

There are certain genetic factors that are associated with alloimmunisation with HPA-1a, and by experiments with the HPA-1a-specific T cells in culture, the functional role for one of the strongest genetic association markers, *HLA-DRB3*01:01*, was demonstrated. The allogeneic residue Leu33 ensures efficient binding to the peptide-presenting MHC molecule DRA/DRB3*01:01, while the epitopes recognized by the T cells are common in both HPA-1a and HPA-1b, thus representing a "self" epitope, that allows for a more diverse T cell response than if the epitope was restricted to the allogeneic amino acid alone. In addition, the HLA class II alleles are located in close proximity on the chromosome, and are therefore inherited in confined entities defined as haplotypes. The *DRB3*01:01* allele seen in HPA-1a immunised women, are associated with only a few DR-DQ haplotypes. By identifying the DR-DQ haplotypes in HPA-1a-immunized women, an overrepresentation of the DR3-DQ2 haplotype was demonstrated compared to the general population, suggesting that other properties in the DR3-DQ2, additional to the *HLA-DRB3*01:01,* can influence immunisation. The understanding of the cellular reactions that results in production of anti-HPA-1a antibodies and subsequent NAIT, are important for potential treatment strategies to prevent immunisation.

Introduction

Alloimmunisation in pregnancy

Allogenicity; differences between individuals in the same species, is an intrinsic property of viviparous reproduction, as the foetus inherits half of its genomic material from its father. Successful pregnancies are a result of maternal tolerance of the allogeneic foetus, and the phenomenon has intrigued scientists for more than a century; the mechanisms that allow a semiallogeneic foetus to grow without compromising the maternal immune response to pathogens. However, pregnancy-related alloimmune disorders, in which maternal immunisation results in the destruction and depletion of foetal blood cells can complicate pregnancies. The manifestation of the conditions depends on the target of the maternal alloantibodies; antibodies against red blood cell antigens may cause anaemia in the newborn, antibodies against neutrophils cause neutropenia, while antibodies against foetal platelets can cause thrombocytopenia.

Neonatal alloimmune thrombocytopenia - NAIT

Neonatal alloimmune thrombocytopenia (NAIT) is a condition in which maternal alloantibodies sensitise foetal platelets during pregnancy and reduce their survival in circulation, rendering the foetus thrombocytopenic and at risk of bleeding. The condition of thrombocytopenia in otherwise healthy newborns, due to platelet isotype incompatibilities were described back in early 1950's.[1]

Thrombocytopenia in the newborn is defined as a platelet count $<150 \times 10^9$/L, while platelet counts $<50 \times 10^9$/L are defined as severe thrombocytopenia. NAIT can result in intracranial haemorrhage and death, and severely affected neonates may suffer brain damage and lifelong severe disability.[2-4] NAIT cannot be foreseen without screening programs; and therefore most cases of alloimmune thrombocytopenia are diagnosed after birth by obvious skin bleedings (petechiae or purpura) or by accidental detection of low platelet counts. NAIT due to anti-HPA-1a antibodies is reported to occur in about 1 of 1100 newborns.[4,5] Without screening programs, the detection rate of NAIT in Norway is poor.[6] In Norway, postnatal treatment by compatible platelet transfusions is given if the platelet count is less than 35×10^9/L, and may be a logistical challenge in unexpected cases.

There is no international consensus on the management of alloimmune thrombocytopenia.[7] Intravenous immunoglobulin (IVIg) with or without additional steroids given to the mother during pregnancy is the recommended intervention in some countries.[7] Good, but variable,

efficiency of this treatment is reported by several studies,[8-11] however the protective mechanism is not clear.

NAIT has been regarded as the platelet counterpart of RhD alloimmunisation - the clinically most important red cell antigen. About 15% of the Caucasian population lack the gene (*RHD*) encoding the protein and thus the structural antigen, rendering RhD-negative women at risk of alloimmunisation in connection with pregnancy. Most cases of RhD alloimmunisation are caused by foetal-maternal bleedings around time of delivery and rarely takes place during the first pregnancy.[12] The incidence of RhD alloimmunisation were reduced from ~13-16% to ~2% by administration of prophylactic post-natal maternal anti-D treatment implemented (in most countries), successfully reducing the incidence of subsequent neonatal alloimmune anaemia (HDN) correspondingly.[12-14]

Most comparisons have emphasised that NAIT, unlike HDN, also frequently occurs in primigravida, implicating that the mechanisms of RhD- and platelet-immunisation are potentially different. The prophylactic approach has therefore not seemed optimal to prevent platelet alloimmunisation and subsequent thrombocytopenia. However, prospective screening studies have found that the incidence of immunisation in primigravida is low (~25%).[4,5,15] A further follow-up of the women included in the Norwegian screening study (1995-2004),[5] showed that several women were alloimmunised in connection with delivery.[16] This matter is under debate, as several other groups have reported previously that ~50% of NAIT cases were detected in the firstborn child.[17-19]

Platelets

Platelets, or thrombocytes, are the smallest of all blood cells, with a 1-3μm disc-shaped form in their resting phase. They originate from megakaryocytes matured under tight regulation by cytokines and hormones in the bone marrow, and the platelets are shed by shear forces in the blood circulation.[20] The size of the released platelets is determined at release, and their size is maintained through their approximately nine days of life.[21] The platelets are anucleate but carry cytoplasmic material such as dense granules, alpha granules, mRNA and miRNA, derived from the megakaryocyte from which they originate. The normal platelet count in healthy individuals is within the range of $150\text{-}450\times10^9$/L. Despite their small size, the platelets serve as critical mediators in maintaining normal haemostasis.[22] Whenever damages in vascular tissue lead to bleeding, the platelets are immediate rescuers by detecting subendothelial tissue and forming an aggregate that stops the bleeding. When activated, the platelets rapidly changes shapes, to a contracted spherical form with dendritic structures essential for adhesion. The clot formation process is initiated by the attachment of platelets as a monolayer on the site of bleeding, due to

the exposure of vWF and collagen, followed by release of chemo-attractants, leading to massive recruitment and aggregation of additional platelets to form a stable plug. In the course of the past decade, interest in platelets has risen, as their functions have been found to extend beyond their role in clot formation following vascular tissue damage.[23]

Platelet surface molecules

The role of platelets in regulation of haemostasis depends on their ability to adhere and aggregate, mediated by glycoprotein receptors on the platelet surface.

The α/β heterodimeric receptors expressed on platelets are $\alpha_{II}\beta_1$ (collagen receptor), $\alpha_V\beta_1$ (fibronectin receptor), $\alpha_{vI}\beta_1$ (laminin receptor), $\alpha_V\beta_3$ (vitronectin receptor) and $\alpha_{IIb}\beta_3$; the latter being primarily a receptor for fibrinogen, but it can also bind vWF, fibronectin and vitronectin.[24] $\alpha_{IIb}\beta_3$, also called GPIIb/IIIa, is the most abundant glycoprotein, with 50-80,000 molecules distributed on the surface of each platelet.[25,26] GPIIb/IIIa is essential for platelet aggregation, and is activated by inside-out signalling, by an intracellular signal cascade caused by signalling from other receptors. The activation of GPIIb/IIIa results in a conformational change in the extracellular domains, forming a high-affinity fibrinogen- and vWF-binding site that allow stable platelet-platelet interaction.[22]

Several single nucleotide polymorphisms (SNP) in genes encoding platelet surface glycoproteins result in single amino acid polymorphisms, which have been shown to be immunogenic.[27] These polymorphisms are defined as Human Platelet Antigen (HPA) systems.[28] Generally, the systems have two allelic variants, with the most common allele being designated *a*, and the other, *b*; however additional infrequent alleles have been described in several systems,[29,30] while not yet studied worldwide. Most HPA-systems are defined as polymorphisms in the GPIIb/GPIIIa encoding genes *ITGA2B* and *ITGB3 (*gene IDs:3674 and 3690, respectively). From an alloimmune point of view, HPA-1 and HPA-5 are the clinically most important systems in Caucasians: anti-HPA-1a antibodies cause most cases of NAIT (85%), whereas 6-15% are caused by anti-HPA-5b antibodies, with other HPA specificities adding in by a few percent.[31,32]

Alloimmunisation to HPA-1a

The genetic basis of the HPA-1 system is a C/T polymorphism (rs5918) in exon 3 of *ITGB3*. This corresponds to mRNA position 196 and amino acid residue 59 in the β3 integrin precursor protein, resulting in a leucine/proline substitution at residue 33 in the mature β3 integrin, where the HPA-1a (Leu33 variant) is immunogenic.[33] HPA-1b (Pro33 variant) is the minor allele, and in the Norwegian population about 2% are homozygous (HPA-1bb),[5] representative for most

Caucasian populations studied; updated information about HPA antigens and their allele frequencies can be retrieved from the IPD - HPA Database.[34] An infrequent Val33 variant is also described.[29] Women homozygous for HPA-1b, and thus lacking the HPA-1a epitope, can become alloimmunised in connection with pregnancy, when carrying an HPA-1a positive foetus. Immunisation can occur during the pregnancy, or in connection with delivery, resulting in anti-HPA-1a IgG antibody production.

