Development of new tissue culture protocols for enrichment of CD4 T cells associated with neonatal alloimmune thrombocytopenia

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"An immune system of enormous complexity is present in all vertebrate animals. When we place a population of lymphocytes from such an animal in appropriate tissue culture fluid, and when we add an antigen, the lymphocytes will produce specific antibody molecules, in the absence of any nerve cells. I find it astonishing that the immune system embodies a degree of complexity which suggests some more or less superficial though striking analogies with human language and that this cognitive system has evolved and functions without assistance of the brain".

Niels K. Jerne

'The Generative Grammar of the Immune System', Nobel Lecture, 8 Dec 1984. In *Nobel Lectures: Physiology or Medicine 1981-1990* (1993), 223.

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Ida Løken Killie

Abbreviations

Ab Antibody

ADAM A disintegrin and metalloproteinase domain

ADCC Antibody-dependent cell-mediated cytotoxicity

AdEtOH Adamantane-ethanol

ADP Adenosine diphosphate

Ag Antigen

AP-1 Activator protein 1

APC Antigen presenting cell

APC Allophycocyanin

APC-Cy7 Allophycocyanin-cyanin7

BCIP-NBT 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium

BCR B cell receptor

BSA Bovine serum albumin

CD Cluster of differentiation

cDNA Complementary deoxy ribonucleic acid

CDR Complementary Determining Region

CFSE Carboxyfluorescein succinimidyl ester

CMV Cytomegalovirus

CTL Cytotoxic T lymphocyte

DEPC Diethyl pyrocarbonate

DNA Deoxy ribonucleic acid

DMSO Dimethyl sulfoxide

EAE Experimental allergic encephalomyelitis

EDTA Ethylenediaminetetraacetic acid

ELISPOT Enzyme-linked immunosorbent spot

Fab Fragment that binds antibody

FACS Fluorescent activated cell sorter

FBS Foetal bovine serum

Fc Fragment that crystallizes

FcR Fc receptor

FcRγn Neonatal Fc gamma receptor

GP Glycoprotein

HDN Haemolytic disease of the newborn

HLA Human leukocyte antigen

HPA Human platelet antigen

HSA Human serum albumin

ICH Intracranial haemorrhage

ICS Intracellular cytokine staining

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IMDM Iscove's modified dulbecco medium

iT_{reg} Induced regulatory T cell

kDa Kilodalton

mAb Monoclonal antibody

MHC Major histocompatibility complex

mRNA Messenger ribonucleic acid

NAIT Neonatal alloimmune thrombocytopenia

NFAT Nuclear factor of activated T cells

NFκB Nuclear factor-kappa B

NK Natural killer

nT_{reg} Natural regulatory T cell

PBMC Peripheral blood mononuclear cell

PBS Phosphate buffered saline

PBSA Phosphate buffered saline with bovine serum albumin

PCR Polymerase chain reaction

PE Phyco-erythrin

PE/A.610 Phyco-erythrin/Alexa 610

PE-Cy7 Phyco-erythrin-cyanin7

PerCP Peridinin chlorophyll protein complex

PerCP-Cy5.5 Peridinin chlorophyll protein complex-cyanin5.5

Rh Rhesus

RNA Ribonucleic acid

RQ-PCR Quantitative RealTime polymerase chain reaction

SNP Single nucleotide polymorphism

TACE TNF alpha converting enzyme

TAPI TNA alpha protease inhibitor

TCR T cell receptor

TGF Transforming growth factor

T_H T helper

TNF Tumour necrosis factor

T_{reg} Regulatory T cell

U Unit

Summary

Neonatal alloimmune thrombocytopenia (NAIT) is most commonly caused by destruction of foetal platelets by maternal antibodies reactive to human platelet antigen (HPA)-1a. The activation of antigen-primed B cells to differentiate to antibody-secreting plasma cells usually requires help from CD4 T cells. The strong association between anti-HPA-1a-production and the MHC allele *HLA-DRB3*0101* supports that this notion is also valid in the context of NAIT, and suggests the activation of HPA-1a-specific T cells as the most critical event of immunization.

In this study, an improved protocol for enrichment, identification and efficient isolation of HPA-1a-specific CD4 T cells is presented. By replacing foetal bovine serum with human serum, enrichment of antigen-specific CD4 T cells improved dramatically. Identification and isolation of HPA-1a-specific CD4 T cells greatly improved when combining the CFSE proliferation assay with a second stimulation with antigen and subsequent assay for surface detection of TNF production. HPA-1a-specific CD4 T cells could also be identified in the CFSE proliferation assay as proliferating T cells with down-regulated expression of CD4.

HPA-1a-specific T cells isolated from immunized women may serve as useful tools for investigating the cellular immune response to HPA-1a, and for developing strategies to prevent immunization in HPA-incompatible pregnancies, e.g. through TCR epitope mapping and examinations of the immunogenicity of the HPA-1a antigen at the amino-acid level.

1. Introduction

Nearly all, living organisms possess some form of defence strategy to gain protection against potentially harmful pathogens. These mechanisms may range from the simple antibacterial peptides found in plants and insects to the highly sophisticated network of molecules, cells and tissues that make up the immune system of higher vertebrates. It is a well-known fact that a functional immune system is essential to human life, as common infections harmless to healthy individuals may be life threatening to people suffering from immune deficiencies.

The human immune system is based on the recognition of non-self structures, enabling discrimination between structures of the individual's own cells and tissues and foreign substances. When a pathogen enters the circulation by defeating the physical barriers made up by the skin and mucosal surfaces, proteins and cells of the innate immune system will target and seek to eradicate the invading pathogen. If necessary, cells of the innate immune system will also recruit cells of the adaptive immune system and activate these to provide specific targeting of the pathogen and induce immunological memory to provide lasting protection to subsequent infections by the same pathogen.

Although essential for human life, the immune system may also cause disease. In some cases the immune system may be activated by self-structures, causing the immune system to target the individual's own cells or tissues, as is the case with autoimmune diseases. In addition, our immune system will not only respond to foreign antigens from pathogens or toxins, but also to non-self structures from other individuals of the same species, in the settings of blood transfusions, organ transplants or pregnancy, resulting in alloimmune responses.

1.1 Neonatal alloimmune thrombocytopenia is caused by foeto-maternal incompatibility in human platelet antigens (HPA)

Neonatal alloimmune thrombocytopenia (NAIT) is a condition caused by destruction of foetal thrombocytes (platelets) by maternally produced platelet-reactive antibodies. In the Caucasian population, NAIT is most commonly caused by an incompatibility between maternal and foetal HPA-1 antigens, where an HPA-1a negative female may produce antibodies specific to HPA-1a when carrying an HPA-1a positive child. IgG anti-HPA-1a may cross the placenta by transcytosis

via the neonatal Fcγ-receptor (FcRγn) expressed on syncytiotrophoblasts in the foeto-maternal interface and enter the foetal circulation where they bind and opsonize foetal platelets (Figure 1.1).

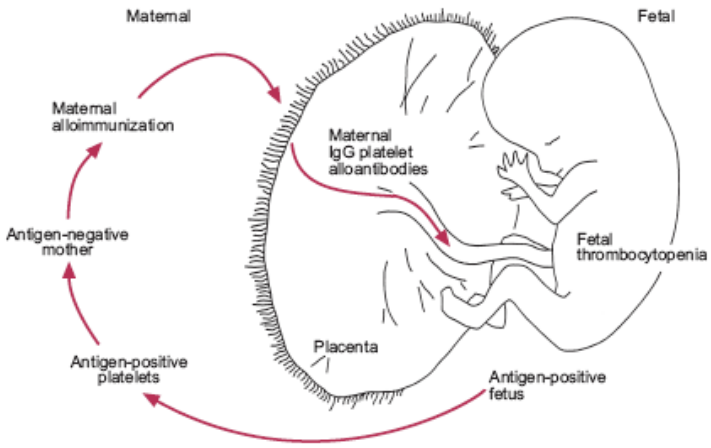

Figure 1.1 Schematic overview of the proposed pathogenesis of Neonatal alloimmune thrombocytopenia (1).

1.1.1 Human platelets

Blood platelets are the smallest blood components ranging from 2.0-5.0 μm in diameter. Platelets are non-nucleated structures produced by fragmentation of megakaryocyte cytoplasm in the bone marrow; where one megakaryocyte can give rise to about 4000 platelets.

In the circulation, platelets are responsible for blood clotting and constriction of damaged blood vessels to prevent bleeding and to promote blood vessel repair. In addition, they are involved in regulating various processes including haemostasis, thrombosis and inflammation. In response to vascular injury, activated platelets will undergo adhesion to exposed subendothelial connective tissue through the glycoprotein (GP) IIb/GPIIIa receptor complex. Platelet adhesion promotes the release of granules containing various substances including ADP, serotonin, fibrinogen, lysosomal enzymes, β-thromboglobulin, heparin neutralizing factor and prostaglandins. In addition, arachidonate is released from the cell membrane and gives rise to thromboxane A_2, which together with ADP promotes platelet aggregation at the site of vessel damage, leading to the formation of a haemostatic plug occluding the damaged vessel.

The normal human platelet count is 150-450x10^9/L. Thrombocytopenia increases the risk of prolonged bleeding and is defined by a platelet count of <50x10^9 /L. NAIT usually presents as petechiae (small subcutaneous haematomas caused by capillary vessel lesions) and a platelet count below 50x10^9 /L in an otherwise healthy neonate. Severe thrombocytopenia (<20x10^9 /L)

dramatically increases the risk of intracranial haemorrhage (ICH). In severely affected neonates surviving intracranial haemorrhage, long term neurological complications including mental retardation, cerebral palsy and cortical blindness may arise (2-5).

1.1.2 Human platelet antigens

Human platelet antigens (HPA) are generated by single nucleotide polymorphisms (SNP) in platelet antigens, and are defined by immune responses between individuals that differ by allomorphisms at these sites. HPA-1 antigens are located on the β3-integrin (GPIIIa) of the GPIIb/GPIIIa complex, the major fibrinogen receptor on human platelets, and are generated by a Leu33/Pro33 amino acid difference (6). The GPIIb/GPIIIa integrins are expressed in normal amounts (50,000 – 80,000 copies) (7) on foetal platelets from approximately 16 weeks of gestation (8). The classification of HPAs is summarized in Table 1.1.

The HPA alloantigens may cause production of HPA-reactive alloantibodies, which cause Neonatal Alloimmune Thrombocytopenia (NAIT) in incompatible pregnancies or post-transfusion purpura (PTP) in HPA-unmatched platelet transfusions. The SNPs that give rise to the different HPAs are listed in Table 1.2.

Table 1.1 Nomenclature of Human Platelet Antigens (HPA). Modified from Metcalfe et al 2003 (9)

System*	Antigen*	Glycoprotein	CD	System*	Antigen*	Glycoprotein	CD
HPA-1	HPA-1a	GPIIIa	CD61	HPA-5	HPA-5a	GPIa	CD49b
	HPA-1b				HPA-5b		
HPA-2	HPA-2a	GPIbα	CD42b		HPA-6bw	GPIIIa	CD61
	HPA-2b				HPA-7bw	GPIIIa	CD61
HPA-3	HPA-3a	GPIIb	CD41		HPA-8bw	GPIIIa	CD61
	HPA-3b				HPA-9bw	GPIIb	CD41
HPA-4	HPA-4a	GPIIIa	CD61		HPA-10bw	GPIIIa	CD61
	HPA-4b				HPA-11bw	GPIIIa	CD61
					HPA-12bw	GPIbβ	CD42c
					HPA-13bw	GPIa	DC49b
					HPA-14bw	GPIIIa	CD61
				HPA-15	HPA-15a	CD109	CD109
					HPA-15b		
					HPA-16bw	GPIIIa	CD61

* HPA-systems are grouped based on the existence of alloantibodies defining a given alloantigen and its antithetical alloantigen. The HPA-systems are numbered chronologically according to their date of discovery. HPAs and their allotypes are designated alphabetically according to their frequency, with the more common variant designated 'a' and the less common variant designated 'b'. A 'w' is added to the antigen name where an alloantibody against the antithetical antigen has not been reported.

Table 1.2 Polymorphisms giving rise to human platelet antigens (HPA). Modified from Metcalfe *et al* 2003 (9)

Antigen	HGNC*	Nucleotide change**	Mature protein***
HPA-1	*ITGB3*	176T > C	L33P
HPA-2	*GP1BA*	482C > T	T145M
HPA-3	*ITGA2B*	2621T > G	I843S
HPA-4	*ITGB3*	506G > A	R143Q
HPA-5	*ITGA2B*	1600G > A	E505K
HPA-6w	*ITGB3*	1544 G > A	R489Q
HPA-7w	*ITGB3*	1297C > G	P407A
HPA-8w	*ITGB3*	1984C > T	R636C
HPA-9w	*ITGA2B*	2602G > A	V837M
HPA-10w	*ITGB3*	263G > A	R62Q
HPA-11w	*ITGB3*	1976G > A	R633H
HPA-12w	*GP1BB*	119G > A	G15E
HPA-13w	*ITGA2B*	2483C > T	T799M
HPA-14w	*ITGB3*	1909_1911delAAG	K611del
HPA-15	*CD109*	2108C > A	S682Y
HPA-16w	*ITGB3*	497C > T	T140I

* HGNC: Name of encoding gene (assigned by The Human Genome Organization (HUGO) Gene Nomenclature Committee; http://www.gene.ucl.ac.uk/nomenclature).

** Nucleotide numbers are derived from the reference sequence in the NCBI database and may differ from numbers in the original publications describing the mutations. Nucleotide and amino acid substitution (mature protein column) is shown from the more common variant (a) to the less common variant (b). "Del" indicates a deletional mutation.

*** Amino acid (single letter abbreviation) change corresponding to the nucleotide change. The number flanked by the amino acids give the positions in the primary sequence of the protein.

1.1.3 No effective treatment or prevention of NAIT is currently available

To this day, no safe or effective treatment, or prevention of NAIT is available, and the detailed natural history of the immunization process is not completely understood. A large prospective screening and intervention study performed on 100 448 pregnant women in Norway revealed that almost 75 % of HPA-1a negative who produce anti-HPA-1a antibodies are immunized around delivery (10), suggesting that immunization in most cases is a consequence of foetomaternal haemorrhage. Another possible route of antigen exposure that needs to be considered is foeto-maternal cell trafficking, which may cause foetal cells expressing HPA-1a (endothelial cells, invasive trophoblasts and thrombocytes) to enter the maternal circulation. In addition, invading foetal trophoblasts expressing the β3-integrin ($\alpha_v\beta_3$) replace endothelial cells of the spiral arteries in maternal placental endomethrium (11) and may also be a source of foetal HPA-1a exposed to maternal antigen presenting cells (APC).

All factors that make a woman susceptible to immunization by HPA-1a are yet to be identified. So far it is known that females who produce anti-HPA-1a during pregnancy or after delivery are HPA-1a negative (HPA-1bb) and that the major histocompatibility complex (MHC) class II allele

*HLA-DRB3*0101* is strongly associated with the development of NAIT (12;13). *HLA-DRB3*0101* is, in fact, present in over 90 % of women who produce anti-HPA-1a antibodies in connection with preganancy (14), compared to less than 30 % in the general population. The Norwegian screening and intervention study showed that anti-HPA-1a was detected in only 10,6 % of HPA-1a negative pregnant women (14), suggesting a missing link in the prediction of antibody production in HPA-1 incompatible pregnancies that requires further investigation.

The main focus of the management of NAIT is to prevent the consequences of ICH. NAIT occurs in approximately 1:1200 pregnancies, of which up to 30 % of the cases present with ICH (5;15;16). Two independent studies showed that 50-80 % of ICH cases due to NAIT occured *in utero* (5;17), meaning that in these cases, prophylactic strategies to prevent NAIT, rather that antenatal treatment is necessary.

1.1.4 NAIT is the platelet counterpart of haemolytic disease of the newborn (HDN)

The Rhesus (Rh) antigen system is the most important blood group system after the ABO system in terms of donor-recipient compatibility. The Rh antigens are located on transmembrane proteins on red blood cells (erythrocytes). The most important Rh antigens are D, C, c and e, where Rh-D is the most immunogenic antigen and the one that defines the Rh-factor of blood type designation. An individual positive for Rh-D will therefore be referred to as Rh positive (Rh^+), while an individual negative for Rh-D will be referred to as Rh negative (Rh^-) regardless of expression of any of the other Rh antigens.

Immunization to the Rh-D antigen may occur in situations where an Rh-D negative woman is pregnant with an Rh-D positive child. Maternally produced antibodies targeting the Rh-D antigen may cross the placenta and cause destruction of the foetal/neonatal red blood cells, with haemolytic anaemia as the clinical consequence of Rh-D immunization, referred to as Haemolytic Disease of the Newborn (HDN). Anti-D transfer from mother to foetus occurs in multigravidae, and HDN rarely occurs in the clinic thanks to an effective anti-D prophylaxis administered to Rh-D negative women after delivery of their first child.

Previously the immunization to HPA-1a was assumed to occur in primigravidae (18), and for this reason, prophylactic strategies similar to that of preventing HDN was dismissed as a possibility to prevent NAIT. However, results from the Norwegian screening and intervention program revealed that in most cases, anti-HPA-1a was not detectable in the mother's circulation until 6 weeks post

partum (10), suggesting that NAIT most likely occurs in the second incompatible pregnancy making it more similar to RhD-immunization than previously suggested.

1.2 Antibody isotypes

Each lymphocyte in the circulation will express identical lymphocyte receptors specific for the same epitope. The great diversity of epitope specificities displayed by the T cell receptor (TCR) and B cell receptor (BCR) repertoires is generated by randomized joining of gene segments encoding the variable regions of the lymphocyte receptors, a process referred to as recombinant gene rearrangement.

The BCR is an immunoglobulin (Ig) transmembrane protein made up by four polypeptide chains; two heavy chains of approximately 50 kDa and two ~25 kDa light chains. The two heavy chains are joined together by two disulfide bonds, and one light chain is linked to each heavy chain via one disulfide bond. The heavy- and light chains each consist of a constant and a variable region, where the variable region of one heavy- and one light chain together make up the antigen-binding motif of the immunoglobulin molecule (Figure 1.2). The two heavy chains and the two light chains of each immunoglobulin molecule are identical, giving each immunoglobulin two identical antigen binding sites, and the ability to bind two identical structures simultaneously.

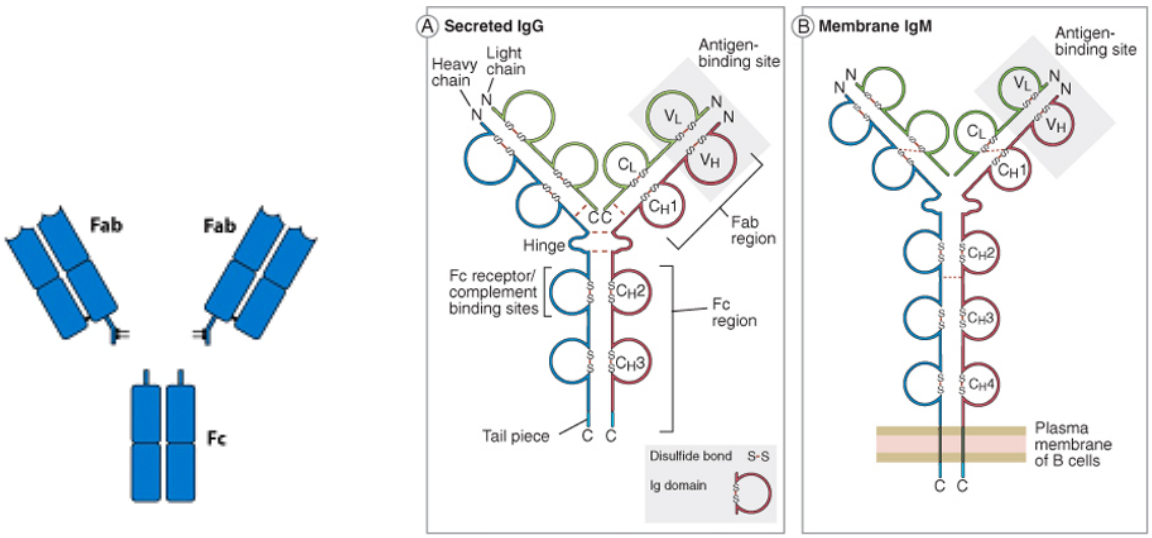

Figure 1.2 An immunoglobulin molecule is a homodimer of two heavy- and two-light chains with constant (C) and variable (V) regions. The variable region of one heavy (V_H) and one light (V_L) forms the antigen-binding site of the immunoglobulin molecule, giving each molecule two identical antigen-binding sites. Modified from Janeway's Immunobiology 7th edition (19) and Abbas' Basic Immunology 3rd edition (20).

B lymphocytes are responsible for the production of antibodies targeting foreign structures recognized by the specific lymphocyte receptors of the adaptive immune system. If an antigen-primed B cell is activated by a helper T cell specific to the same antigen, the activated B cell will differentiate to a plasma cell producing large amounts of secreted immunoglobulin, referred to as antibodies, of the same specificity as the membrane bound BCR. The three main effector functions of antibodies are neutralization, e.g. of viral antigens preventing virus entry to healthy cells, opsonization of bacteria and other foreign structures for uptake by phagocytes and activation of the complement system of the innate immune system.

Several antibody isotypes may be produced by plasma cells (Figure 1.3), serving different effector functions, summarized in Table 1.3. The isotypes transferred from a mother to a foetus or neonate, are IgA and IgG.

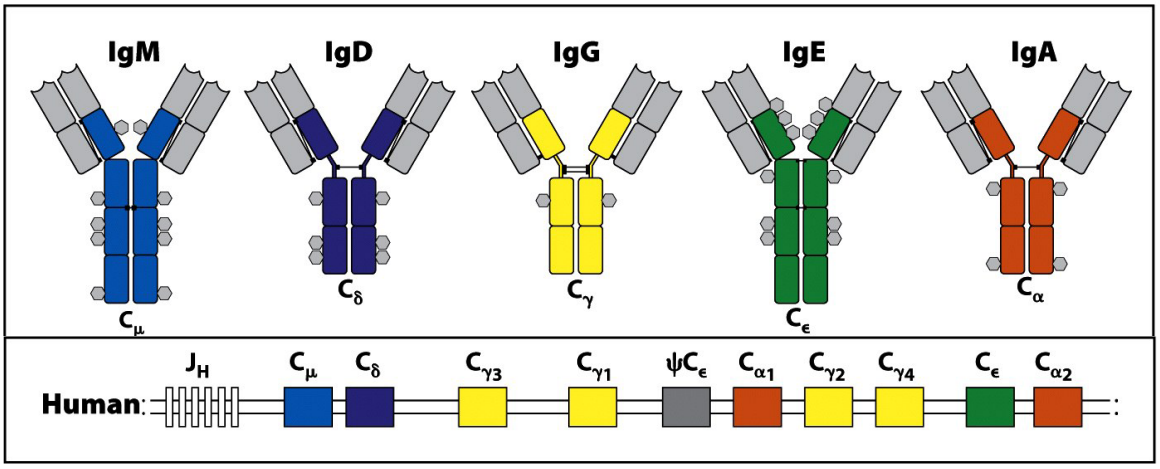

Figure 1.3 Schematic overview of the five different antibody isotypes produced by B cells, with their gene coding segments. The different isotypes are determined by the genes encoding the constant heavy chain region. Modified from Janeway's Immunobiology 7th edition (19).

Monomeric IgA will not be subject for transport across epithelial barriers, since transport of IgA is dependent on a specific joining segment containing a secretory signal that links IgA molecules to their dimeric form. Dimeric IgA is only released in bodily secretions such as tears, breast milk and mucus. However, IgA dimers with specificities for platelet antigens will not be of concern if transferred to the neonate via breast milk, as the only effector function of dimeric IgA is neutralization. In addition, IgA will remain in the gut mucosal layer and will not be transported over the gut epithelia of the neonate to enter the neonatal circulation.

Table 1.3 Specialized function and distribution of the different antibody isotypes. Modified from Janeway's Immunobiology 7th edition (19)

	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Functional activity								
Neutralization	+	-	++	++	++	++	++	-
Opsonization	+	-	+++	*	++	+	+	-
Sensitization for killing by NK cells	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+++
Complement activation	+++	-	++	+	+++	-	+	-
Distribution								
Transepithelial transport	+	-	-	-	-	-	+++ (dimer)	-
Transplacental transport	-	-	+++	+	++	+/-	-	-

*) IgG2 may excert opsonizing functional activity in the presence of an Fc receptor of a certain allotype found in approximately 50 % of the Caucasian population.

As mentioned, most cases of NAIT are caused by an incompatibility in the expression of HPA-1 antigens between an HPA-1a negative mother and an HPA-1a-positive child. During pregnancy, IgG antibodies are continuously transported across the placenta from the mother to the foetus via the FcRγn receptor. The trans-placental transport of IgG antibodies is meant to protect the foetus from infectious agents. If anti-HPA-1a IgG is present in the mother's circulation during pregnancy, these will be transported along with these beneficial antibodies and enter the foetal circulation. Here, they may bind and opsonize foetal platelets expressing HPA-1a, rendering them for destruction. The destruction of antibody-coated platelets occurs mainly by Fcγ expressing phagocytes in the spleen, but may also be lysed by FcγRII (CD16)-expressing NK cells in antibody-dependent cell-mediated cytotoxicity (ADCC).

1.3 T cells are involved in the generation of antibody-mediated immune responses

NAIT is caused by destruction of foetal platelets coated with anti-HPA-1a produced by an HPA-1a-negative female. The initiation of an antibody mediated immune response normally requires both helper T (T_H) cells and B cells with specificity for epitopes on the same antigen.

An immune response typically starts with activation of the innate immune system when a pathogen enters the body. When cells of the innate immune system encounter the pathogen they

will become activated and secrete chemoattractant molecules (chemokines) and cytokines to recruit and activate other cells of the immune system.

Antigen presenting cells of the innate immune system are responsible for recruiting the highly specific adaptive immune system, through the activation of lymphocytes. These phagocytic cells are able to ingest pathogen, process antigenic protein to peptide fragments and present them on their cell surface bound to MHC molecules. Viral peptides and other intracellular antigens are usually presented on MHC class I molecules, expressed by most nucleated cells in the body, to CD8 T cells. Most bacterial and other extracellular antigens are usually presented on MHC class II molecules, expressed on professional antigen presenting cells (macrophages, dendritic cells and B cells), to CD4 T cells.

After activation, CD8 T cells will proliferate, differentiate to cytotoxic T lymphocytes (CTL) and kill virus infected cells presenting viral peptide bound to MHC class I. Activated CD4 T cells will proliferate and differentiate into one of several different subtypes of T lymphocytes, determined by which cytokine stimuli they receive at the time of activation through the T cell receptor (TCR). Common to all subtypes is that they help orchestrate the adaptive immune response by activating and directing other effector functions of the immune system, and are therefore referred to as helper T (T_H) cells.

1.3.1 Activation of T cells

In vivo, T cells are activated by antigenic peptide presented on MHC molecules on the surface of antigen presenting cells. The T cell receptor resembles one Fab-fragment of an antibody, and has one binding site for peptide:MHC. The T cell receptor is linked to several activation subunits designated CD3, that orchestrate the signalling events that lead to transcription of genes that promote T cell survival and proliferation upon TCR ligation. The most important genes that are transcribed are Interleukin (IL)-2 genes, that encode the T cell growth factor IL-2 and a high-affinity IL-2 receptor. The transcription of these genes are activated by the transcription factors NFκB, NFAT and AP-1 that are activated and transported to the nucleus in response to activation signalling through the TCR.

Naïve T cells require two signals for activation and proliferation. Signal one, which is an antigen-specific activation through the TCR upon binding to peptide:MHC, and signal two which is given upon binding of CD28 on the T cell surface to the co-stimulatory molecule B7 expressed on APC.

Signal one activates intracellular signal pathways that induce transcription of IL-2 and the α-chain of the IL-2 receptor (IL-2Rα) that associates with the IL-2 receptor to make a high-affinity IL-2 receptor. Signal two increase the initiation of IL-2 mRNA transcription and stabilisation of IL-2 mRNA.

Memory T cells are already differentiated to exert a specialized effector function after being activated through the TCR and CD28 in a previous immune response, and have undergone several phenotypic alterations that differ them from the naïve T cells. A memory T cell that already has overcome an activation barrier through antigen priming, selection and clonal expansion will only require ligation of the TCR and will not be dependent of a co-stimulatory signal through CD28 when recruited to subsequent immune responses. For the same reason, stimulating peripheral blood mononuclear cells (PBMC) with antigen *in vitro*, should only lead to activation and specific proliferation of memory T cells with specificity to the given antigen.

1.3.2 CD4 T cell lineages

The two major subsets of CD4 T cells -T_H1- and T_H2 cells- have been well known since the early 1990s. These subtypes are differentiated from naïve CD4 T cells in the periphery. In 2003 a third subtype of T_H-cells differentiated from naïve peripheral CD4 T cells was described (21;22) and later designated T_H17 cells due to their interleukin(IL)-17 dominated cytokine profile (23-25). In addition, a fourth subtype differentiated from naïve CD4 T cells, induced regulatory T cells (iT_{reg}), has been described (26-29). Natural killer (NK-) lymphocytes and natural regulatory T cells (nT_{reg}) are also CD4 cells, but these do not differentiate from naïve peripheral CD4 T cells. Instead, they belong to distinct cell lineages differentiating in the thymus parallel to the cells that differentiate to become the naïve peripheral CD4 T cells that later give rise to the subtypes already described (30;31). More CD4 T cells subsets are likely to be discovered. In fact, an IL-22-secreting subset has recently been suggested as a separate subset designated T_H22-cells (32;33).