Anti-HPA-1a antibodies – clinical relevance

Maternal anti-HPA-1a antibodies are transported across the placenta during pregnancy by FcRn receptor-mediated transfer.[35-37] The international gold standard for detection and quantification of anti-HPA-1a antibodies has been the Monoclonal Antibody Immobilization of Platelet Antigen assay (MAIPA).[38-40] A correlation of the maternal anti-HPA-1a antibody level and the severity of thrombocytopenia in the newborn is reported in several studies.[4,16,18,41] It has also recently been shown that low-avidity anti-HPA-1a antibodies in the plasma of women with an affected neonate can be detected by plasmon-surface resonance measurements.[42] These antibodies cannot be measured by MAIPA, due to their low avidity for the platelet antigen, as MAIPA requires several washing steps and these antibodies are probably washed away. The frequency of such low-avidity antibodies is difficult to determine, as their presence is potentially masked by high-affinity antibodies in many cases.

The cellular immune response as a premise for antibody production

The maternal platelet-reactive IgG antibodies are indisputably the effector molecules that can cause thrombocytopenia in the foetus or neonate. However, IgG antibodies are classically produced by plasma cells differentiated from activated B cells that have received stimulating signals from antigen-specific $CD4^+$ T cells. The role for such antigen-specific T cells in NAIT has been plausible for several reasons. First, the antigen is derived from a protein, and most immune responses to peptide antigens are T cell-dependent. Second, there is a strong HLA association with this condition, which implies that an HLA molecule plays a functional role in antigen presentation. Third, the maternal antibodies are of IgG class, and T cell help is generally required for class transition and isotype switching, from IgM to IgG isotypes.

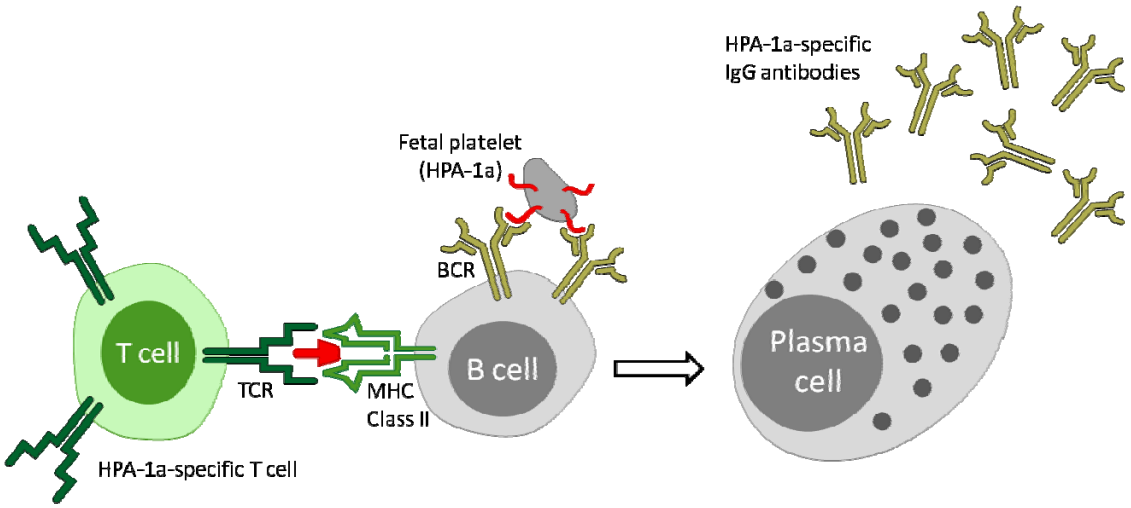

Figure 1. A hypothetical scenario in which a primed HPA-1a-specific T cell helps a HPA-1a-specific B cell, which subsequently differentiates into an antibody-secreting plasma cell.

Processing foreign antigens

Our immune system protects us against pathogens through its constant hunt for foreign antigens in the body. The innate and adaptive immune systems and their interplay, by direct interaction via membrane-bound receptors or via secreted molecules, are the key to fully efficient functional immune responses against pathogens. Innate response mechanisms are essential for immediate alertness to foreign antigens and for efficient presentation to the adaptive system, which then can contribute with specificity and memory to the immune defence process. The T cells function as very critical surveyors, scanning proteolytic peptide fragments presented to them on MHC molecules on other cells. Most of the proteolytic waste presented, obviously consists of fragments of self-proteins as cellular proteins are degraded due to misfolding during synthesis or by down-regulation of proteins. The capability of T cells to discriminate foreign from self is therefore fundamental to the normal immune response.

The two main mechanisms for dealing with proteins of both self and foreign origin are proteasomal and lysosomal degradation. Ubiquitinated intracellular antigens are processed by proteosomal degradation in the cytoplasm, and loaded and presented on MHC class I molecules ready for recognition by CD8 cytotoxic T cells in order to eliminate the infected cell. On the other hand, extracellular antigens taken up by endocytosis are subject to lysosomal degradation. The newly synthesized MHC class II molecules encounter these antigens in the acidic lysosomal-

like compartment MIIC, from which only stable peptide/MHC complexes are transported to the surface.[43-45]

Whenever an MHC molecule on the surface has lost its peptide, the heterodimer rapidly converts to an inactive "non-receptive" state,[46] meaning that the peptide-binding groove is not accessible for other exogenous peptides. This appears to be a safety mechanism that ensures that the antigens presented in the MHC molecule on the surface, actually represent the peptide repertoire present in the MIIC compartment, not just any extracellular peptide binding to empty MHC molecules.

HLA polymorphisms

The human MHC, also named Human Leukocyte Antigen (HLA), are highly polymorphic, with numerous allelic variants.[47] The number of reported alleles is continuously increasing, although most of the new MHC class II alleles reported are infrequent, or represents subdivisions of previously grouped alleles.

The various HLA alleles are divided into classes (I/II), genes (A/B/C, DR/DQ/DP etc) and allelic groups and are then further subdivided on the basis of genetic/phenotypic differences. In April 2010, an update of the HLA nomenclature was released by the WHO Nomenclature Committee for Factors of the HLA System.[48] Due to the rapid increase in described alleles, colons have now been officially introduced as delimiters to separate the allelic designating fields. According to the new nomenclature, the gene and protein names are identical, however discriminated by italicisation of gene names. The nomenclature system is illustrated in Figure 2. According to the Nomenclature Update 2010, there are 3249 class I alleles and 1198 class II alleles; and newly designated alleles are published monthly.[49]

Although the various alleles are designated according to their serological properties, the nature of the polymorphisms in MHC class II causes difficulties for intuitive logical grouping. This diversity also complicates the matter of HLA genotyping. For detection of specific alleles; allele- or group-specific primers and probes can be used. For overall typing, commercial kits with validation of the detection of new or newly designated alleles should preferably be used, as control DNA is not commercially available.

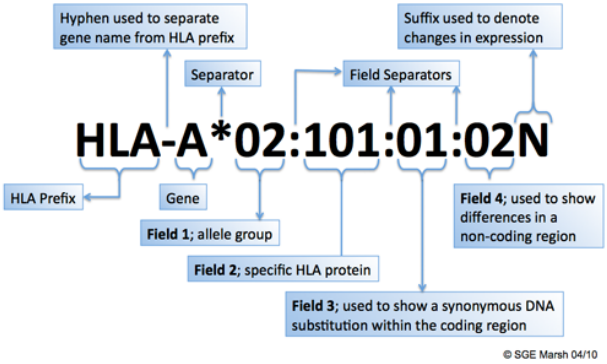

Figure 2. Naming of HLA alleles according to the WHO Nomenclature Update April 2010.[48]

The set of HLA molecules in an individual is defined genetically by, and limited to, allelic variants in each HLA-locus. This implies that there are variations in the antigen-specific immune response in different individuals, depending on their inherited set of MHCs. The polymorphisms in the MHC thus contribute to disease susceptibility. As every individual can have only a limited number of different MHC alleles, functional immunity depends on the ability of a wide range of peptides to bind to each MHC molecule.

Particular HLA molecules are associated with different immune-related diseases, and in many cases only a genetic association can be observed. In most cases there is a positive association, in which the associated allelic variants increase susceptibility to the disease. However, negative associations have also been reported, which means that the given allelic variants protect against the disease. Reports of HLA class II associations have sometimes been surprisingly indefinite and occasionally conflicting, due to problems in defining the specific associated allele, since the region displays extensive linkage disequilibrium.

Peptide binding to MHC class II

Substantial efforts have been made to understand the "rule" of which, and how, peptides bind to the different HLA class II molecules, ever since the first crystal structure of an MHC class II molecule, DRA/DRB1*01:01, was reported in 1993 by Brown et al., who described the entire three-dimensional structure of the an α/β heterodimer.[50] Since then, a number of different allelic variants of DRA/DRB1 has been structurally resolved,[51,52] as well as DRA/DRB3 (DRB3*01:01,[53] DRB3*03:01,[54]) DRA/DRB5,[55] a few DQ molecules,[56,57] and the structure of a DP molecule has recently been reported.[58] Several assessments have attempted to computationally predict MHC class II binding.[59,60] It has turned out to be particularly difficult to

make evaluations of this sort, as the peptide-binding-motifs appear to be less conserved than initially believed.

The ectodomains of the alpha and beta chains within each subgroup of MHC class II together form an allele-specific peptide-binding groove; which resides the determination of which peptides that can be presented by the given MHC class II molecule.
In contrast to MHC class I, that can fit peptides of 8-10 amino acids length, the MHC class II molecules are generally thought to bind peptides of 12-25 amino acids; however, the binding core is restricted to nine to twelve amino acids, and the open ends of the MHC class II molecule allow the extending residues to continue outside of the peptide-binding groove.
Within the binding groove of the class II molecules, the general major sites of peptide interaction are pockets P1, P4, P6 and P9, according to the peptide amino acid residue with which they interact.[61] These pockets accommodate the peptide amino acid anchor residues according to their intrinsic properties; size, charge, hydrophobicity, and steric conformation. In addition, non-anchoring peptide residues can also interact with the MHC by hydrogen bonds, thus stabilising the complex.

The core peptide residues are certainly required for the peptide to bind to a given MHC class II molecule. Nevertheless, peptide-flanking residues (PFRs) also play a role. Studies in which the naturally processed peptides have been eluted from their presenting MHC have shown that the core peptide residues have conserved PFRs.[62] These PFRs can interact with the MHC molecule and influence the strength of the peptide-binding conformation of the peptide epitope,[63] stability and presentation time.[64] PFRs can also influence recognition by TCR.[65]

The *DRA* locus has only three different alleles, which encode two different DRA proteins; DRA*01:01 and DRA*01:02. However, they are identical in the region relevant for peptide binding, and the locus is therefore considered to be functionally invariant.
The DRB3 locus has 52 different described alleles (November 2010) that encode 14 different DRB3*01 proteins (*01:01 to *01:14), 25 different DRB3*02 proteins (*02:01 to *02:25) and 3 different DRB3*03 proteins (*03:01 to *03:03). However, there are only three frequent alleles in Caucasians; *DRB3*01:01*, *DRB3*02:02* and *DRB3*03:01*. They are very similar, although the few differences in DNA sequences result in amino acid changes in the peptide-binding pockets, which confer different antigen-presenting properties. The regions that interact with the TCR are identical in the three allelic variants.[54]

Previous reports have shown that the *DRB3* alleles are remnants due to gene duplications,[66,67] and it has been suggested that they are only weakly expressed,[68] and that peptides presented by these molecules are cross-presented by DRB1 molecules.[69] A recent report emphasises that this is not the case, and that the DRB3 is present in significant amounts on the cell surface on $CD19^+$ and $CD14^+$ cells, and that the antigenic peptides presented are, at least to a large extent, not cross-presented by any of the *DRB3*-associated DRB1 molecules.[70]

Despite that the MHC molecules on the surface, that have lost their low-affinity ligands, are thought to be converted into a non-receptive state, the concept of *in vitro* peptide-pulsing is very useful. As no prediction model of peptide-binding to MHC molecules is perfect, peptide-binding needs to be determined for any given peptide before any TCR-recognition can been studied.
In order to measure the binding efficiency of individual peptides to MHC class II molecules on APCs, MHC loading enhancers can be used, assisting the exchange of naturally bound peptides with the exogenous experimental peptides, by interacting with pocket P1, thus stabilising the DR heterodimer in a receptive state to allow efficient peptide exchange.[71-76] By this method, peptide binding can be directly compared to potential T cell activation, since the same peptide-pulsed APCs can be employed in both assays.

*HLA-DRB3*01:01* association in HPA-1a induced NAIT

The HLA class II allelic variant *HLA-DRB3*01:01* has been reported to be genetically associated with immunisation against HPA-1a in 1990,[77] and it has been confirmed by several studies, that 90-95% of HPA-1a-immunised women carry this allelic variant. [3-5,78,79] The association of the DRA/DRB3*01:01 molecule was also addressed on a structural basis, as the molecule was crystallized – with the HPA-1a peptide in the peptide-binding-grove.[53] The complex of an HPA-1a peptide and the DRA/DRB3*01:01 is illustrated in Figure 3.

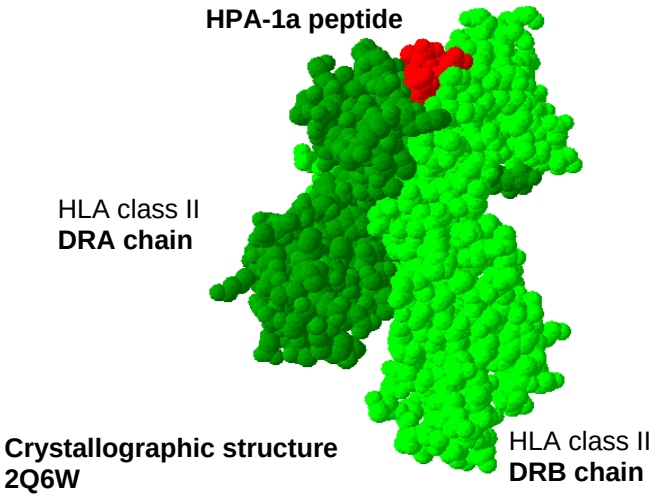

Figure 3. The crystal structure of DRA/DRB3*01:01 with the HPA-1a peptide in the peptide-binding groove. Adapted from the Protein Data Bank Entry 2Q6W[53] at www.pdf.org.

HLA-DR-DQ haplotypes

The HLA genes are located in such close proximity within the MHC region that they are inherited as confined entities, and different allelic combinations on each chromosome can subsequently be defined as haplotypes.[80] *HLA-DR-DQ* haplotypes are defined by the presence of specific allelic variants in *HLA-DRB1*, *-DQA1* and *-DQB1* loci, illustrated in Figure 4. The extended polymorphism seen in MHC genes is thought to be an important intrinsic property of the adaptive immune system. Interestingly, even though numerous different alleles have been reported,[81] most are rare, and there are only 24 common (present in more than ~1% of the population) *HLA-DR-DQ* haplotypes in the Norwegian population.[82]

Evolutional investigations have shown that not only are HLA-DR-DQ haplotypes inherited as specific units, but also that there are a number of conserved extended haplotypes (CEH) in a region from HLA-A (MHC class I) to HLA-DQ (MHC class II) that includes non-MHC loci (MHC class III).[80]

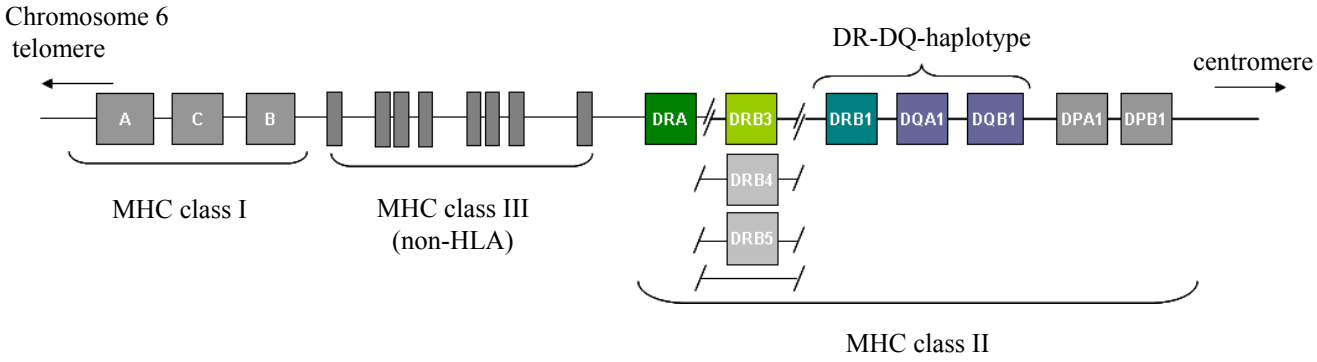

Figure 4. A simplified illustration of the human MHC genes. The presence of an additional functional DRB locus is associated with specific DR-DQ haplotypes. DR-DQ haplotypes with no associated additional DRB carry pseudogenes in this region. Distances are not to scale.