The cytokine stimuli received by CD4 T cells at the time of activation, determines their differentiation and commitment to a given lineage. If the cell is stimulated by IL-4 and IL-2 at the time of activation, it will commit to the T_H2 lineage (34-37), while differentiation to the T_H1 subtype occurs in response to IL-12 and Interferon(IFN)-γ (38;39). Differentiation to the T_H17 lineage is stimulated by transforming growth factor (TGF)-β and IL-6 (25;40;41).

T_H1 and T_H2 cells may be identified based on their pattern of cytokine production after activation, which is dominated by IFN-γ and IL-4, respectively. The cytokine profile of these two lineages is of importance to their effector functions. IFN-γ will stimulate macrophages to increase phagocytosis and antigen presentation, while IL-4 serves as a co-stimulatory factor during the activation of B cells.

1.3.3 Evidence for involvement of T cells in immunization to HPA-1a

The production of IgG antibodies directed towards HPA-1a in immunized females indicates that T cells are involved in the cellular immune response that may result in NAIT. This hypothesis is supported by several evidences obtained from prospective studies as well as investigations of cells derived from women who have given birth to affected children (42-44).

T cells will only recognize antigen when presented by MHC molecules expressed on the surface of antigen presenting cells. The MHC variant HLA-DRB3*0101, which in association with the invariant HLA-DRA forms the MHC class II molecule HLA-DR52a, is present in over 90 % of women who produce anti-HPA-1a (14). By comparison, less than one third in the general population carry this allele, and the strong association between a specific MHC allele and IgG antibody production in response to HPA-1a, supports the notion that T cells with specificity to HPA-1a are involved in the immunization process (Figure 1.4). In addition, it has been shown that HLA-DRB3*0101 is able to bind and present HPA-1a-derived peptide (45).

The SNP that defines the HPA-1a system is responsible for generating the immune response leading to the production of anti-HPA-1a. The immunogenicity of HPA-1a lies likely in the ability of the L33 residue to bind to a pocket in the peptide binding groove of HLA-DRB3*0101 (45), anchoring the antigen to the HLA-DR52a molecule. In 2007, the crystallographic structure of the HLA-DR52a molecule with HPA-1a L33 peptide in the groove, was published, predicting the binding (46). HPA-1b with a proline residue in position 33 fails to bind HLA-DR52a (45), suggesting that T cells reactive to HPA-1a are not eliminated from the T cell repertoire during thymic selection in HPA-1bb individuals.

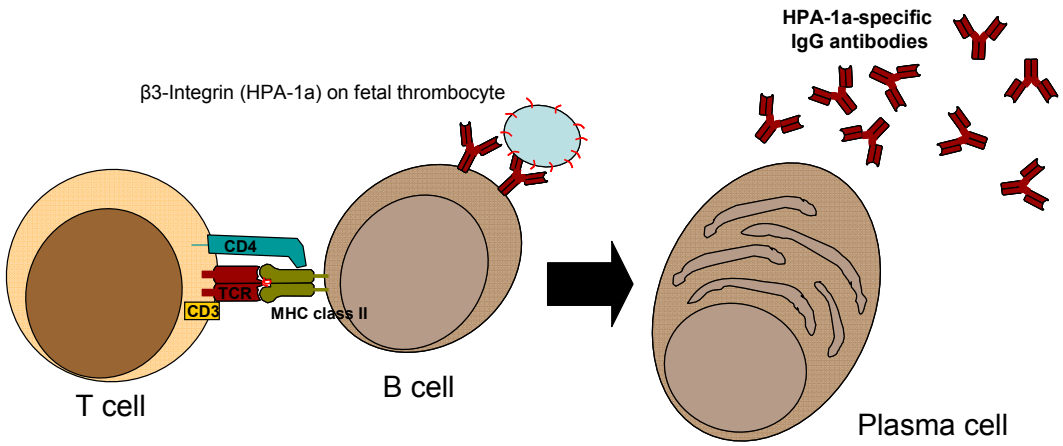

Figure 1.4 T cells specific to HPA-1a are believed to activate HPA-1a antigen primed B cells. After activation by an HPA-1a-specific T cell, the B cell is believed to proliferate and differentiate to anti-HPA-1a secreting plasma cells.

The involvement of T cells in the immune response leading to NAIT has previously been suggested by studies demonstrating HPA-1a specific proliferation of peripheral blood mononuclear cells (PBMC) from immunized mothers (42-44). In 2009 HPA-1a-specific T cell clones were isolated from women who had previously given birth to a child with NAIT (47;48), providing the first formal evidence that T cells with reactivity to HPA-1a are indeed present in the circulation of women who have produced HPA-1a-reactive antibodies in relation to a HPA-1-incompatible pregnancy.

1.4 Aim of study

T cell clones isolated from HPA-1a-immunized women may serve as useful tools for investigating the cellular immune response to HPA-1a and as tools for developing potential strategies to prevent immunization in incompatible pregnancies i.e. through TCR epitope mapping and examinations of the immunogenicity of the HPA-1a antigen at the amino acid level.

Cloning of antigen specific T cells is time consuming and with low cloning efficiency, larger patient samples (which may be difficult to obtain) are required in order to isolate specific clones. The main challenge in cloning HPA-1a specific T cell clones lays in the low frequency of T cells specific to a certain alloantigen in the circulation. In addition, spontaneous proliferating T cells of irrelevant specificities in *in vitro* proliferation assays makes the identification of antigen-specific T cells more challenging.

The aim of this study was to develop an improved protocol for enrichment, identification and isolation of HPA-1a specific T cells associated with NAIT. The main goal was to improve the cloning efficiency in order to isolate more clones from patient samples in a shorter period of time by seeking answers to the following questions:

1. Can HPA-1a-specific T cells in PBMCs from alloimmunized women be enriched and isolated after a single stimuli and expansion in bulk culture?
2. Can modifications in the culturing conditions help reduce the proliferation of T cells that proliferate without any obvious source of antigen-specific stimulation?
3. Is the phenotype of HPA-1a-specific T cells distinguishable from the proliferating T cells with irrelevant specificities?

2. Materials and methods

2.1 Materials

Table 2.1 Cell lines used in the study

			HLA Genotype								
Cell line	**Cell type**	**Source**	**A**	**B**	**C**	**DRB1**	**DRB3**	**DQA1**	**DQB1**	**DPA1**	**DPB1**
						*0301	*0101	*0501	*0201	unknown	*0401
D4BL4	B lymphoblast	Peripheral blood				*1454	*0202	unknown	*0503	unknown	*3201
								*0501	*0201	unknown	unknown
D18BL	B lymphoblast	Peripheral blood				*0301	*0101	unknown	unknown	unknown	unknown

Table 2.2 Peptides used in the study

Antigen	**Peptide**	**Amino acid sequence***	**Length of peptide**
HPA-1a	L33 (integrin β3 19-38)	VSPMCAWCSDEALPLGSPRC	20-mer
HPA-1b	P33 (integrin β3 19-38)	VSPMCAWCSDEALPPGSPRC	20-mer
Rye grass pollen	Lol P1(191-210)	ESWGAVWRIDTPDKLTGPFT	20-mer
Cytomegalovirus (CMV)	pp65 41-55	LLQTGIHVRVSQPSL	15-mer
Cytomegalovirus (CMV)	pp65 281-295	IIKPGKISHIMLDVA	15-mer
Cytomegalovirus (CMV)	pp65 485-499	PPWQAGILARNLVPM	15-mer

*) Underlined amino acids serve as anchor residues for binding to HLA-DRB3*0101 (45).

Peptides are synthesized by Eurogentec, Seraing, Belgium.

Table 2.3 Cell culture medium and reagents used in the study

Medium/reagent	**Distributor**	**Usage**
Iscove's Modified Dulbecco Medium (IMDM)	Lonza BioWhittaker (Verviers, Belgium)	Cell culturing and handling
Fetal Bovine Serum (FBS)	Lonza BioWhittaker (Verviers, Belgium)	Cell culturing and handling
Human Serum (HS) from HPA-1bb donors	Blood Bank and transfusion service, UNN	Cell culturing and handling
Penicillin-Streptomycin	Lonza BioWhittaker (Verviers, Belgium)	Cell culturing and handling
Recombinant IL-2 (25 U/µl)	PreproTech (London, UK)	Cell culturing
Recombinant IL-15 (500 ng/ml)	PreproTech (London, UK)	Cell culturing
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen (Carlsbad, CA)	Cell proliferation tracking assay
Dimethyl sulfoxide (DMSO)	WAK-chemie medical GMBH (Steinbach, Germany)	Cell cryopreservation, reagent solute
Brefeldin A	Fluka (Milwaukee, WI)	Intracellular cytokine staining assay
Adamantane-ethanol (AdEtOH)		MHC-peptide loading enhancer
Lymphoprep density gradient medium	Axis-Shield (Oslo, Norway)	Isolation of PBMCs and platelets
Optiprep density gradient medium	Axis-Shield (Oslo, Norway)	Isolation of monocytes and platelets
Streptavidin-Alkaline Phosphatase conjugate	Caltag Laboratories (Burlingame, CA)	ELISPOT
BCIP-NBT	Moss (Pasadena, MD)	ELISPOT

Table 2.4 Buffers used in the study

Buffer	Usage
Phosphate buffered saline (PBS)	General buffer and diluent
PBS 0.2 % Bovine Serum Albumin (0.2 % PBSA)	General buffer and diluent
PBS 0.2 % Human Serum Albumin (0.2 % PHSA)	General buffer and diluent
PBS 0.5 % Fetal Bovine Serum	Antibody diluent for ELISPOT
PBS 0.01 % Tween20	Wash buffer for ELISPOT
Saponin permeabilisation buffer	Intracellular cytokine staining
Fixation buffer (4 % paraformaldehyde)	Intracellular cytokine staining

Table 2.5 Monoclonal antibodies (mAbs) used in the study

mAb	Conjugate	Distributor	Usage	Amount (µl) used in 100 µl staining volume
anti-IFNγ	purified	BD Biosciences (San Jose, CA)	ELISPOT	-
anti-IFNγ	biotin	BD Biosciences (San Jose, CA)	ELISPOT	-
anti-IL-4	purified	BD Biosciences (San Jose, CA)	ELISPOT	-
anti-IL-4	biotin	BD Biosciences (San Jose, CA)	ELISPOT	-
anti-CD3	purified	Caltag Laboratories (Carlsbad, CA)	Expansion of T cell clones	-
anti-CCR7	PE	R&D Systems (Minneapolis, MN)	Flow cytometry	5.0
anti-CD14	APC-Cy7	BD Biosciences (San Jose, CA)	FACS/Flow cytometry	5.0
anti-CD25	PE	BD Biosciences (San Jose, CA)	Flow cytometry	4.0
anti-CD27	APC-Cy7	BioLegend (San Diego, CA)	Flow cytometry	4.0
anti-CD29	APC-Cy7	BioLegend (San Diego, CA)	Flow cytometry	4.0
anti-CD3	APC	Invitrogen (Camarillo, CA)	Flow cytometry/FACS	1.0
anti-CD3	Horizon-V450	BD Biosciences (San Jose, CA)	Flow cytometry	1.0
anti-CD38	APC	Invitrogen (Camarillo, CA)	Flow cytometry	1.0
anti-CD4	PE-Alexa 610	Caltag Laboratories (Carlsbad, CA)	Flow cytometry/FACS	1.0
anti-CD4	Horizon-V500	BD Biosciences (San Jose, CA)	Flow cytometry	1.0
anti-CD45RA	biotin	BioLegend (San Diego, CA)	Flow cytometry	0.2
anti-CD49d	PE	BioLegend (San Diego, CA)	Flow cytometry	4.0
anti-CD56	biotin	BioLegend (San Diego, CA)	Flow cytometry/FACS	0.2
anti-CD62L	PE-Cy7	BioLegend (San Diego, CA)	Flow cytometry	4.0
anti-CD69	APC-Cy7	BioLegend (San Diego, CA)	Flow cytometry/FACS	4.0
anti-CD8	PE	Caltag Laboratories (Carlsbad, CA)	Flow cytometry/FACS	0.5
anti-CLA	biotin	BioLegend (San Diego, CA)	Flow cytometry	0.2
anti-HLA-DR	PE-Cy7	BioLegend (San Diego, CA)	Flow cytometry/FACS	2.0
anti-IFNγ	APC	Invitrogen (Camarillo, CA)	Intracellular cytokine staining	0.5
anti-TNF	PE	BD Biosciences (San Jose, CA)	Flow cytometry/FACS	0.75
anti-β7 integrin	APC	BioLegend (San Diego, CA)	Flow cytometry	4.0
7- Amino-actinomycin D (7-AAD)	-	BD Biosciences (San Jose, CA)	Flow cytometry/FACS	10.0

Table 2.6 Primers used in analysis of T cell receptor β-chain variable regions (TCRBV). Forward primers from two different publications were used. "MCOLA" primers published by Lee *et al* (49) and "Aka" primers published by Akatsuka *et al* (50). Common reverse primer BC63 and internal BC primers from Stuge *et al* (51)