T cell populations

The thymus is populated by a large pool of immature thymocytes that can proceed to the very strict T cell developmental program within the thymus. Every single, naïve, T cell has achieved successful rearrangement of its T cell receptor genes, has gone through positive selection, (ensuring recognition of self-MHC molecules) as well as negative selection (ensuring no high-affinity binding of self peptides in complex with MHC), in order to function as a potential productive member of the adaptive immune response. The vast majority of potential T cells will be deleted due to failure during the positive or negative selection processes. Phenotypically, the two major T cell lineages are defined by their co-receptor expression, $CD8^+$ and $CD4^+$, determining their antigen-recognition in the context of MHC class I or II, respectively. While the $CD8^+$ T cells will acquire a cytotoxic function upon antigen-specific priming, the faith of $CD4^+$ T cells as T helper (Th) cells is more indefinite. The classical Th1/Th2 paradigm were initially launched by Mosmann et al in 1986,[83] and has been acknowledged during the years, supported by reports of different chemokine receptors and cytokine-production patterns,[84,85] characteristic transcription factors,[86] as well as a skewed balance in many immune-related conditions.

The classical Th1 cells, secreting pro-inflammatory cytokines, IL-2 and IFNγ, were originally thought to mainly be involved in antiviral immunity, while the Th2 cells, secreting IL-4, IL-5 and IL-13, were associated with humoral immune responses. Antibody production is seen in both Th1- and Th2- dominating responses. Generally, the antibody responses have been thought to be induced by Th2-responses; however, Th1 cytokine IFNγ is required for IgG antibody class switching, leaving neither of the two helper profiles as the perfect sole mediator of B cell

differentiation. During the past decade, several additional T helper lineages have been reported, complicating the view of T helper cells, but at the same time contributing to the known unknowns in T cell helper function, particularly regarding mechanisms that stimulate B cells to differentiate. The follicular helper T cells are strong candidates for this.[87,88] However, all novel theories of lineages and mechanisms of differentiation are in line with the dogma; that the commitment to a certain lineage is given by the stimulating signals during the activation of the naive T cell.

The T cell receptor

The T cell receptor (TCR) is a transmembrane heterodimer, where the pair of protein chains form a unique receptor for recognizing peptide:MHC complexes on APC. The TCR loci (*TCRA, TCRB, TCRG, TCRD*) contain all the genetic information required to generate all the TCRα/β (and TCRγ/δ) receptors needed to ensure a diverse T cell response in the individual; by numerous variable (V), diverse (D) and joining (J) gene segments and the process of genetic recombination. The generation of the receptor takes place during the early development of the thymocyte, by recombination of V and J segments at the *TCRA* locus and V, D and J segments at the *TCRB* locus, and additional random nucleotide sequences at the recombination sites.[89]

Both TCRα and TCRβ contain three complementarity determining regions (CDR). The CDR1 and CDR2 are determined by germ line sequences, as they are encoded completely by the variable gene sequences used (*TRAV* and *TRBV).* In contrast, the CDR3 regions are derived from the VJ and VDJ, including random regions. The CDR3 regions of the TCRα/β molecules are the parts that primarily interact with the antigenic peptide, centrally positioned over the peptide. However, the CDR1 of the TCRα-chain interacts with the N-terminal part of the peptide, and the CDR1 of the TCRβ-chain interacts with the C-terminal part of the peptide,[90] while the CDR2 loops recognize the MHC.[91] The interaction of an MHC class II and a T cell receptor is illustrated in Figure 5.

The immunological synapse, the local long-lasting junctional structure formed between the T cell and the antigen-presenting cell, also includes the interaction of TCR-associated molecules, as co-receptors CD8 and CD4.

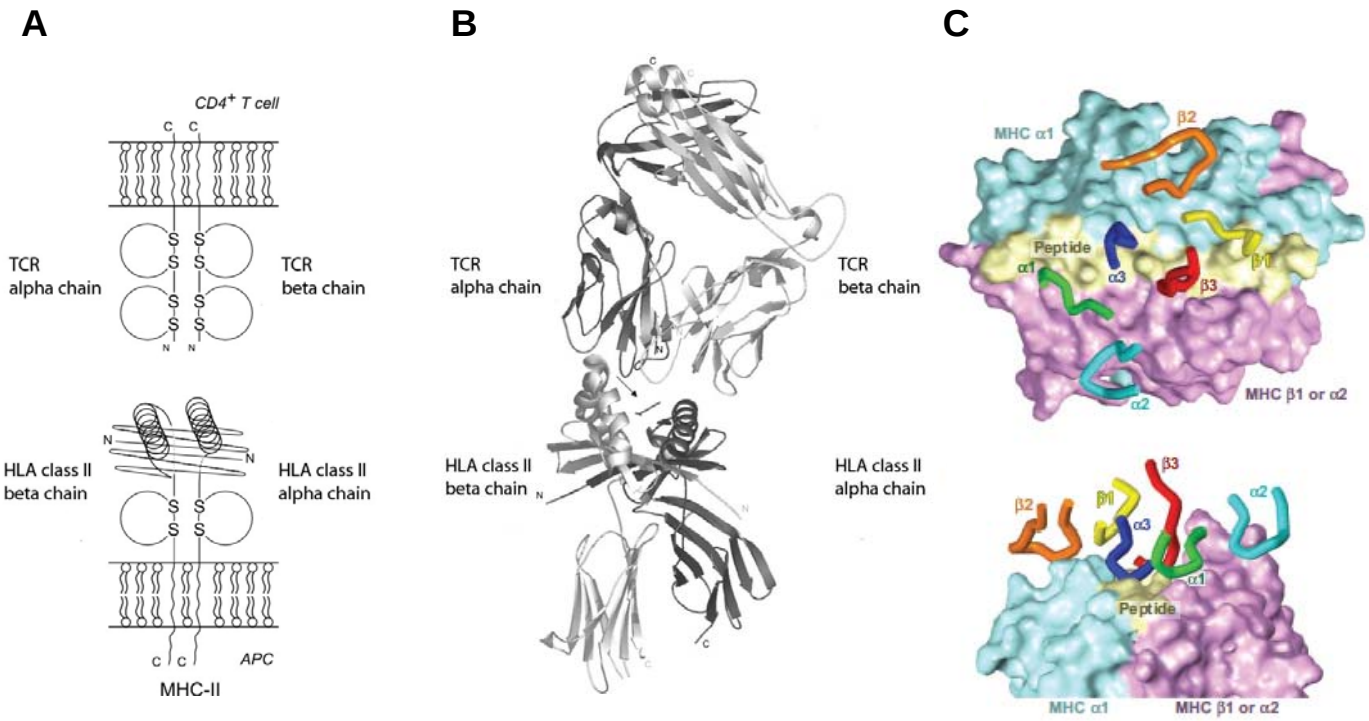

Figure 5. Interaction of an MHC class II molecule and a TCR. (A) Schematic view of variable domains, constant domains and transmembrane part. (B) Structural view of ectodomains only. Modified from Kaas and Lefranc.[90] (C) Schematic overview of the TCR-peptide:MHC sites.[91] Top view (upper structure) and side view (lower structure)

Aims of the study

The Department of Laboratory Medicine at the University Hospital of North Norway has hosted the *National Reference Laboratory for Advanced Platelet Immunology* since 1995, where the main area of research has been neonatal alloimmune thrombocytopenia, which has intrigued the researchers in the group since the 1980s. The largest prophylactic screening and intervention study that has been carried out to date was performed in collaboration with Ullevål University Hospital in 1995-2004, and focused on maternal anti-HPA-1a antibodies. In the wake of this study, the cellular mechanism that results in the generation of the platelet reactive alloantibodies in these women was addressed, as the majority of women at risk of immunization (HPA-1bb women that carry *HLA-DRB3*01:01*) are not immunised in connection with pregnancy. The main focus of this work has been on maternal T cells specific for the HPA-1a epitope, which are thought to play an important role in the underlying cellular immune response that may result in anti-HPA-1a antibodies and subsequent NAIT in the foetus and newborn. At the origin of this project there was no formal evidence for the existence of these cells. In order to study this topic in more depth, the following questions were raised and studies initiated to find answers:

- The frequency of antigen-specific T cells in circulation is low, so the hunt for potential HPA-1a-specific cells requires sensitive detection methods. Do HPA-1a-specific helper T cells exist, and can such cells be detected in peripheral blood from immunised women who have given birth to a child with NAIT?
- The HLA class II association with HPA-1a alloimmunisation is likely to be due to presentation of the HPA-1a derived peptide by the MHC molecule encoded by the associated HLA alleles. Can the HLA restriction of HPA-1a-specific T cells be determined?
- The HLA class II allele *DRB3*01:01* is associated with only a few DR-DQ haplotypes. Can the *DRB3*01:01*-associated DR-DQ-haplotype influence the risk of immunisation?
- What is the nature of T cell recognition of HPA-1a; is the allogeneic residue Leu33 recognized by HPA-1a-specific T cells? Is the recognition of antigen related to specific motifs in the T cell receptors?

Summary of papers

Paper I

T cell responses associated with neonatal alloimmune thrombocytopenia: Isolation of HPA-1a-specific, HLA-DRB3*0101-restricted CD4+ T cells

The idea of HPA-1a-specific T cells in HPA-1a-immunised women has been acknowledged for years in the field, as the antigen is a peptide epitope, and there is a strong association with a specific HLA class II allele (*DRB3*01:01*), both being characteristic features of T cell-dependent immune responses. Several reports have supported this hypothesis; however the cells had never been unambiguously detected. In this paper, we report isolation and characterization of HPA-1a-specific T cells from an HPA-1a alloimmunised woman who gave birth to a child with severe neonatal alloimmune thrombocytopenia. This was done by stimulating maternal PBMCs with synthetic HPA-1a-derived peptide in culture, followed by cloning of proliferating cells using fluorescent activated cell sorting (FACS). Two long-term clonal T cell cultures were established. We confirmed their specificity with both synthetic (peptide) and native platelet antigen (platelets), and demonstrated that the recognition of antigen by these cells is in fact restricted by the very same HLA variant, *DRB3*01:01*, which is genetically associated with immunisation. These data served as formal evidence of the existence of maternal HPA-1a-specific T cells, and the clonal T cells are valuable tools for further research to understand the cellular mechanisms that result in anti-HPA-1a antibody production that can cause NAIT.

Paper II

T cell responses associated with neonatal alloimmune thrombocytopenia: HPA-1a-specific T cell clones recognize a "self"-epitope that does not include the allogeneic Leu33 residue.

In paper II, the antigen recognition of HPA-1a-specific T cells was investigated in more detail. The repertoire of clonal T cells to use for such studies was increased by the isolation of several clones from additional donors. We addressed the question whether or not the allogeneic β3-integrin residue Leu33 itself is a part of the T cell epitope recognized by HPA-1a-specific T cell clones. A model for measuring both peptide-binding to antigen-presenting cells and the corresponding activation of T cells were developed. With this model, we determined that substitution of the Leu33 residue with other small hydrophobic amino acids did not disrupt binding of these peptides to DRA/DRB3*01:01-positive APCs and subsequently still activate T cells clones. These data demonstrate that residues specifically recognized by the T cells are present in both HPA-1a and HPA-1b variants and do not necessary include the Leu33 residue. The implication of these findings is that immunogenicity of the HPA-1a peptide lies in the anchoring to the MHC and not in the distinction between these epitopes by the T cells. Furthermore, by using a panel of peptides with selected amino acid substitutions, we revealed that the isolated HPA-1a-specific T cell clones are unexpectedly heterogeneous in their recognition of peptide-MHC. In line with thus finding, the TCRs of these clones do not share any specific CDR3 sequences, or any characteristic V segment usage.

Paper III.

HLA-DR-DQ haplotypes in HPA-1a-immunised women: DR3-association is stronger than expected by random distribution.

The finding reported in paper I, that the HPA-1a-specific T cells isolated were restricted by DRA/DRB3*01:01, raised further interest in this particular MHC molecule. There are only a few common HLA-DR-DQ haplotypes in the Norwegian population that carry the *DRB3*01:01* allelic variant. HLA-DR-DQ investigations were initiated according to our hypothesis that one DR-DQ haplotype is overrepresented among HPA-1a-immunized women. High-resolution typing of individual HLA class II molecules includes numerous group- and sequence-specific tests. HLA-DR-DQ haplotype identification strategies were chosen, followed by typing of 167 HPA-1a immunised women and 782 healthy volunteers from the blood bank at the University Hospital of North Norway (control group of *DRB3*01:01* positive individuals). In this paper, we report that the *DRB3*01:01*-associated DR3-DQ2 haplotype is overrepresented among the HPA-1a immunised women, compared to the general population of *DRB3*01:01*-positive individuals. Also the *DQB1*02* has previously been reported to be associated with immunisation. Here, we demonstrate that ~80% of the *DQB1*02* alleles among the immunized women in our study, are present due to the DR3-DQ2 haplotype. The data in the present study suggests that another, not yet identified, genetic element within this haplotype can influence HPA-1a-immunisation. Identification and understanding of such novel factors are important to attempt prevention of this condition, and may also allow more accurate prediction of women at risk of alloimmunisation.

Discussion

Most of the results are discussed in the papers. Some major aspects are further discussed here.

Methodological Considerations

In vitro experimental models, using peripheral blood cells from alloimmunised women in cell culture systems, can never perfectly mimic the network of biological mechanisms taking place *in vivo*. Still, it allows us to explore characteristic properties of the *in vivo* players, when it comes to genotyping (HPA, HLA and T cell receptors) and biological specificity (T cell recognition and HLA-restriction) which are intrinsic properties of the cells, and therefore also representative *ex vivo*. All methods are described in detail in the papers, and potential methodological biases are discussed in their appropriate/specific setting in the following discussion of results.

Flow cytometry and flow cytometry-based cell sorting has been particularly important tools for the cellular studies included in the work summarized in this thesis. We have attempted to describe the flow cytometry data according to the *Minimal Information about T cell Assays* (MIATA) recommendations as far as reasonable.[92]

Furthermore, flow cytometry analyses of clonal T cells in culture differ from regular analysis of peripheral blood, by the clonal nature of the cells. Due to the intrinsic homogeneity of a clonal culture, the characteristics and dynamics of their activation responses can be evaluated in a stable and highly reproducible manner. We have chosen to assess the activation responses of each clone by measuring median fluorescence intensities and/or percentage of responding cells.

Any given established antigen-specific clonal cell strain has limited life-time, as they are not transformed like cell lines. They are instead maintained by monthly re-stimulation and expansion. Therefore, it is inevitable that a given clone will reach senescence or lose its potential to divide, after numerous expansions in culture. The different clones have different growth/expansion rates in culture, and occasionally, newly identified clones show very poor expansion and die out after some weeks in culture. For successfully established clonal cell cultures, a number of vials are cryopreserved for future use.

The existence of HPA-1a-specific T cells

The notion that HPA-1a-specific T cells exist in HPA-1a-alloimmunised women has been recognized for several years, due to the characteristic features of T cell-dependent immune responses; a peptide epitope antigen and a strong association with a specific HLA class II allele (*HLA-DRB3*01:01*), resulting in IgG antibody responses. Several reports have supported this hypothesis, as cell proliferation in bulk PBMC cultures has been demonstrated following stimulation with HPA-1a-derived peptides.[93-95] Detailed studies of HPA-1a-specific T cells nevertheless require the isolation and long-term culture of these cells. In Paper I, we report formal evidence of the existence of these cells, confirming their specificity with both synthetic antigen (peptide) and native platelet antigen (platelets), and demonstrating that the recognition of antigen is in fact restricted by the very same HLA-molecule DRA/DRB3*01:01 as is associated with immunisation. This is important, since it further supports the functional role for this MHC in the alloimmune response, previously indicated by genetic association as well as biochemical peptide-MHC interaction studies.[3,53,77,96] Shortly after the appearance of our paper, our results were confirmed by Rayment et al, who also reported HPA-1a-specific T cell clones from alloimmunised women.[97] Synthetic peptides were used in both studies in the primary stimulation PBMC culture, followed by confirmation of recognition of native platelet antigen by the established clones. We showed that only *HLA-DRB3*01:01*-positive, HPA-1bb monocytes co-cultured with HPA-1a$^+$ platelets overnight, but not HPA-1bb platelets, could stimulate the T cell clones, supporting both the idea that the native HPA-1a antigen on platelets can be naturally processed and presented, and that the isolated T cells efficiently recognize the presented epitope. This was further supported by Anani Sarab et al., who demonstrated that naturally processed HPA-1a peptides are displayed on *DRB3*01:01*-positive antigen-presenting cells.[98] This was the first demonstration of cellular processing and presentation of a human alloantigen; by exposing a DRB3*01:01-positive B-LCL to the recombinant antigen (the PSI domain of β3 integrin), cell lysis and isolation of DRB molecules, followed by elution and characterization of the peptides. Together, these findings establish fundamental support for the functional activation of the maternal HPA-1a-specific T cells on exposure to the HPA-1a antigen presented by DRA/DRB3*01:01 *in vivo*. However, the role of these T cells in subsequent anti-HPA-1a antibody production and profile *in vivo* has not yet been elucidated. While induction of *in vitro* anti-HPA-1a production in cultures with proliferative responses after stimulation with HPA-1a peptides has been reported,[95] better controlled experiments and more sophisticated methods will certainly be required to unambiguously demonstrate the role of HPA-1a-specific T cells in anti-HPA-1a antibody production.