	Primer	**Sequence (5' - 3')**
PCR control	UpActin	CCGGGACCTGACTGACTA
PCR control	LoActin	TACAGGTCTTTGCGGATGT
Internal control	UpBC	CGCTGTGTTTGAGCCATC
Internal control	LoBC	TGCTCAGGCAGTATCTGGA
Reverse primer	BC63	GTGTGGCCTTTTGGGTGT
Forward primers	MCOLA BV1	CCTCCAGTTCCTCATTCAG
	MCOLA BV2	ATGCTGATGGCAACTTCCA
	MCOLA BV3	GGGCTACGGCTGATCTATTTC
	MCOLA BV4	CACTGATCGCAACTGCAA
	MCOLA BV5.1	GCCTTCAGTTCCTCTTTGA
	MCOLA BV5.2	GGCCCCAGTTTATCTTTC
	MCOLA BV5.4	TCCAGTTCCTCCTTTGGTATG
	MCOLA BV 6.1	GAGTTTTTAATTTACTTCCAAGGCA
	MCOLA BV 6.3	CCCAGAGTTTCTGACTTACTTC
	MCOLABV 6.2	GGCCAGAGTTTCTGACTTATT
	MCOLA BV7.1	GAAAGCTAAGAAGCCACCG
	MCOLA BV7.2	TACAAGCAAAGTGCTAAGAAGC
	MCOLA BV8.1	GGACTGGAGTTGCTCATTT
	MCOLA BV8.3	CAGACAGACCATGATGCAA
	MCOLA BV9	ATTTCTGAAGATAATGTTTAGC
	MCOLA BV11	CTACACCTCATCCACTATTCCTA
	MCOLA BV12.1	GGCTGAGGCTGATCCATTAC
	MCOLA BV12.3	CATGGGCTGAGGCTGATCTA
	MCOLA BV13.1	GGCTGAGGCTGATTCATTAC
	MCOLA BV13.3	TGGGACTGAGGCTGATTT
	MCOLA BV13.5	GGCTAAGGCTCATCCATTAT
	MCOLA BV13.6	GGCTGAAGCTGATTTATTAT
	MCOLA BV14	GGGCTTAAGGCAGATCTACT
	MCOLA BV15	CCTACGGTTGATCTATTACTCCTT
	MCOLA BV16	TCGACGTGTTATGGGAAA
	MCOLA BV17	GACAGGACCCAGGGCAAG
	MCOLA BV18	GGTCTGAAATTCATGGTTTATCT
	MCOLA BV20	CCTCCAGCTGCTCTTCTA
	MCOLA BV21.1	GAGCTTCTGGTTCAATTTCA
	MCOLA BV21.2	GAGCTTCTGATTCGATATGAGA
	MCOLA BV21.3	CCAAAGCTTCTGATTCAGTT
	MCOLA BV22	CGAGTTTCTGGTTTCCTTTT
	MCOLA BV23	CCCAGTTCCTCATTTCGTT
	MCOLA BV24	CAAAGCTGCTGTTCCACTACTA
	MCOLA BV25	GGTCCTGAAAAACGAGTTCAAG
Reverse primer	Aka TCRBC	CTAAAACACAGCGACCTC
Forward primers*	Aka BV1	CTTGCACTCTGAACTAAACC
	Aka BV2	TACCGTTCCCTGGACTTTC
	Aka BV3	CAAAGTAACCCAGAGCTCG
	Aka BV4	CCTGGACAGAGCCTGACA
	Aka BV5	GAGWVRVARAGGAAACTTCCCT
	Aka BV6	RMKCTCAGGTGTGATCCAA
	Aka BV7a	AACCTTCACCTACACGCCC
	Aka BV7b	TBCCTTCACCTACACACCC

Table 2.6 continued

Primer	Sequence (5' - 3')
Aka BV8	ATGCRRGGACTGGAGTTG
Aka BV9	AATGAAACAGTTCCAAATCGC
Aka BV11	CGAGGAATGGAACTACACC
Aka BV12a	TGAGATGTTCACCAGACTGA
Aka BV12b	TGACGTGTCACCAGAACTTG
Aka BV13	ACTCAGACCCCAAAATTCC
Aka BV13.5	ATCACCCAGGCACCAACATCT
Aka BV14	ATAAGGGAGATGTTCCTGAA
Aka BV15	ATTCTCCCTGTCCCTAGAG
Aka BV16	TCAGTTCCCCAGCCACAG
Aka BV17	CAGAAAGGAGATATAGCTGAA
Aka BV18	GAGGAAGGTCTGAAATTCAT
Aka BV20	AGTTCATCCTGAGTTCTAAG
Aka BV21	CTCTCAAGATCCAGCCTG
Aka BV22	CCAGACTCCCAGCCATCA
Aka BV23	AAATGCTATCCTATCCCTAG
Aka BV24	CAATGAAGCAGACACCCCT
Aka BV25	ACAGGTCCTGAAAAACGAGT

*)Nucleotide mismatches introduced to avoid primer-dimer formation are underlined. K=G+T, M=A+C, R=A+G, W=A+T, B=C+G+T, D=A+G+T, V=A+C+G (50).

Table 2.7 1x SuperScript III reverse transcriptase master mix

Reagent	Distributor	Volume for 1x master mix (µl)
Random primers (500 µg/ml)	Promega (Madison, WI)	2.0
dNTP 10 (10 mM)	QIAGEN (Hilden, Germany)	2.0
DTT (0.1 M)	Invitrogen (Carlsbad, CA)	2.0
5x first strand buffer	Invitrogen (Carlsbad, CA)	4.0
RNase inhibitor 40 u/µL)	Promega (Madison, WI)	0.5
SuperScript III (200 u/µL)	Invitrogen (Carlsbad, CA)	0.5

Table 2.8 1x master mix for PCR amplification of TCRBV cDNA

Reagent	Distributor	Volume for 1x master mix (µL)
10x PCR buffer mix	QIAGEN	2.5
dNTP	QIAGEN	0.5
H2O		9.5
BC63 (rev primer)		5.0
MCOLA BV (fwd primer)		5.0
HotStarTaq	QIAGEN	0.25

Table 2.9 1x Master mix for TCRBV sequencing using BigDye 3.1

Reagent	Distributor	Volume for 1x master mix (µL)
Sterile H2O		12.0
BigDye 3.1	Applied Biosystems (Foster City, CA)	1.0
5x Sequencing buffer	Applied Biosystems (Foster City, CA)	4.0

2.2 Methods

2.2.1 Preparation of cells and platelets

Isolation of PBMCs from blood buffy coats

Peripheral blood mononuclear cells (PBMCs) were isolated from blood buffy coats provided by the blood bank at the University hospital North Norway from donors giving written consent to the usage of their blood products for research purposes. The blood was diluted 1:1 in phosphate buffered saline (PBS) and layered on lymphoprep density gradient medium. After centrifugation at 800g for 15 minutes without brakes, the leukocyte interface was collected and suspended in PBS with 0.2 % bovine serum albumin (0.2 % PBSA) or 0.2 % human serum albumin (0.2 % PHSA). The cells were pelleted at 400g for 6 minutes to remove platelets (platelet-rich supernatant were used for isolating platelets, see below), washed twice in 0.2 % PBSA or 0.2 % PHSA and resuspended in appropriate cell culture medium.

Isolation of platelets

When needed, platelets were isolated from the supernatant collected after isolation and washing of PBMC (above) by layering platelet rich 0.2 % PBSA on top of a 1.047 g/mL gradient made of Optiprep medium and PBS and centrifuging at 400g for 10 minutes. The platelet interface was collected and washed in 0.2 % PBSA by centrifugation at 950g for 10 minutes.

Isolation of monocytes

Monocytes were isolated from PBMCs using Optiprep medium. A working solution (WS) was prepared by mixing 4 mL Optiprep medium with 2 mL IMDM medium supplemented with 10 % FBS. A 1.070 g/L density gradient was prepared from the working solution by mixing 2.1 mL WS with 5.0 mL IMDM 10 % FBS. PBMCs were suspended in 2.5 mL IMDM 10% FBS + 2.5 mL WS and cooled on ice. 5.0 mL 1.070 g/mL density gradient was layered on top of the PBMC suspension, and 0.5 mL IMDM 10 % FBS was layered on top of the 1.070 g/L gradient. The preparation was centrifuged at 700g for 30 minutes without brakes, and the top interface (Figure 2.1) containing monocytes was collected and washed twice in 0.2 % PBSA at 1400 rpm for 4 minutes.

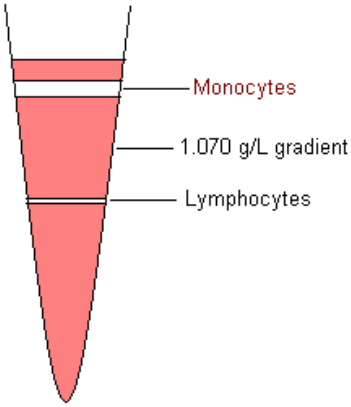

Figure 2.1 A 1.070 g/L density gradient made from Optiprep medium was used to enrich for monocytes.

Generation of B-lymphoblast cell line

The B lymphoblast cell line D4BL4 was generated in our laboratory by Epstein-Barr virus (EBV) transformation of IgM^- IgD^- $CD22^+$ (memory IgG^+ B cells) cells from an *HLA-DRB3*0101*-positive donor, as described by Ahlen *et al* (47).

2.2.2 Peptide stimulation

For studying the HPA-1a-specific T cell response, synthetic β3-integrin peptides spanning the L33/P33 polymorphism were used. 20-mer β3-integrin L33 peptide was used to resemble HPA-1a stimuli, while 20-mer β3-integrin P33 peptide was used to resemble the HPA-1b variant. As a negative control, 20-mer peptide derived from rye grass pollen protein, $LolP1_{191-210,}$ was used, as $LolP1_{191-210}$ has been experimentally shown to bind the HLA-DR52a molecule (45).

For control proliferation studies of T cells from cytomegalovirus (CMV)-positive donors, three different peptides derived from the CMV-associated protein pp65 were used. The selected peptides (Table 2.2) were chosen due to their ability to engage CD4 T cell responses (52).

2.2.3 CFSE proliferation assay

Carboxyfluorescein succinimidyl ester (CFSE) is an intracellular fluorescence dye used in cell proliferation assays to track cell divisions by flow cytometry (53). When incubated with cells, CFSE will penetrate the plasma membrane and bind cytosol components. When cell division takes place, CFSE bound components will be equally distributed to both daughter cells, leaving the

daughter cells with half the original amount of CFSE. Thus, when analyzing the cultured cells in flow cytometry, dividing cells will emit lower CFSE fluorescence than non-dividing cells, with a decrease CFSE amount proportional to the number of cell divisions.

PBMCs were washed in 0.2 % PBSA or 0.2 % PHSA and stained with CFSE at 10 μg/mL in 1 mL 0.1 % PBSA or 0.1 % PHSA at 37 °C for 10 minutes. 5 mL ice-cold culture medium was added, followed by 5 minutes incubation on wet ice to stop the staining process. Cells were pelleted at 300 g for 4 minutes, washed three times in 0.2 % PBSA or 0.2 % PHSA and resuspended in culture medium.

Cells were seeded out in 24 well plates at a density of $5 \cdot 10^6$ cells per well and stimulated with 1 μM peptide or platelets (10^7 platelets per well) using HPA-1bb platelets as negative control. Cells were cultured for enrichment of antigen-specific cells at 37 °C in 7.5 % CO_2 and atmospheric or 5 % O_2. Cells were cultured in IMDM medium supplemented with either 10 % FBS + 4 % human serum and Pen-Strep or 10 % human serum and Pen-Strep. The T cell growth factor interleukin (IL)-2 was added on days 5 and 10 (10 U/mL), and cells were analyzed by flow cytometry for proliferation and expression of selected surface markers by staining with fluorochrome-conjugated mAbs (Table 2.5). Single proliferating CD4 T cells were then sorted with the FACSAria (Becton Dickinson, NJ, USA) fluorescence activated cell sorter (FACS).

2.2.4 Flow cytometry

Flow cytometry is a fluidic- and laser-based method used for multiparameter analysis of single cells in suspension. Parameters measured can be cell size and complexity, as well as expression of various molecules – both extracellular and intracellular – with the use of fluorochrome-conjugated antibodies. In addition, cell function and responses can be measured by flow cytometry, using a number of different protocols and reagents (e.g. the CFSE proliferation assay described above).

Data collected from each cell, which is up to ten different parameters in some of the experiments performed in this study, are analyzed consecutively in two-dimension plots where each cell is represented by a coordinate. Usually, 10,000 or more cells (events) are analyzed simultaneously in a single plot.

For flow cytometric analysis of cultured cells, cells were washed and resuspended in IMDM 10 % FBS in 4 mL Falcon tubes (Becton Dickinson, NJ, USA) and incubated with fluorochrome-conjugated monoclonal antibodies (Table 2.5) in a final staining volume of 100 µl for 10 minutes in room temperature protected from light. When biotinylated mAbs were used, cells were stained with biotinylated mAbs for 10 minutes at room temperature in 100 µl IMDM 10 % FBS, washed and incubated with fluorochrome-conjugated streptavidin for 10 minutes at room temperature in 100 µl IMDM 10 % FBS protected from light. Stained cells were finally washed in 4 mL PBS 0.2 % BSA and resuspended in ~300 µl 0.2 % PBSA for flow cytometric analysis.

2.2.5 Fluorescent activated cell sorting

In this study the FACSAria (Becton Dickinson) fluorescent activated cell sorter (FACS) was used to sort single proliferating T cells from peptide- or platelet-stimulated donor PBMCs. The FACS method is based on the principle of flow cytometry, with the option to isolate cells from chosen populations visualized by fluorochrome-conjugated monoclonal antibodies. When sorting cells, the cells are passing a laser, one by one, in a thin fluid stream. After passing the laser, a vibrating nozzle releases the fluid stream in open air and breaks up the fluid stream to droplets containing a single cell to be sorted. Each droplet receives an electric charge at the break-off-point from the fluid stream. The charged droplets pass through an electric field and are diverted into a designated container, e.g. a centrifuge tube or a 96-well plate.

2.2.6 Expansion of T cell clones

Single FACS-sorted CD4 T cell clones were expanded in round bottom 96 well plates (NUNC, Roskilde, Denmark) in IMDM 10 % FBS, 4 % HS (HPA-1bb) supplemented with Penicillin-Streptomycin and purified monoclonal anti-CD3 (30 ng/mL). Irradiated D4BL4 B-lymphoblasts (2,500 cells/well) and irradiated PBMCs (20,000 cells/well) were used as feeder cells. Recombinant IL-2 (50 units/mL) was added at 4 day intervals, starting at day 1 of stimulation.

Clonal HPA-1a specific T cells were expanded in flat bottom 24 well plates (NUNC) in IMDM 10 % FBS, 4 % HS, PenStrep. T cells ($5 \cdot 10^5$ cells/well) were expanded with purified monoclonal anti-CD3 (30 ng/mL), IL-15 (5 ng/mL) using irradiated B-lymphoblasts (10^6 cells/well) and irradiated PBMCs ($4 \cdot 10^6$ cells/well) as feeder cells. IL-2 (50 U/mL) was added at 4 day intervals, starting at day 1 of stimulation. Cells were washed to remove anti-CD3 on day 7. Cells were expanded for 14 days before they were used in other assays.

2.2.7 ELISPOT assay

The Enzyme-linked immunosorbent spot (ELISPOT) assay is a highly sensitive assay for detecting cytokine producing cells. In this study the ELISPOT assay was used to detect antigen-specific T cell clones based on cytokine secretion after stimulation with HPA-1a antigen.

MultiScreen HTS 96-well plates with nitrocellulose membrane (Millipore, Billerica, MA) were prepared with 30 µl 35 % Ethanol per well, washed three times in 200 µl PBS and coated with purified IFNγ or IL-4 monoclonal capture antibodies (10 µg/mL in 25 µl PBS per well). After incubation at 4 °C for 20 hours or longer, the mAb solution was decanted and the membranes were blocked with IMDM 10 % FBS 4 % Human HPA-1bb serum (HS), 200 µl per well at 37 °C for 2 hours.

Clonal T cells were added at a density of 1000-2000 cells/well in ~50 µl IMDM 10 % FBS 4 % HS with L33 peptide (5 µM) pulsed D4BL4 cells (50,000 cells per well) or autologous monocytes (50,000 cells per well) pulsed with HPA-1a^{+} platelets. D4BL4 lymphoblasts pulsed with LolP1 peptide or non-pulsed autologous monocytes were used as controls. After incubation over night at 37 °C, the cell suspensions were decanted and the wells were washed six times with 200 µl 0.01 % Tween20 in PBS and once in 200 µl PBS. Secondary biotinylated anti-IFNγ or anti-IL-4 antibodies were added (1 µg/mL in 50 µl 0.5 % FBS in PBS) and the plates were incubated at 37 °C for 2 hours. The wells were then washed 6 times in 0.01 % Tween20 in PBS and once in 200 µl PBS. Streptavidin-alkaline phosphatase conjugate was diluted 1:10,000 in PBS and 50 µl was added per well. After incubating in room temperature for 45 minutes, wells were washed 3 times with 200 µl 0.01 % Tween20 in PBS and 4 times in 200 µl PBS. After washing, 50 µl of the colourless alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP-NBT) was added to each well and incubated for 10 minutes protected from light. The substrate is cleaved by alkaline phosphatase to yield a coloured product that precipitates on the nitrocellulose membrane in concentric spots that correspond to cytokine secreting cells (Figure 2.2).

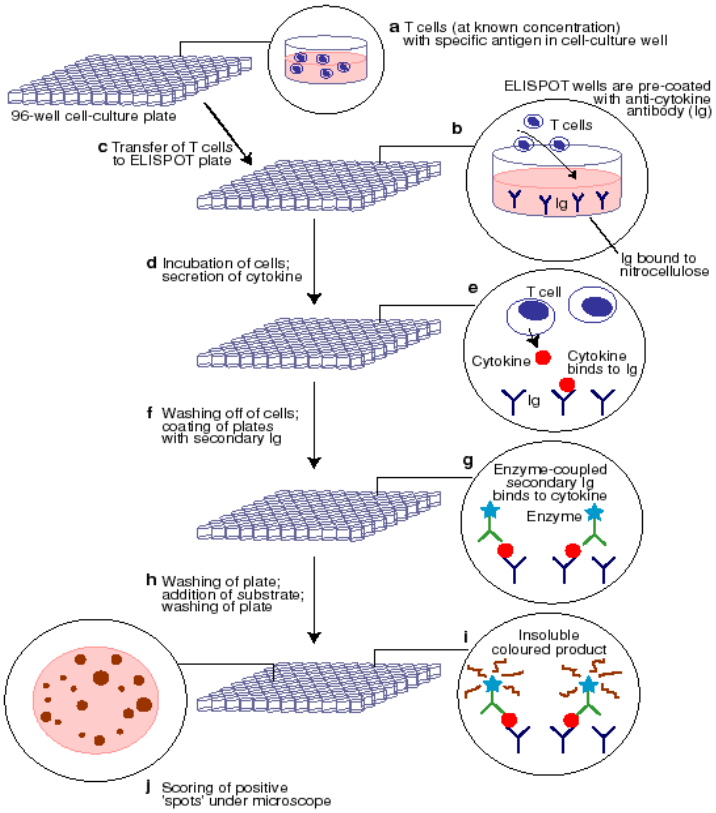

Figure 2.2 Steps of the Enzyme-Linked Immunosorbent SPOT (ELISPOT) assay. Figure from Hickling, J.K, 1998 (54).

2.2.8 Intracellular cytokine staining

The intracellular cytokine staining-assay allows the detection of cytokine secreting cells with the use of flow cytometry. In this assay, cells are stimulated with antigen and reacted with Brefeldin-A to block the secretory pathway of the cell, resulting in an intracellular accumulation of the cytokines produced in response to activation. Cells are then fixed in 4 % paraformaldehyde buffer and permeabilized with a saponin buffer to allow entry of cytokine-specific, fluorochrome-conjugated monoclonal antibodies before flow cytrometic analysis.

Intracellular cytokine staining of isolated T cell clones was performed to determine and confirm HPA-1a specificity:

10^5 clonal T cells were labelled with CFSE to distinguish them from the peptide-pulsed B lymphoblasts used as antigen presenting cells. 10^5 HLA-DRB3*0101-positive B-lymphoblasts (D4BL4) were pulsed with L33 or LolP1 20-mer peptide for 3.5 hours at 37 °C in IMDM

(supplemented with 10 % FBS, 4 % HS and pen-strep) with 10 μM AdEtOH as a peptide loading enhancer.

After pulsing, D4BL4 cells were irradiated for 800 s (receiving 12000 RAD) using a radioactive caesium source. Irradiated, peptide-pulsed D4BL4 cells were incubated with T cell clones in 100 μL T cell medium with 10 μM Brefeldin-A over night at 37 °C, 7,5 % CO_2 in 96-well round bottom cell culturing plates (NUNC).

Cells were pelleted by centrifugation of the plates at 800g for 5 minutes and the supernatant was decanted. Cells were fixed in 100 μl fixation buffer (4 % paraformaldehyde) for 10 minutes at room temperature and washed once in PBS 0.2 % BSA before permeabilization with 100 μl saponin-buffer for 10 minutes at room temperature. Cells were pelleted by centrifugation and the supernatant was discarded. Cells were stained with 0,5 μl APC-conjugated anti-IFNγ in 20 μl permeabilisation buffer per well, and after 10 minutes incubation in room temperature protected from light, cells were washed in 200 μl permeabilisation buffer and resuspended in 50 μl permeabilisation buffer + 150 μl PBS 0.2 % BSA for flow cytometry.

Lymphocytes were gated using side-scatter and forward-scatter parameters, and $CFSE^{hi}$ cells (T cell clones) were analysed for IFNγ expression (mean APC fluorescence intensity).

2.2.9 TCRBV analysis

Total RNA was isolated from T cell lysates using RNeasy Mini Kit (QIAGEN), and cDNA was synthesized from RNA by reverse transcription using SuperScript III Reverse Transcriptase. TCRBV family genes were determined by RQ-PCR and TCRBV genes were amplified by Polymerase Chain Reaction (PCR) (55) and sequenced as follows:

RNA extraction from T cells

Cells were washed twice in 1 ml PBS 0.3 % Ethylenediaminetetraacetic acid (EDTA) 0.1 % Diethylpyrocarbonate (DEPC) by sentrifugation at 6,000 g for 2 minutes. Cells were resuspended in 350 μl lysis buffer (buffer RLT, RN Easy MiniKit, Qiagen) with β-mercaptoethanol and lysed by vortexing for 30 seconds before addition of an equal volume of 70 % Ethanol.

The suspension was transferred to an RNeasy Spin Column (QIAGEN) placed in a collection tube. After centrifugation at 16,000 g for 30 seconds, flow through was discarded and 700 µl wash buffer RW1 (RNeasy Mini Kit) was added followed by centrifugation at 16,000 g, 30 seconds and flow through was discarded. 500 µl wash buffer RPE (RNeasy Mini Kit) was added. After centrifugation (16,000 g, 30 seconds), the RNeasy Spin Column was transferred to a new collection tube and 500 µl buffer RPE was added follwed by centrifugation (16,000 g, 30 seconds) and the spin column was placed into a RNAse free 1.5 mL eppendorf tube and centrifuged at 16,000 g, 30 seconds. 50 µl RNAse free H_2O was added to the membrane and after 3 minutes incubation, the tube was centrifuged at 16,000 g for 30 seconds, and the RNA was used further for cDNA synthesis by reverse transcription.

cDNA synthesis from total RNA

The RNA eluate was incubated at 70 °C for 10 minutes followed by 10 minute incubation on ice. 11 µl SuperScript III master mix (Table 2.7) was added and cDNA synthesis was performed by the following program using a PCR thermal cycler: 22 °C 10 minutes, 42 °C 45 minutes, 99 °C 3 minutes and 4 °C termination. Synthesized cDNA was eluted in sterile H_2O to 300 µL for Real time PCR.

Quantitative Real time PCR

Quantitative Real time polymerase chain reaction (RQ-PCR) was performed using 5' primers for all known functional genes encoding the variable region of the TCR β-chain (TCRBV) and a common 3' primer (BC63 or Aka-TCRBC) for a specific sequence located in the β chain constant region (Table 2.6) using QuantiTect SYBR green PCR kit (QIAGEN) to detect the amplified PCR product. Primers amplifying a segment of the gene encoding the cytoskeleton molecule actin were used as PCR control, since actin is constitutively expressed by most cells. β-chain constant region primers were used as an internal control for amplification of TCR genes (Table 2.6).

The following reactions were set up in 96 well MicroAmp plates (Applied Biosystems):

Table 2.10 RT-PCR reactions for TCRBV analysis

Reagent	Volume (µL)	Final
Quantitect PCR SYBR mix (2x)	12.5	1x
Forward BV primer (1 µM)	5.0	200 nM
Common reverse primer BV63 (1 µM)	5.0	200 nM
cDNA	2.5	
Total volume	25.0	

The following cycles were run on ABIPrism 7900HT (Applied Biosystems) and data was analyzed using the SDS 2.2.2 software (Applied Biosystems):

1 cycle	95 °C
50 cycles	95 °C
	58 °C
	72 °C
1 cycle	95 °C
	60 °C
	95 °C

After determination of the TCRBV gene family used by the given T cell clone, TCRB PCR reactions were set up using the forward primer for the given TCRBV gene to amplify TCRBV cDNA for sequence analysis. PCR reaction was set up in two parallels in 0.2 mL PCR tubes with one non-template control (NTC) using water instead of cDNA.

22.5 µL 1x master mix (table 2.8) and 2.5 µL cDNA were mixed and PCR was performed by the following cycles:

1 cycle	95 °C	15 minutes
50 cycles	94 °C	30 seconds
	58 °C	30 seconds
	72 °C	15 seconds
1 cycle	72 °C	10 minutes
End	4 °C	∞

Sequencing reactions were set up by mixing 17 µl BigDye 3.1 master mix (Table 2.9), 1 µl primer (forward or reverse primer) and 2 µl PCR product. The reverse primer and forward primers were used in separate reactions. The gene products were amplified by PCR with the following cycles:

1 cycle	95 °C	10 seconds
1 cycle	58 °C	20 seconds
	72 °C	30 seconds
20 cycles	94 °C	15 seconds
	55 °C	20 seconds
	72 °C	30 seconds
1 cycle	72 °C	10 minutes
End	4 °C	∞

Sequencing was performed by personnel at the sequencing facility at the University Hospital North Norway, by Sanger chain termination DNA sequencing (56) using BigDye 3.1 enzyme and nucleotide mix (Applied Biosystems).

2.2.10 Phenotyping of HPA-1a specific T cells using monoclonal antibodies

To determine if HPA-1a T cell clones display a phenotype distinguishable from other populations of proliferating cells, an extensive monoclonal antibody-panel to various activation-, subset-, and homing markers was designed and tested on clones and various control cell populations. Known surface markers for T cell activation, T cell subsets and tissue homing (Table 2.11) were chosen. Monoclonal anti-CD3 and anti-CD4 were included in all panels for gating purposes.

Table 2.11 Surface markers included in phenotyping of HPA-1a specific T cell clones and controls

Activation markers	CD25	CD56	HLA-DR	CD69	
Subset markers	CCR7	CD45RA	CD62L	CD38	CD27
Tissue homing markers	CD49d	CLA	β7-integrin	CD29	

To compare the phenotype of HPA-1a specific T cells to control populations of cells that may be present as background in CFSE proliferation assays, the following control populations were also phenotyped: $CD3^+CD4^+$ untreated PBMCs from a random blood donor, proliferating $CD3^+CD4^+$ cells from a random HLA-DRB3*0101-positive donor studied in a CFSE proliferation assay with HPA-1a antigen stimuli and proliferating $CD3^+CD4^+$ cells from two HPA-1a immunized donors studied with in a CFSE proliferation assay with HPA-1a antigen.

2.2.11 Surface TNF assay

In response to stimulation through the TCR, T cells secrete the cytokine TNF. TNF is synthesized as a 26-kDa protein first inserted to the plasma membrane immediately followed by an Ala^{76}-Val^{77} cleavage of the extracellular domain releasing the secreted 17 kDa TNF molecule, catalysed by the metalloprotease TNFα converting enzyme (TACE), also known as ADAM-17 (57).

Inhibition of TACE using the TACE inhibitor TAPI-0, allows the detection of TNF on the surface of activated T cells by flow cytometry. This has already been done effective in studies of antigen-specific CD8 T cells (unpublished data by Haney and Betts *et al* (58;59)), and here we tested if TACE inhibition to detect TNF producing cells is also applicable to CD4 T cells. Protocols for this assay were generously provided by (Danielle Haney at the University of Pennsylvania).

In order to test if TACE inhibition allowed the detection of TNF producing CD4 T cells, HPA-1a specific T cell clones were stimulated with L33 peptide and LolP1 control peptide in the presence of TAPI-0 (10 μM) and 1.5 μl PE-conjugated anti-TNF in 200 μl culture medium in 15 mL conical polyethylene tubes. After 4.5 hours of incubation at 37 °C, cells were washed in 15 mL culture medium and once in 15 mL PBS 0.2 % BSA. Cells were resuspended in ~300 μl PBS 0.2 % BSA and transferred to 4 mL FACS tubes for flow cytometric analysis.

For incorporation of the surface TNF assay in the sorting of single proliferating cells in the CFSE proliferation assay (section 2.2.3), cultured donor PBMCs were restimulated with peptide or platelets in the presence of TAPI-0 (10 μM) and anti-TNF for 4.5 hours before staining with fluorochrome-conjugated antibodies (anti-CD4, anti-CD8, anti-CD14) for FACS analysis and single cell sorting of proliferating TNF-producing CD4 T cells.