In the present study we used different synthetic β3-derived peptide (Leu33/Pro33) variants; in the initial stimulation experiments 20-mers $\beta3_{19\text{-}38}$ (VSPMCA**W**CS**D**EALP(**L/P**)GSPRC) were used, and in the following epitope studies mainly 12-mer $\beta3_{24\text{-}35}$ (A**W**CS**D**EALP(**L/P**)GS). The choice of peptide for stimulations in different studies is interesting. To date, only a few studies have reported T cells associated with HPA-1a-related alloimmunsation. Indications of HPA-1a-specific T cells in PBMCs from alloimmunised women were first reported in 1996 by Gorski's group, employing the spectratyping method.[94] While this study was inventive and proposed a very important property of HPA-1a-derived peptide in the alloimmunisation, an obvious contradiction in the paper needs to be addressed. The study was performed with bulk proliferation of PBMC following stimulation with β3 integrin-derived peptide (Leu33/Pro33) variants. However, the peptide spans the residues $\beta3_{26\text{-}38}$ (CSDEALP**L/P**GSPRC) and thus does not include the Trp25 residue that the same group showed to be an essential P1 anchor residue a year later.[96] This certainly complicates the interpretation of the results, and questions the specificity of the proliferation seen. Although the cells may have been truly HPA-1a-specific T cells, the later finding further emphasizes the importance of isolating the actual antigen-specific cells in order to conclusively study their specific recognition and HLA restriction. Moreover, the peptide concentration in the stimulation of PBMCs that resulted in detection of specific clonotypes was 12.5-50 μM, which is about 10-1000 times higher than was required to activate our HPA-1a-specific T cells. In our hands, the clones are over-stimulated and die if challenged with L33 peptides (although not LolP1 or P33 peptides) at concentrations ~25 μM (unpublished observations). A plausible explanation for the proliferation seen at these high concentrations might therefore be the lack of the $P1^{Trp}$ anchor residue in the stimulating peptide, as considerably higher peptide concentrations were required for sufficient peptide binding to induce proliferation of specific cells, with merely the P4 and P9 anchor residues.

In 2005, two studies reported HPA-1a-specific T cell proliferation on stimulation of PBMC from immunised donors, detected by ^{3}H-thymidine incorporation assays.[93,95] Both studies were open-minded in their choice of peptides used for stimulation, in the way that all potential binding motifs from the β3 integrin that include the allogeneic residue 33 were taken into account. Thus, T cell stimulation due to potential peptide presentation by other MHC class II molecules than the DRA/DRB3*01:01 was not excluded. Sukati et al[95] used a panel of 15 β3-derived 15-mers, allowing the (Leu33/Pro33) residue to occupy all the different positions in the peptide, from C-terminal to N-terminal, using a peptide concentration of 20 μg/mL (corresponding to ~12 μM). Jackson et al.[93] used 20-mer $\beta3_{20\text{-}39}$ (SPMCA**W**CS**D**EALP(**L/P**)GSPRCD) and 22-mer $\beta3_{20\text{-}39}$

(A**W**CS**D**EALP(**L/P**)GSPRCDLKENLI) peptides at ~0.5-15 μM, probably as an alternative strategy aimed at including all the potential binding motifs from the β3 integrin. Hence, considering that the core-peptide-binding sequence can be as short as nine amino acids, there is a theoretical risk that C/N-terminal peptide-binding motifs from the 20- and 22-mers may leave the allogeneic residue 33 outside on either side of the MHC peptide-binding groove.

In these studies we pointed out that the isolation of HPA-1a-specific T cells serves as evidence for the existence of such cells in the alloimmunised women from whom they were isolated. The implication of these finding is that these cells originates from clonally expanded populations of T cells that have participated in previous immune responses. In theory, HPA-1a-specific T cells could be developed *in vitro* from naïve T cells. The priming of antigen-specific T cells *in vitro*, however, depends on interaction with mature dendritic cells (mDC) to present any given antigen and the signal 2 (CD28-B7 interaction), by which the signalling cascade induced, ensures the T cell that the antigen was presented by a professional APC. The induction of DCs from monocytes in PBMCs requires specific protocols, and different maturation cocktails have been described as generating mDCs *in vitro.*[99-101] Furthermore, a total of eight of the HPA-1a-specific T cell clones (from two donors) were isolated using only pre-FACS isolated $CD4^+$ T cells in the stimulation culture, which rules out a fundamental role being played by any professional APCs in the culture. Additionally, the fact that the identical clones have been isolated several times from the same donor, but from different samples, further supports the priming and activation of these cells *in vivo*.

Traditionally, Th1 and Th2 responses have been associated with inflammatory and non-inflammatory immune responses respectively. The phenotypic characteristic of the isolated cells is informative, even though the *in vivo* functional Th1/Th2 paradigm is apparently undergoing re-evaluation. The T cell clones isolated (Papers I and II) have all been shown to be of a Th1 type, secreting IFNγ (and TNFα) on stimulation with specific antigen. T cell clones are thought to keep their effector profile when established in culture, and we have not seen any changes in cytokine production over time. However, during isolation and expansion of clones, we cannot ignore the possibility of bias resulting from which cells are successfully expanded. On the other hand, some of the expanded clones of irrelevant specificities have shown an IL-4-secreting profile, suggesting that the method is not discriminating any potential cells with Th2 profile. Several arguments suggest that the cloning and expansion protocol can certainly be optimized. First, there is proliferation of T cells with irrelevant specificities in the primary stimulation cultures, which reduces the efficiency of cloning of HPA-1a-specific cells (background). Second,

although we do isolate identical clones from the same experiments, their frequency should be higher than we actually observe, as the cloning sort is performed after a minimum of 12 days of proliferation (detected by reduced CFSE intensity). During this time, all HPA-1a-specific T cells present in the primary stimulation culture will have divided several times, resulting in a larger number of cells with this clonality. Third, the strength of the stimulation is also a matter of attention, and there is a risk that some *in vivo* clones may have been over-stimulated in our *in vitro* experiments, as we have seen that the successfully isolated clones have different stimulation thresholds.

Polyclonality of the HPA-1a-specific T cells

The HPA-1a-specific T cell response appears to be broad (polyclonal). In one of the immunised women participating in this study, from whom we have repeatedly received peripheral blood, we have so far isolated a total of 13 different clonal T cells, in which the TCRα/TCRβ sequence analyses revealed unique CDR3s.

The actual number of HPA-1a-specific T cells clones in a given donor is probably higher than the number identified so far, as repeated experiments typically result in the isolation of further unique clones, in addition to re-isolation of previously identified ones. To what extent the number of different clonal antigen-specific cells is of importance for the antibody production and clinical outcome of the neonate is not yet known. Some alloimmunised women, but not all, present continuously high levels of anti-HPA-1a antibodies for years, without any known re-exposure to antigen. The donor from whom we have isolated the majority of clones is an example of this, as she was alloimmunised in 1980 and still presents relatively high levels of anti-HPA-1a IgG antibodies (~30 IU/mL, in 2008 and 2010). Whether the permanently high antibody levels and the broad T cell response are connected, or the antibody production is due to long-lived plasma cells, is not known.

The recognition of the antigenic peptide by all T cells isolated in this study has been restricted by the DRA/DRB3*01:01 molecule. This finding suggests that HPA-1a-specific DRA/DRB3*01:01-restricted T cells are dominating the T cell response associated with NAIT. However, the isolation of T cells restricted by this molecule may have been biased by the use of HPA-1a-derived peptides that are known to bind this MHC. The possibility of isolating HPA-1a-specific T cells restricted by an MHC class II molecule other than DRA/DRB3*01:01 depends on the use of autologous antigen-presenting cells to stimulate PBMCs, as well as intact platelet antigen or peptides that span all possible peptide-binding motifs by other MHC class II

molecules. It also requires autologous or matched, antigen-presenting cells for specificity testing of growing clones to express all the relevant MHC class II molecules. So far, the few efforts that we have made to isolate HPA-1a-specific T cells restricted by an MHC class II molecule other than DRA/DRB3*01:01 using autologous APCs have been unsuccessful; also using DQA1*05:01/DQB1*02:01-positive donors. Indeed, the existence of HPA-1a-specific T cells restricted by other MHC class II molecules than DRA/DRB3*01:01 is plausible, as not all women producing anti-HPA-1a IgG antibodies carry the *DRB3*01:01* allele. However, in a hypothetical setting where the Leu33 residue does not function as an anchor residue, the residues docking the HPA-1a peptide to a given MHC class II molecule is shared by both HPA-1 variants, rendering the allogeneic residue the sole difference between self and allogeneic peptide:MHC epitopes, limiting the pattern of recognition dramatically for potential T cells. This could explain the low levels of HPA-1a antibodies often seen in *DRB3*01:01*-negative immunised women, that do not result in severe NAIT.

Interestingly, CD4 T cells also express MHC class II molecules,[102] and HLA-DR expression on these cells was first described as an activation marker. It has also been shown that T cells, *in vitro*, can take up and process antigens, and present on the MHC class II on their surface.[103,104] The MHC class II DR molecules are suggested to be more than activation markers of $CD4^+$ T cells, but no evidence for any antigen-presenting mechanism *in vivo* has yet been found.[104]
Proliferation assays with T cell clones stimulated with intact *DRB3*01:01*-negative, HPA-1aa platelets have not shown any stimulation, indicating that the native platelet antigen is not endocytosed, processed and presented efficiently enough (unpublished data). However, exogenous peptide loading can be the mode of action in T cell activation assays, where peptides alone are efficiently stimulating the T cells, as we have detected peptide-binding on the surface of the cells, indicating that direct extracellular peptide loading of DRA/DRB3*01:01 is taking place in these experiments (unpublished data).

Reconsidering the immunisation status

It is possible that there can be HPA-1a-specific T cell responses in women, without B-cell activation and anti-HPA-1a antibody production. We have not yet had the opportunity to investigate this. This will require screening/identification of truly primigravida, HPA-1bb and *HLA-DRB3*01:01* women, succeeded by antibody testing during and after pregnancy, genotyping of the newborn to confirm the incompatible HPA-1ab genotype to ensure a potential antigenic exposure, and finally, a very reliable and sensitive method for measuring the HPA-1a-specific T cells directly in peripheral blood. By monitoring the T cell responses and generation

of antibodies during pregnancies, the progress of events can be followed in detail. Some women become immunised in subsequent pregnancies, and it is quite possible that the maternal T cells were primed in a prior pregnancy or in connection with delivery, and that generation of antibodies is the result of an additional and possibly larger antigenic load.

Today, we define HPA-1a immunisation as detectable anti-HPA-1a IgG antibodies in maternal plasma. In our screening and routine follow-up programme for women at risk at HPA-1a immunisation, only the presence of IgG antibodies is analysed in the laboratory. The rationale for this is obviously that only antibodies of IgG class are transported over the placenta by the receptor FcRn. The trans-placental transport of IgG normally protects the foetus from pathogens.[36] However, platelet-reactive alloantibodies, if present, can cause harm in the alloimmune setting during pregnancy by causing NAIT. From a biological point of view, the presence of anti-HPA-1a IgM antibodies would also indicate alloimmunisation. By also monitoring potential IgM antibody production in the women at risk, the process of immunisation could be followed. IgM responses are generally transient and antibodies present in low levels, and conversion to IgG responses requires sustained antigenic exposure; the antigenic platelets transferred to maternal circulation at delivery, might not be sufficient.
Additional IgM analyses of IgG-negative women ("non-immunised") exposed to foetal HPA-1a antigens in connection with pregnancy would add substantial information to the debate regarding the incidence of alloimmunisation in primigravida. The IgG isotypes of anti-HPA-1a antibodies are not routinely tested, but one study reports a tendency of higher levels of IgG3 in cases with severely thrombocytopenia compared to mildly thrombocytopenic or compensated cases.[105]

T cell recognition of the HPA-1a:DRA/DRB3*01:01

The allogeneic Leu33 residue is the central focus of paper II. As this is the only difference between the HPA-1a and HPA-1b, it obviously plays a critical role for the epitopes recognized by the maternal immune system. The HPA-1a epitope for B cells has been shown to be a conformational epitope.[106] The T cell epitope, however, is necessarily a linear epitope, but might also have conformational properties by its docking to the MHC molecule. The idea that the Leu33 residue functions as an anchor residue for binding DRA/DRB3*01:01 is not novel. This finding was a landmark in HPA-1a-related research when published in 1997, and was beautifully illustrated by the crystallographic structure of the HPA-1a-derived peptide bound to DRA/DRB3*01:01 in 2007.[53,96] Indeed, this notion was the basis for studying the T cell responses associated with HPA-1a immunisation. Our studies elucidate the recognition of HPA-1a by maternal T cells in detail. By demonstrating that the Leu33 residue can be substituted by

other small, aliphatic, hydrophobic amino acids, without diminished peptide-binding to DRA/DRB3*01:01 and stimulation of HPA-1a-specific T cells, a required specific recognition of the Leu33 side chain, can be excluded. We were open to the possibility that even the substitution of the Leu33 residue to other aliphatic hydrophobic residues could disrupt the conformation of the T cell epitope by docking slightly differently into the P9 pocket, but this was only observed for one of the T cell clones. The implication is that the specific amino acid residues that make up the epitope recognized by the HPA-1a-specific T cells are in fact present in both HPA-1 variants, but that the HPA-1b-derived peptide is not naturally presented in DRA/DRB3*01:01 molecule. This binding discrepancy might explain the immunogenicity of this epitope; the amino acid polymorphism is intriguingly immunogenic only in this setting. Our data strongly support the theory regarding a lack of self-tolerance to the missing epitope; as the maternal T cells have probably never encountered the maternal β3 integrin-derived peptide variant in the negative selection process during development in the thymus, as the self-variant is so poorly bound. The opposite situation, in which the pregnant women are HPA-1aa and carry an HPA-1ab foetus, is quite frequent; however anti-HPA-1b antibodies have only been reported occasionally, with no clear HLA association.[78,107,108] While such HPA-1b antibodies are rare, the fact that they do exist rules out the possibility that the HPA-1b B cell epitope is the limiting factor. This is in accordance with the notion that a T cell response to an immunogenic T cell epitope is required for efficient antibody production.

As the Leu33 residue in the antigenic HPA-1a peptide is not directly recognized by the T cell clones, we studied the role of the other residues in the HPA-1a-derived peptide by amino acid residue substitutions (Paper II). First, we determined the effect of a given amino acid substitution on DRA/DRB3*01:01-binding by peptide-binding assays; this information is essential for determining the recognition by the T cell clones.
In the study by Rayment et al., particular T cell contact sites were indicated,[97] a finding based on computational modelling of the previously published crystal structure,[53] in agreement with other T cell epitope-mapping studies with peptide:HLA-DR molecules.[90,109] Our results, using a panel of modified HPA-1a peptides, presented by DRA/DRB3*01:01, clearly demonstrate that the T cell recognition of the peptide:MHC is heterogeneous, as the different clones show individual patterns of recognition of the modified peptides.

The *TCRA* and *TCRB* loci have been reported to have several SNPs that result in amino acid polymorphisms.[110,111] Gras et al. recently showed that a single amino acid difference between the two common allelic variants of the *TRBV9* gene resulted in differential TCR binding affinity and

functional recognition of a viral epitope, demonstrating that allelic polymorphisms within the TCR loci can influence the immune response.[112] Furthermore, specific TCRα/TCRβ loci have been reported to be associated with increased susceptibility to immune disorders.[110,111]
As the HPA-1a-specific T cells used in this study were isolated from only three women, the information that can be deduced from the analyses of T cell receptor genes is limited. However, as the T cell recognition of the peptide:MHC was shown to be heterogeneous among the T cell clones, this may explain why no conserved TCRα/β CDR3 "motifs" are seen in the T cell clones. Furthermore, the number of TRAV/TRBV and TRAJ/TRBJ regions used to build a TCR specific for this peptide:MHC-epitope indicates that no particular TCRα/TCRβ allelic variants are required for alloimmunisation, thus ruling out this as a possible risk factor.

The *DRB3*01:01* allele is the only *DRB3* allele predisposing HPA-1a alloimmunisation, rationalized by the predicted lack of antigen presentation by other DRB3 variants. The amino acid differences in three different HLA-DRB3 variants *01:01, *02:02 and *03:01 are comprised to the peptide binding regions, indicating that T cell recognition of these molecules themselves should not be discriminated.[54] Peptide-binding assays confirm that the DRA/DRB3*02:02 and DRA/DRB3*03:01 bind the original L33 peptide poorly. By substituting the P1 and P4 anchor residues in the HPA-1a derived peptide (residues 25 and 28), for favourable docking to DRA/DRB3*02:02 and DRA/DRB3*03:01 molecules, we could demonstrate peptide-binding to APCs expressing these MHC variants, as well as corresponding T cell activation after stimulation with these peptide-pulsed DRB3*02:02- and DRB3*03:01-positive APCs (Ahlen et al, 2010 unpublished data).

In contrast to the activation of antigen specific B cells, the priming event of antigen specific T cells *in vivo*, the β3-integrin-derived HPA-1a antigen is not necessarily processed from the $\alpha_{IIb}\beta_3$ on foetal platelets. It may be processed from any other β_3 integrins on other cells of foetal origin.[113] The role of $\alpha_v\beta_3$ on invasive trophoblasts,[114] remodelling of the spiral arteries during early pregnancy to ensure increased blood flow to nourish the placenta, was suggested as a source of antigen years ago.[115] However, also leukocytes and especially monocytes, also carry β_3 on their surface.[116]

The very obvious idea of platelets as the source of antigen causing immunisation in connection with pregnancy probably originates from the Rh(D) immunisation mechanism as a counterpart for HPA-1a alloimmunisation. However, the Rh(D) antigen is exclusively expressed on red cells in adults, and human trophoblast cells are reported not to carry any Rh(D) antigen in any

trimester,[117] and therefore the foetal red cells are the certain antigenic origin. For comparison of sustained exposure, the lifespan of red cells is ~100 days, compared to ~9 days for platelets. The notion that incompatible platelet transfusions can cause alloimmunisation also supports the idea that the foetal platelets can function as a source of antigenic exposure to the mother.