3. Results

3.1 Stimulation with HPA-1a positive platelets resulted in down-regulation of CD4 co-receptor expression on proliferating T cells

To determine if HPA-1a-specific CD4 T cells should be isolated from proliferating cells following a single stimulation with antigen, $5 \cdot 10^6$ peripheral blood mononuclear cells (PBMC) from a previously immunized donor (Donor 8) who gave birth to a child with NAIT were labelled with CFSE and cultured in two parallels with HPA-1aa platelets in 24-well culture plates for 14 days. HPA-1bb platelets were used as negative control:

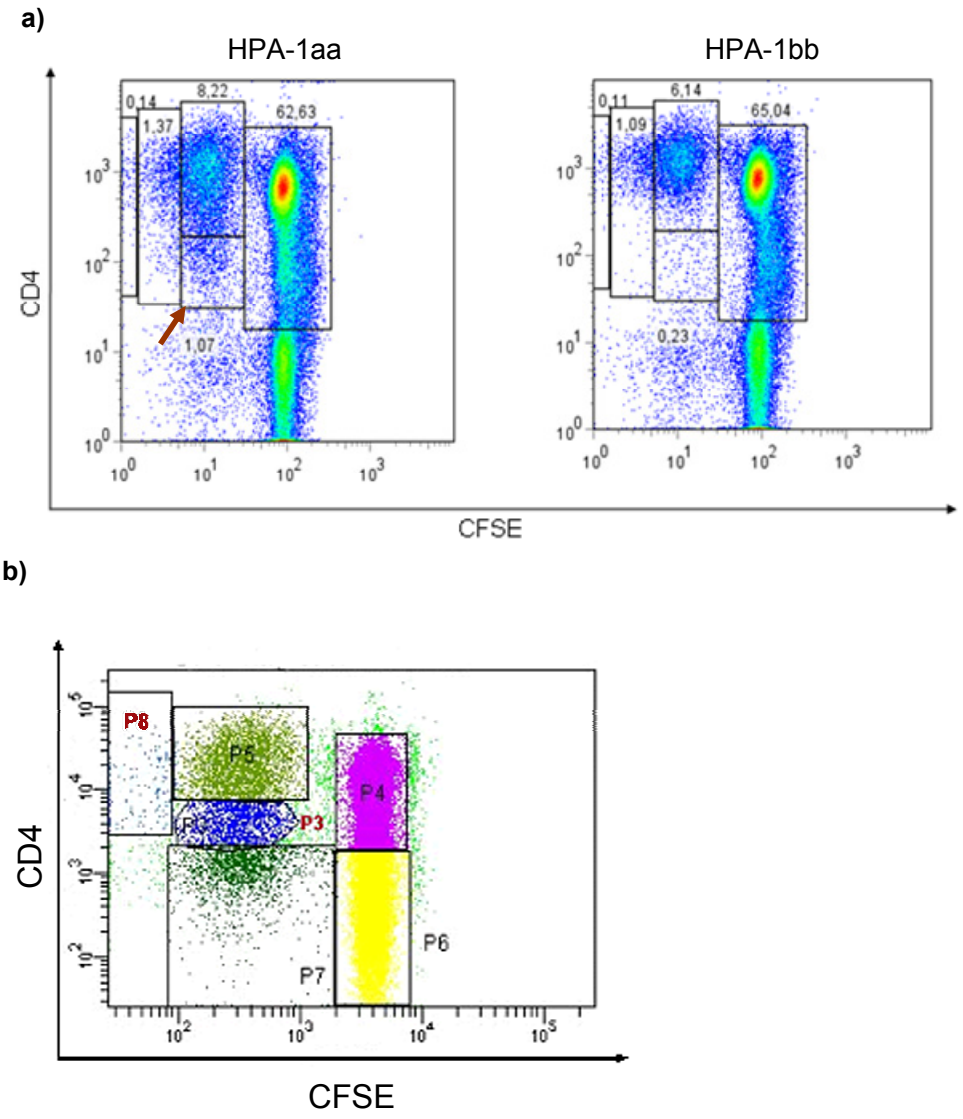

Figure 3.1 a) CFSE Proliferation assay of Donor 8 PBMCs performed on day 14 after a single stimulation with HPA-$1a^+$ platelets and HPA-$1a^-$ platelets. Proliferating cells with down-regulated surface CD4 expression are indicated (arrow). b) Single cells were sorted from gates P8 and P3 with FACSAria and expanded in culture.

A marked down-regulation of CD4 cell surface expression was observed in a subpopulation of cells proliferating in response to HPA-1a-positive platelets (Figure 3.1 a). Single cells were sorted using FACSAria from two populations of proliferating CD4 T cells (Figure 3.1 b); one population of high proliferation, and one population of slight lower proliferation, with marked CD4 down regulation.

3.2 HPA-1a-specific T cell clones can be isolated from a population of CD4-down-regulated T cells

Single sorted CD4 T cells were expanded in culture and proliferating clones were tested for HPA-1a-specificity by IFNγ and IL-4 ELISPOT. Clones secreting cytokines in response to HPA-1a antigen and not to control antigen were defined as specifically activated by HPA-1a.

Regions P8 and P3 (Figure 3.1 b) gave rise to 50 and 30 proliferating clones, respectively. Of the 50 clones from the region of higher proliferation (P8), no clones showed specificity to HPA-1a antigen. Of the 30 clones originating from the P3 region of proliferating cells with down regulated CD4 expression, 3 clones were specific to HPA-1a (Figure 3.2).

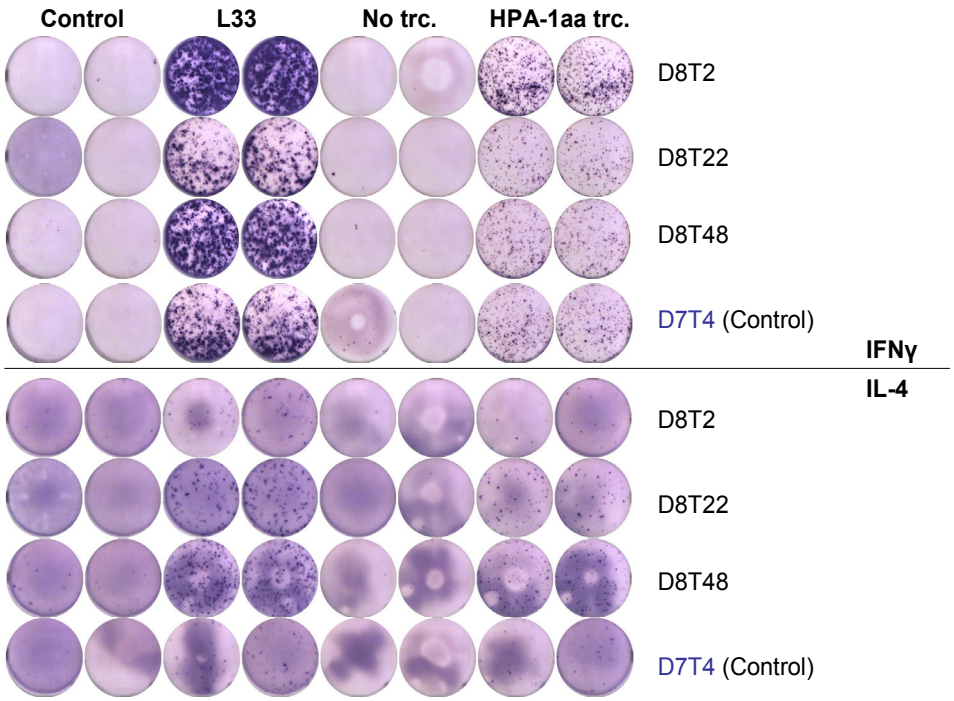

Figure 3.2 HPA-1a-specificity of proliferating T cell clones was determined by the ELISPOT assay detecting IFNγ (top) and IL-4 (bottom) secretion in response to HPA-1a antigen stimuli. Clone D7T4 used as positive control has previously been isolated in our laboratory and published (47).

The IFNγ- and IL-4-secretion in response to HPA-1a platelets and HPA-1a peptide only, confirms the specificity of these clones to the HPA-1a antigen. To determine the clonality of the isolated HPA-1a-specific T cell clones, sequencing of the variable region of the T cell receptor β-chain (TCRBV-sequencing) was performed. Sequences were analyzed using Sequence Scanner v1.0 (Applied Biosystems) and the IMGT/V-quest software available at http://www.imgt.org (60). Analyzed sequences were aligned in BioEdit 7.0.3 (61). As shown in figures 3.3 and 3.4, the TCRBV region of clones D8T2 and D8T22 were identical, while the D8T48 clone was a unique clone. Figure 3.5 shows a schematic illustration of the predicted CDR regions of the T cell receptor, which are the domains making contact with MHC:peptide, with the CDR3 domain being the most variable motif.

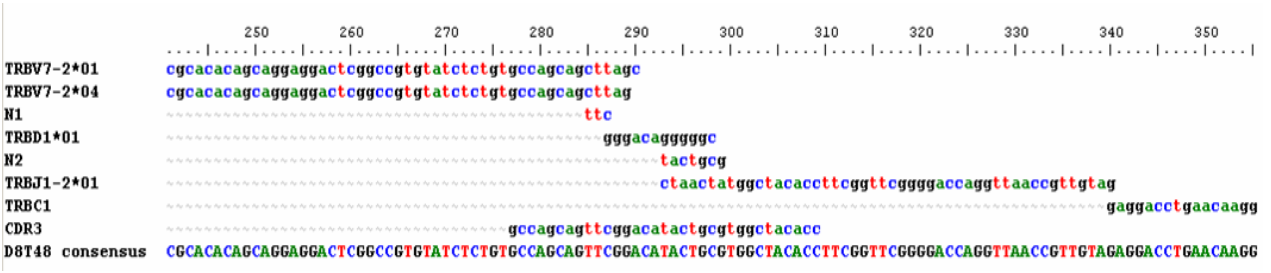

Figure 3.3 Sequence alignment of D8T48 TCR encoding genes.

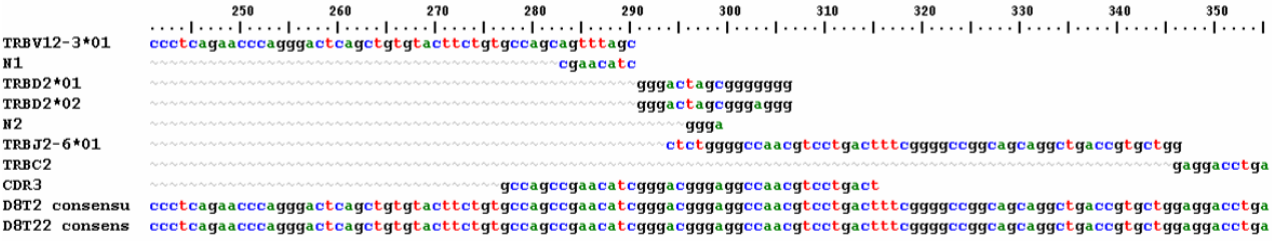

Figure 3.4 Sequence alignment of D8T2 and D8T22 TCR encoding genes.

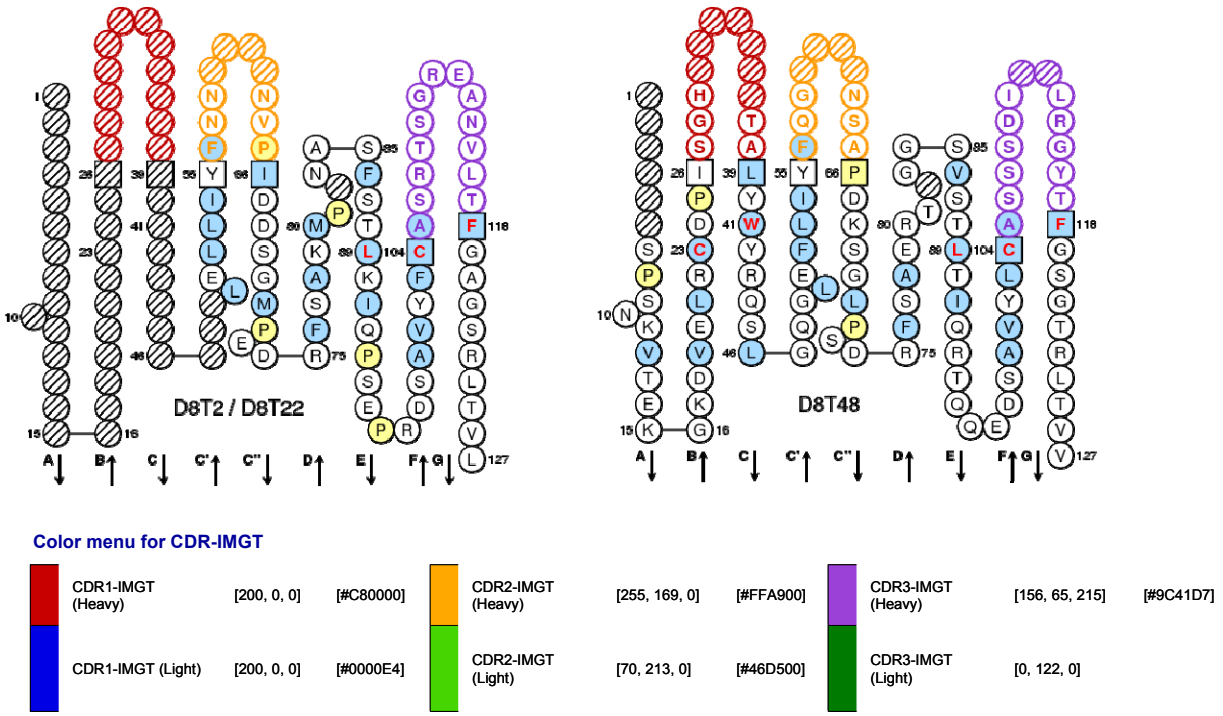

Figure 3.5 Schematic visualizations of the proposed amino acid sequences of the T cell receptor CDR regions reveal the CDR3 regions (purple domain) of the D8T2 / D8T22 and D8T48 T cell receptor. This analysis was performed using the IMGT/Collier-de-Perles tool available at http://www.imgt.org. IMGT unique numbering for V-domains determines the lengths of CDR-IMGT (62).

Table 3.1 Summary of TRBV rearrangements for T cell receptors of clonal cell lines, using IMGT nomenclature (63)

	***TCRB* rearrangements**				**CDR3**	
HPA-1a-specific clone	***TRBV***	***TRBD***	***TRBJ***	***TRBC***	**Length**	**Amino acid sequence**
D8T2	12-3*01	2*01 or 2*02	2-6*01	2	13	ASRTSGREANVLT
D8T22	12-3*01	2*01 or 2*02	2-6*01	2	13	ASRTSGREANVLT
D8T48	7-2*01 or 7-2*04	1*01	1-2*01	1	11	ASSSDILRGYT

The specificity of the T cell clones to HPA-1a was also confirmed by intracellular cytokine staining measuring the production of IFNγ (as APC fluorescence intensity) in response to HPA-1a antigen stimuli (Figure 3.6). These findings demonstrate that HPA-1a-specific T cells can be isolated following a single stimulation of PBMCs from immunized donors, and CD4 down-regulation can be used as a marker for identification of antigen-specific CD4 T cells.