To what extent the antigenic load caused by foeto-maternal bleeding is of importance is not clear, but it is certainly plausible, as the volume of the foeto-maternal bleeding is known to be critical for RhD alloimmunisation. Small foeto-maternal bleedings in the second and third trimesters are considered normal, while larger bleedings are more infrequent.[118,119]

Other factors influencing the HPA-1a alloimmunisation

The functional link between the *HLA-DRB3*01:01* allele and reactivity of maternal platelet-reactive T cells further rationalises the typing of HLA-*DRB3*01:01* in HPA-1a-negative pregnant women at risk, although ~5% of all HPA-1a alloimmunised women do not carry this allele.[5] Generally, these women present relatively low HPA-1a-antibody levels, but occasionally deliver affected newborns.

It is important to keep in mind, that the majority of women at risk (HPA-1bb and *HLA-DRB3*01:01*-positive) are not alloimmunised by the allogeneic exposure in connection with an incompatible pregnancy. Hence, other factors yet unknown, apparently influence the alloimmunisation process, and the severity of thrombocytopenia and bleeding in the foetus or newborn. Several factors implicated in other immune- or pregnancy- related disorders have been studied to further understand the mechanism, and to improve the prediction of women at particular high risk. Data from the Norwegian screening and intervention study, showed that HPA-1a alloimmunised women with blood group A had higher risk (~50%) of giving birth to a child with severe NAIT than women with blood group O (~20%), and further that this was associated to their *ABO* genotype (Ahlen et al. Manuscript in preparation), however by far not sufficient to rationalise why some women are alloimmunised and others are not. In contrast, a recent retrospective study, showed no ABO association.[8]

Polymorphisms in killer immunoglobulinlike receptor (KIR) genes have been implicated in preeclampsia, by the binding of foetal HLA-Cw to particular KIRs on maternal natural killer cells. However, no maternal-KIR/foetal-HLA-Cw gene combinations involved in foeto-maternal tolerance appeared to be important for HPA-1a alloimmunisation.[120]

The presence of anti-idiotypic antibody was investigated for potentially reducing the anti-HPA-1a level during the pregnancy, but was not found to play a role.[121]

The overrepresentation of the *DRB3*-associated DR3-DQ2 haplotype (paper III) indicates that there may be immune-regulating genes within this haplotype that, although not absolutely essential, promote immunisation. A quite small study by Hildén et al. suggested an association of the *DQB1*02:01* allele with severe RhD immunisation (high titre of maternal anti-D antibodies).[122] It is interesting that pregnancy-related alloimmunisation with both HPA-1a and RhD indicates a specific role for this haplotype. *DQB1*02* has also been associated with HPA-1a-related NAIT.[3] Although the authors recognized the linkage disequilibrium between *DRB1* and *DQB1*, the effect of the DR-DQ haplotype does not seem to be taken into account. However, no HPA-1a-specific T cells that are restricted by DQA1*05:01/DQB1*02:01 or any other DQ2 molecule have so far been isolated or unambiguously detected. This might be due to the fact that few serious attempts have been made to hunt for these, or simply because they do not exist. DQ2 molecules have been shown to play a functional role in immune responses by presenting antigens to T cells; gluten antigens in celiac disease[56] and streptococcal superantigens.[123] Whether DQ2 molecules can present HPA-1a peptides is not yet studied in detail.

It is worth reflecting that the overrepresentation of the DR3-DQ2 haplotype reported in paper III, were indirectly suggested in the earliest years of HPA-1a immunisation research, as HLA allele associations were reported in the 1980s. First, the association with HLA-B8 was reported in 1981 by Reznikoff-Etievant et al.[124] and confirmed by Taaning et al. in 1983.[125] However, during the next years the association to DR3 *(DRB1*03:01)* was acknowledged.[126] In, 1990, Valentin et al. showed that the *DRB3*01:01* was in fact the associated genetic factor.[77] This suggested that the associations to the B8 and DR3 was observed due to linkage disequilibrium with the *DRB3*01:01*. This was further rationalized in 1997, when Wu et al presented a role for the HPA-1 allogeneic polymorphic residue in peptide binding to the DRA/DRB3*01:01 molecule. Now, these early HLA association data come together with our recent studies (paper III), and may shed light on functional biology of the DR3-DQ2 haplotype.

The antigen-specific T cells are thought to be crucial for stimulation of the antigen-specific B cells, through "signal 2"; binding of CD154 to the CD40 receptor on the B cells. CD154 has been thought to be uniquely expressed by activated T cells. However, platelets and platelet-derived membrane vesicles have been shown to deliver the CD154 signal to B cells and thus stimulate IgG production in mice.[127] Interestingly, expression of CD154 on the surface of activated platelets is increased in ITP patients, and in vitro experiments have demonstrated that the platelet-associated CD154 can induce CD40-dependent B cell proliferation and production of anti-platelet antibodies.[128] Whether such mechanisms can contribute to alloimmune responses is not yet known.

Whether inflammation plays a role in the alloimmunisation process has yet to be determined. However, several aspects of the mechanisms unravelled to date suggest that it does. First, the apparent inflammatory Th1 profile of the HPA-1a-specific T cells, that secrete the pro-inflammatory cytokines IFNγ and TNFα. Second, the overrepresented DR3-DQ2 haplotype, which is reported to be an "inflammatory" haplotype. Especially the conserved extended ancestral haplotype (AH) 8.1,[129] which has also been reported by many studies to have higher levels of TNFα in serum, although the specific role of the AH 8.1 haplotypic marker promoter polymorphism *TNF-308A* is debated.[130,131] Third, signalling from the innate immune response is the key to activation of the adaptive immune response to any given antigen.

Further use of HPA-1a-specific T cell clones

Nothing is yet known about the presence, time of onset, kinetics or magnitude of *in vivo* HPA-1a-specific CD4 T cell responses during or after affected pregnancies, as no techniques are yet available for such accurate measurements. In order to gain knowledge about this, and to further optimise the T cell detection efficiency, we aim to develop peptide:MHC tetramer reagents. By employing such reagents, screening and characterisation of *in vivo* NAIT-related T cell responses can be measured in blood samples from a large number of women with affected pregnancies. The peptide-MHC tetramer technology is commonly used, and a very efficient method to accurately measure antigen-specific T cell responses *in vivo*,[132,133] which is by nature complicated due to the low frequency of these cells in circulation.

With the isolation of more clones we can study the TCRs further, as well as the characteristics of the T helper cells. Do women with high antibody levels have a different T cell profile response than those with low levels, and how do the diversity of the T cell responses correlate with the antibody titres, subclasses of IgG, and severity of thrombocytopenia in the newborn in terms of platelet counts or bleeding tendency?

By the demonstration that the recognition of antigen by HPA-1a-specific T cells, from alloimmunised women with affected newborns, is restricted by the MHC class II molecule DRA/DRB3*01:01, the potential treatment inducing T cell tolerance to HPA-1a, may approach a prophylaxis for NAIT.

Tolerization of T cells with specificity for the HPA-1a:DRA/DRB3*01:01 complex will likely prevent any specific T cell activation at subsequent exposure to the HPA-1a antigen. Thus, tolerized HPA-1bb women will predictably not produce any anti-HPA-1a antibodies in

connection with their first HPA-1 incompatible pregnancy, or only limited amounts, insufficient to induce severe thrombocytopenia in the foetus or newborn.
However, knowledge of *in vivo* T cell responses and sophisticated methods to measure them directly in patient samples will be essential to monitor and evaluate the effectiveness of any prophylactic treatment.

Historically, MHC class II tetramers have proven to be more complicated to successfully produce than MHC class I tetramers which have been commercially available for years.[134] T cell clones with known specificity and MHC-restriction are absolutely required to test the quality of these reagents, which we aim to develop in our laboratory in near future. We have already tested tetramers provided to us in a limited amount as a part of collaboration with Dr. William Kwok at Benaroya Research Institute (Seattle, USA). These reagents reacted specifically with clonal HPA-1a-specific T cells (unpublished data), thereby proving the feasibility of synthesizing tetramer reagents with a HPA-1a derived peptide in complex with a synthetic DRA/DRB3*01:01 molecule.

Concluding remarks

From a philosophical point of view, it is intriguing that a single nucleotide difference in the maternal and foetal DNA can have such profound effect of the health of the newborn, considering that every pregnancy is in fact semi-allogeneic, and normally proceeds without alloimmune complications. There is now evidence for a diverse T cell response in these alloimmunised women, which most likely is crucial for the development of the platelet-reactive antibodies transferred to the foetus, and subsequently may cause NAIT. Only by elucidating the mechanisms in detail, a preventive therapy for HPA-1a alloimmunisation, and possibly also for other alloimmune conditions in association with pregnancy can be introduced, in order to successfully reach the goal of healthy newborns.

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