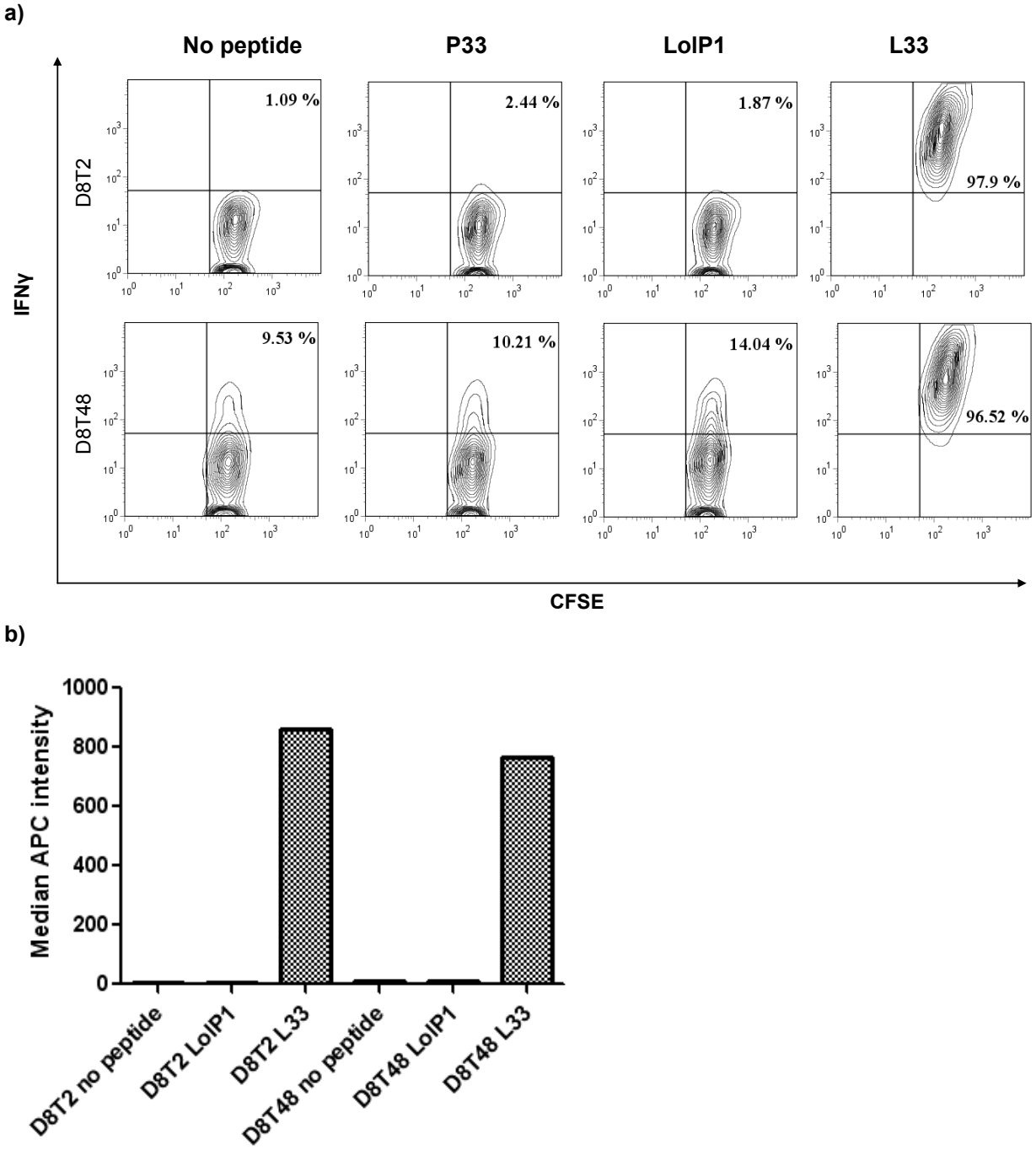

Figure 3.6 Intracellular cytokine staining measuring IFNγ production (as APC fluorescence intensity) in response to peptide stimuli confirmed the specificity of T cell clones D8T2 and D8T48 to HPA-1a antigen.

Table 3.2 Summary of HPA-1a-specific CD4 T cell clones isolated from immunized donor # 8

Stimuli	Population	Proliferating clones	HPA-1a specific clones
HPA-1a trc	CFSE low, CD4 high	50	0
HPA-1a trc	CFSE low, CD4 low	30	3 (10 %)

3.3 Modifications of the composition of cell culture medium resulted in a higher proportion of proliferating T cells in cultures stimulated with specific antigen compared to control cultures

Since many of the proliferating T cells were not HPA-1a-specific (only 3 clones out of 30 from the $CFSE^{low}$, $CD4^{low}$ population), various culture conditions were tested to determine if background proliferation of T cells with irrelevant specificities could be reduced.

In order to optimize conditions for enrichment of antigen-specific CD4 T cells *in vitro*, and considering the relatively small number of valuable patient samples, a larger source of PBMCs with specific T cell responses against a known antigen was needed. Since many people (around 40 %) are infected with cytomegalovirus (CMV) and commonly targeted T cell epitopes in the CMV protein pp65 are known, PBMCs from random blood donors were screened using pp65 peptide epitopes to identify donors who had such responses. CMV-positive donors were identified by assaying donor PBMCs by IFNγ ELISPOT (Figure 3.7). Donors with cells secreting IFNγ in response to CMV derived peptides only were defined as CMV-positive. CFSE proliferation assays were performed on PBMCs from these donors.

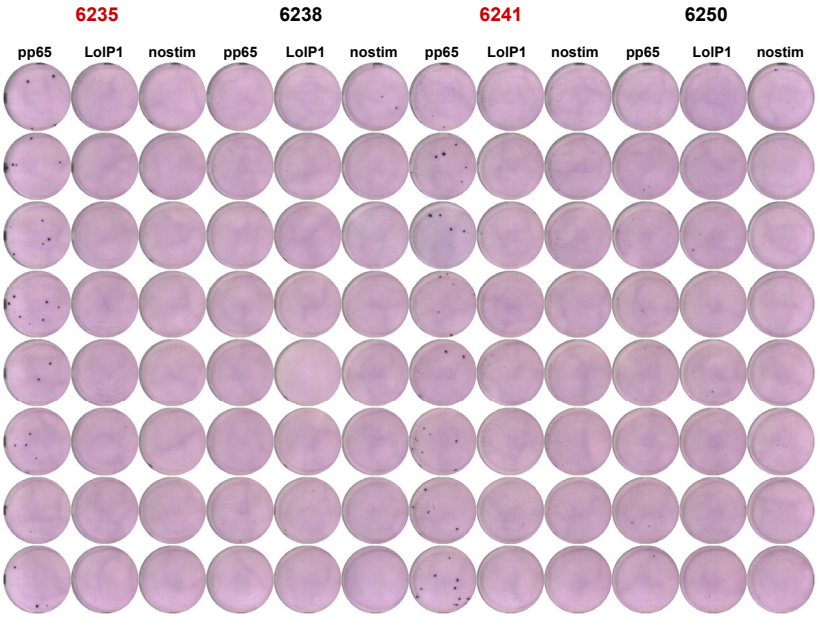

Figure 3.7 IFNγ ELISPOTS of PBMCs ($3 \cdot 10^5$ cells/well) from random blood donors stimulated with pp65 derived peptides at 3 μM for 3 days. Donors 6235 and 6241 were defined as CMV-positive based on their release of IFNγ in response to pp65 peptide only. Each column represents eight replicates of the given stimuli.

PBMCs from CMV-positive donors 6235 and 6241 were labelled with CFSE and stimulated with a peptide pool of the CMV pp65-derived peptides $pp65_{41-55}$, $pp65_{281-295}$ and $pp65_{485-499}$ at a final peptide concentration of 3 μM. Cells were grown for 14 days in culture under different culturing conditions (Table 3.3). Recombinant IL-2 (10 U/mL) was added to the cultures on days 5, 8 and 11 and analyzed for proliferation by flow cytometry on days 3, 6 and 14 (Table 3.4, Figure 3.8). The difference in size between CMV peptide-stimulated and control stimulated proliferating populations was interpreted as antigen-specific responses.

Table 3.3 Variations in serum- and oxygen content were compared to test determine optimal conditions for enrichment of antigen-specific T cells

Conditions	Medium	Serum	O_2 level	CO_2 level
1	IMDM	10 % FBS 4 % Human serum	Atmospheric	7.5 %
2	IMDM	10 % Human serum	Atmospheric	7.5 %
3	IMDM	10 % FBS 4 % Human serum	5 %	7.5 %
4	IMDM	10 % Human serum	5 %	7.5 %

Table 3.4 Proportion of proliferating CD4 T cells in response to CMV antigen (pp65 peptide pool) compared to non-stimulated controls (no peptide). Data are collected from CFSE proliferation assay performed on days 3, 6 and 14

				Proliferating CD4 T cells (%)		
Day	Serum	Oxygen	Donor	no peptide*	pp65 peptide pool*	Fold increase**
3	10 % FBS, 4 % HS	Atmospheric	6235	1,03	1,04	1.0
			6241	0,68	0,72	1.1
		5 %	6235	1,16	1,06	0.9
			6241	1,16	1,10	0.9
	10 % HS	Atmospheric	6235	1,28	1,02	0.8
			6241	0,68	0,58	0.9
		5 %	6235	1,02	1,15	1.1
			6241	0,62	0,98	1.6
6	10 % FBS, 4 % HS	Atmospheric	6235	2,37	5,89	2.5
			6241	1,31	1,83	1.4
		5 %	6235	2,96	3,69	1.2
			6241	1,26	2,66	2.1
	10 % HS	Atmospheric	6235	1,27	2,78	2.2
			6241	0,99	2,13	2.2
		5 %	6235	1,47	1,45	1.0
			6241	1,03	1,55	1.5
14	10 % FBS, 4 % HS	Atmospheric	6235	95,88	91,94	1.0
			6241	89,94	93,50	1.0
		5 %	6235	86,69	85,19	1.0
			6241	82,05	84,06	1.0
	10 % HS	Atmospheric	6235	30,01	76,28	2.5
			6241	50,35	93,57	1.9
		5 %	6235	29,10	25,51	0.9
			6241	55,82	88,46	1.6

* Numbers indicate percent proliferating CD4 T cells of the total population of CD3 positive cells assayed.

** Fold increase of CD4 T cells proliferating at various days after stimulation with CMV peptide relative to negative control.

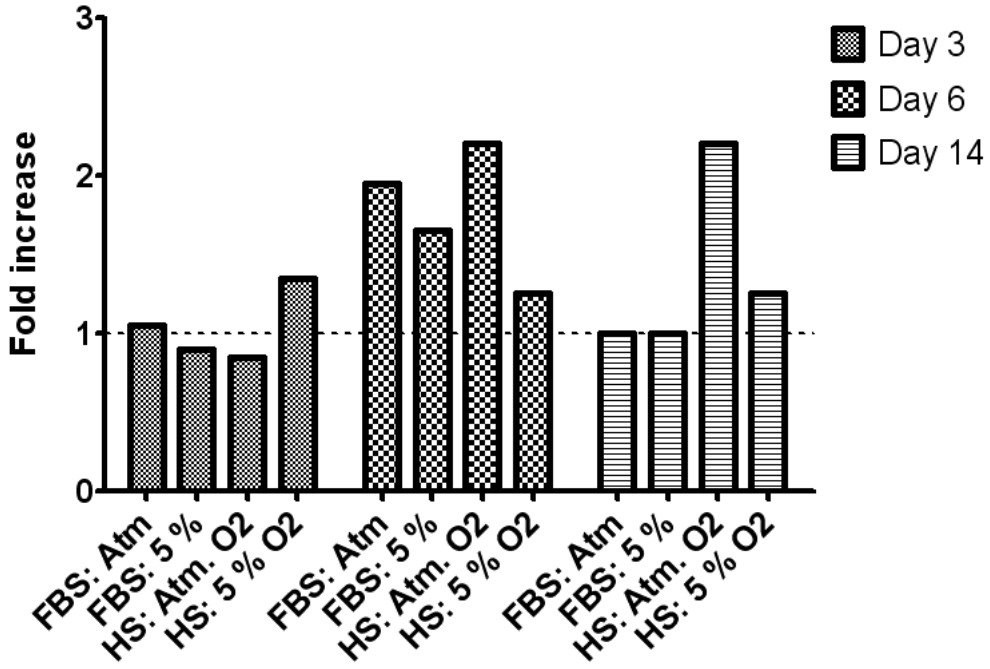

Figure 3.8 Fold increase of proliferating CD4 T cells (% of total CD3 cells) in response to pp65 peptide stimuli relative to negative control (no stimuli) for different enrichment conditions at day 3, 6 and 14 in CMV positive blood donors. A fold increase of 1.0 (indicated by a horizontal line) refers to no increased proliferation in pp65-stimulated cultures relative to the control.

The findings from these experiments show that replacing FBS with human serum reduced proliferation of T cells with irrelevant specificities (an increased difference between "no peptide" and pp65 peptide was observed) and this difference was most prominent on day 6 after stimulation. No effect was observed by reducing O_2 levels.

3.4 No obvious phenotypic differences were detected when comparing non specific/spontaneous proliferating T cells with antigen-specific T cell clones

Since CD4 down-regulation was found to be a potentially useful marker for identification of cells responding to specific antigen, a panel of monoclonal antibodies was used to determine if there were other phenotypic changes that could be helpful in distinguishing antigen-specific T cells from the proliferating T cells with irrelevant specificities.

Table 3.5* HPA-1a specific T cell (D8T2 and D8T48) phenotype compared to phenotype of $CD3^+CD4^+$ cells from random PBMCs and proliferating $CD3^+CD4^+$ cells from random donor (T42) PBMCs after stimulation with HPA-1a L33 peptide and LolP1 control peptide. Random donor T42 expresses the MHC class II molecule HLA-DRB3*0101, which is able to bind and present HPA-1a L33 peptide. Values represent median fluorescence intensity measured by Flow Cytometry

	D8T2	D8T48	PBMCs	T42 nostim	T42 LolP1	T42 L33
CD25	1077.61	346.52	180.58	6005.00	8370.00	9977.29
CD56	1297.26	1293.18	227.07	298.79	335.67	364.25
HLA-DR	17060.98	23075.62	153.26	2856.54	4541.25	3881.19
CD69	3595.29	1961.88	45.88	1136.38	1193.51	965.95
CCR7	67.42	75.93	37.57	49.45	50.46	48.07
CD45RA	184.92	170.42	417.96	205.59	175.38	213.65
CD62L	53.71	43.51	10869.68	405.86	385.61	325.57
CD38	656.77	772.88	182.27	9515.62	7408.27	3703.52
CD27	3.01	3.01	1328.69	587.93	349.46	321.10
CD49d	2127.50	6525.67	676.40	7067.21	8139.81	5891.55
CLA	636.48	948.35	272.41	7068.85	8137.58	5890.29
β7-integrin	31515.50	43161.62	686.95	19849.95	18645.69	18311.55
CD29	14338.79	11740.90	1610.80	5210.36	6734.42	4708.33

Table 3.6* HPA-1a specific T cell (D8T2 and D8T48) phenotype compared to phenotype of proliferating $CD3^+CD4^+$ PBMCs from HPA-1a immunized donor 18 after stimulation with HPA-1a platelets or L33 peptide and HPA-1b / LolP1 controls. Values represent median fluorescence intensity measured by Flow Cytometry

	D8T2	D8T48	D18 HPA-1bb	D18 HPA-1aa	D18 LolP1	D18 L33
CD25	1077.61	346.52	54.05	53.80	12930.41	12825.25
CD56	1297.26	1293.18	631.57	616.43	620.94	555.64
HLA-DR	17060.98	23075.62	1299.04	1446.80	1277.44	1734.54
CD69	3595.29	1961.88	126.68	140.86	129.79	116.23
CCR7	67.42	75.93	45.29	49.06	52.47	51.23
CD45RA	184.92	170.42	347.38	351.51	368.53	349.14
CD62L	53.71	43.51	2120.23	1707.06	1682.05	2366.39
CD38	656.77	772.88	7010.90	6490.56	8973.65	8796.80
CD27	3.01	3.01	431.31	537.70	300.78	394.97
CD49d	2127.50	6525.67	4104.34	5993.64	5935.54	5918.98
CLA	636.48	948.35	878.32	1070.06	1040.80	1124.31
β7-integrin	31515.50	43161.62	12380.35	13724.48	14364.02	11907.35
CD29	14338.79	11740.90	981.14	1437.02	1277.31	1105.73

Table 3.7* HPA-1a specific T cell (D8T2 and D8T48) phenotype compared to phenotype of proliferating $CD3^+CD4^+$ PBMCs from HPA-1a immunized donor 19 after stimulation with HPA-1a platelets or L33 peptide and HPA-1b / LolP1 controls. Values represent median fluorescence intensity measured by Flow Cytometry

	D8T2	D8T48	D19 HPA-1bb	D19 HPA-1aa	D19 LolP1	D19 L33
CD25	1077.61	346.52	13181.55	11842.93	13120.15	11447.17
CD56	1297.26	1293.18	665.13	633.07	614.77	608.16
HLA-DR	17060.98	23075.62	9021.33	10214.70	6833.04	5901.81
CD69	3595.29	1961.88	124.19	126.24	113.03	108.25
CCR7	67.42	75.93	56.31	51.48	53.10	57.43
CD45RA	184.92	170.42	4905.24	4026.20	3842.95	3890.33
CD62L	53.71	43.51	6715.27	7176.95	5312.02	5990.55
CD38	656.77	772.88	5941.62	5169.87	6400.89	6249.81
CD27	3.01	3.01	270.41	180.98	179.09	199.61
CD49d	2127.50	6525.67	5669.49	6202.52	5366.96	6247.47
CLA	636.48	948.35	1081.75	1023.56	928.13	535.06
β7-integrin	31515.50	43161.62	12799.80	13286.56	13325.79	16595.89
CD29	14338.79	11740.90	2121.67	2353.33	2028.68	2441.08

*) Legend Figures 3.5, 3.6 and 3.7:

0-999
1000-1999
2000-9999
> 10 000

No obvious differences were found between the HPA-1a-specific T cell clones and spontaneous proliferating T cells from PBMCs from either random donors or HPA-1a-immunized donors.

3.5 The TACE inhibitor TAPI-0 can be used to allow detection of TNF-production in activated HPA-1a-specific T cells as surface pro-TNF expression

A feature that can be used to identify antigen-activated T cells is secretion of cytokines. While IFNγ measurement by flow cytometry requires fixation of the cells, a new method for surface staining for TNF could be performed on live cells (58;59). This method was tested on HPA-1a-specific T cell clones in order to examinate the feasibility of this method in a strategy to increase the efficiency of identification and isolation of HPA-1a-specific T cells.

T cell clones were labelled with CFSE (to distinguish them from B-lymphoblast cells in flow cytometry) and incubated with peptide-pulsed D4BL4 B-lymphoblasts or peptide only for 4.5 hours in the presence of the TACE inhibitor TAPI-0 and monoclonal PE-conjugated anti-TNF and analyzed by flow cytometry (Figure 3.9).

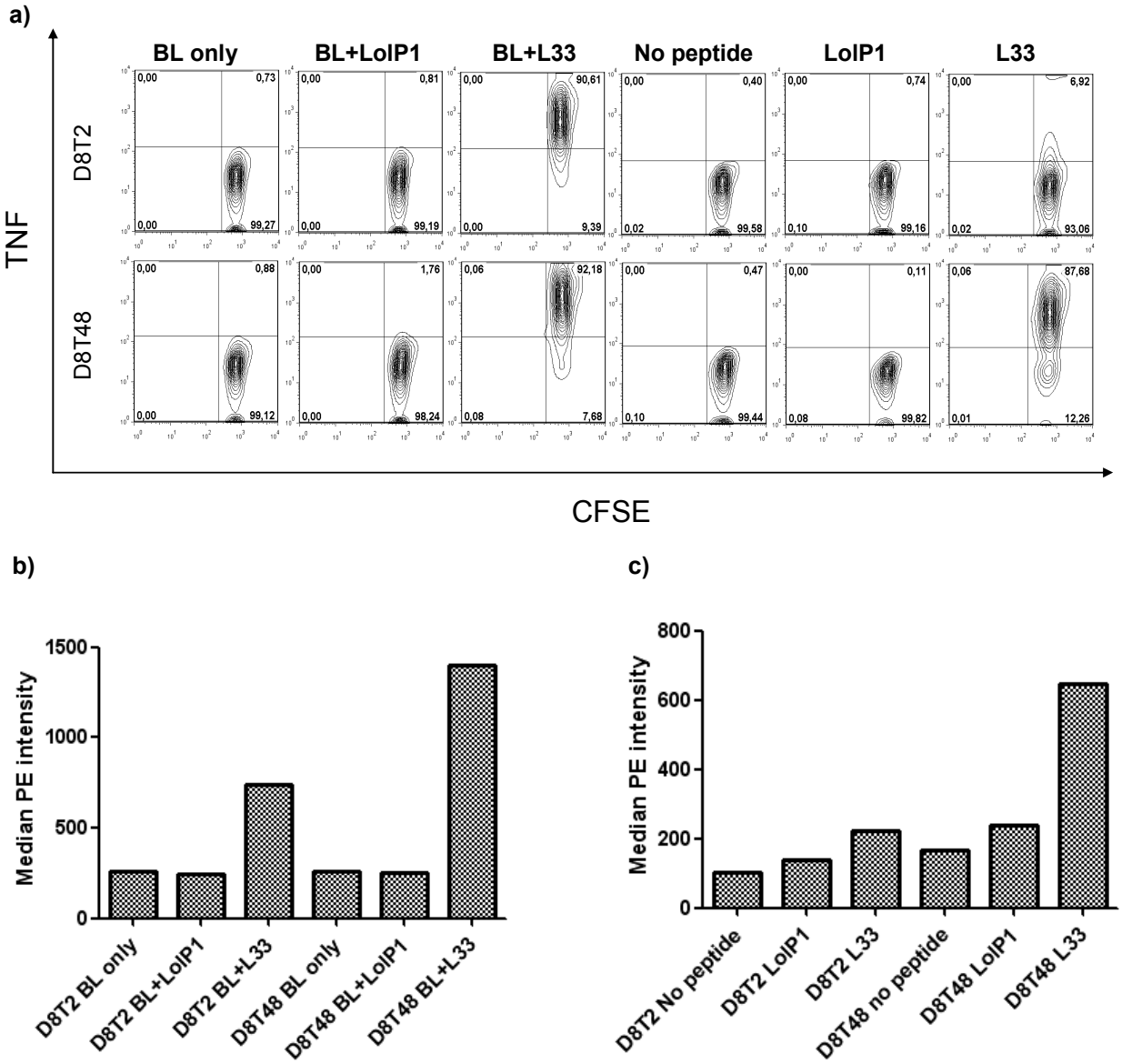

Figure 3.9 TNF-production, measured as PE fluorescence intensity by flow cytometry in HPA-1a-specific T cell clones after stimulation with HPA-1a peptide-pulsed HLA-DRB3*0101-positive B lymphoblasts (D4BL4) (a, b) and in HPA-1a-specific T cell clones after stimulation with HPA-1a peptide only (a, c).

As seen in Figure 3.9 a) and c), T cell clone D8T48 produce TNF in response to HPA-1a L33 peptide stimuli in absence of antigen presenting cells, suggesting that the peptide can bind HLA-DR molecules expressed on the T cell surface directly, and that the T cells can stimulate each other in culture. Three parallels of this experiment revealed that the extent of TNF-production in response to activation is different for these two clones. Still, this method was found to be suitable for viable identification of T cells responding to specific antigen-stimulation, and could likely be combined with the CFSE proliferation assay for enhanced efficiency in identification of HPA-1a-specific T cells in donor PBMCs, as shown later.

3.6 Enhanced culture conditions resulted in a higher proportion of potential antigen-specific cells

Following optimalization of culture conditions using PBMC from CMV-positive donors, these conditions were tested in donor PBMCs to see if the detection of HPA-1a-specific T cell proliferative responses would improve.

Donor 8 PBMCs were stained with CFSE and stimulated with HPA-1aa platelets or L33 20-mer peptide using HPA-1bb platelets or LolP1 20-mer peptide as controls, respectively. The cells were cultured for 11 days at the optimal culturing conditions defined by the CMV-study (section 3.3) using IMDM cell culture medium supplemented with 10 % Human (HPA-1bb) serum and Pen-Strep. Cells were incubated at 37 °C using atmospheric O_2 content and 7.5 % CO_2. Recombinant IL-2 (10 U/mL) was added to all wells on day 5.

On days 3, 6 and 10 donor 8 PBMCs were collected and stained with anti-CD8 (PE), anti-CD3 (APC), anti-CD14 (APC-Cy7) and anti-CD4 (PE-Alexa610) and analyzed for proliferation by flow cytometry. Flow data was analyzed using the FlowJo software (Treestar, Ashland. OR). A large lymphocyte gate was drawn using forward- and side-scatter parameters since stimulated T cells tend to increase in size. To avoid CD8 T cells and monocytes, CD8 and CD14 was used as "dump" markers. The CFSE proliferation assay was studied for $CD3^+CD8^-CD14^-$ lymphocytes*:

Table 3.8 CFSE proliferation assay performed on stimulated Donor 8 PBMCs on days 3, 6 and 10 of stimulation

	Proliferating CD4 T cells (%)			Proliferating CD4 T cells (%)		
Day	LolP1**	L33**	Fold increase***	HPA-1bb trc.**	HPA-1aa trc.**	Fold increase***
3	0,16	0,58	3.6	1,07	2,23	2.1
6	0,17	0,59	3.5	1,08	2,21	2.0
10	15,39	24,48	1.6	3,69	67,93	18.4

* On day 10, cells were not stained with anti-CD3 to avoid background secretion of TNF by cells that may be activated by the anti-CD3 antibody.

** Numbers indicate percent proliferating CD4 T cells of the total population of CD3 positive cells assayed.

** *Fold increase of CD4 T cells proliferating at various days after stimulation with HPA-1a antigen relative to negative control (LolP1 or HPA-1bb trc.).

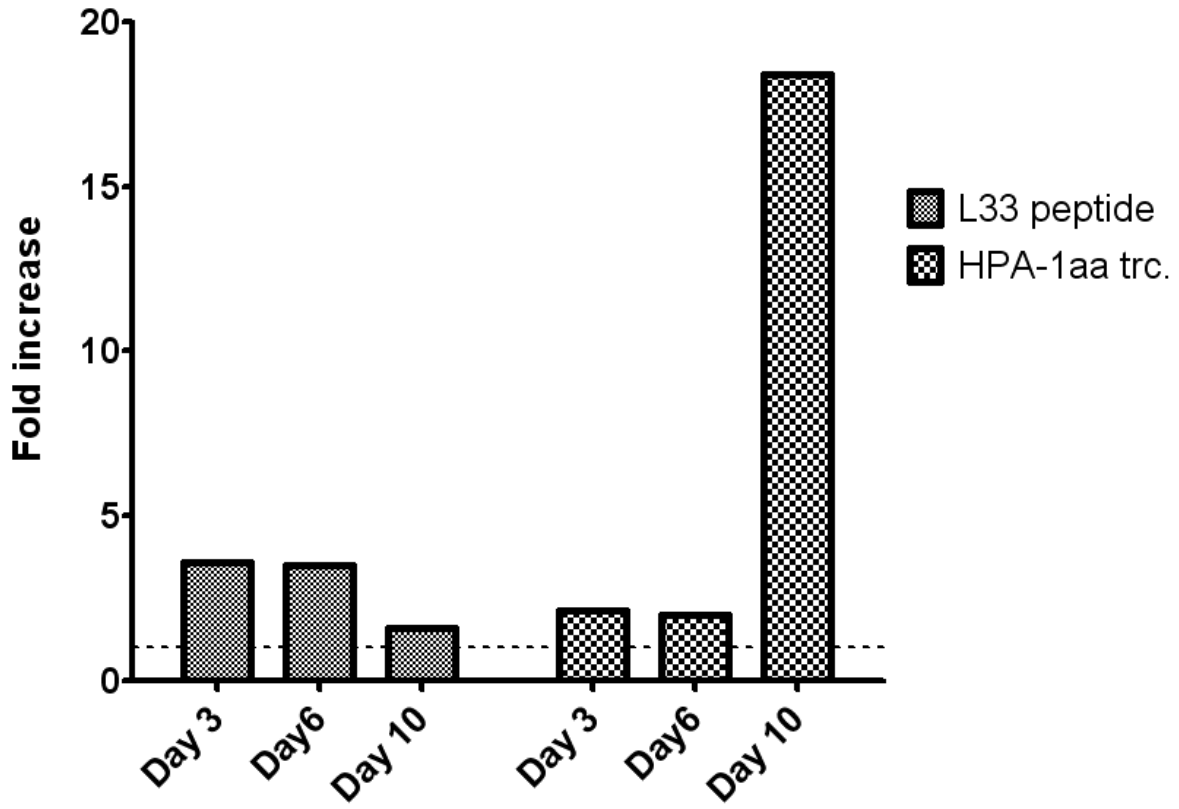

Figure 3.10 Fold increase of proliferating CD4 T cells (% of total) from an HPA-1a-immunized donor in response to HPA-1a antigen stimuli relative to negative control at days 3, 6 and 10. A fold increase of 1.0 (indicated by a horizontal line) refers to no increased proliferation in HPA-1a antigen-stimulated cultures relative to the controls.

CFSE proliferation assay performed on Donor 8 T cells on days 6 and 10 (Table 3.8, Appendix II and Figure 3.11) of stimulation revealed that the proportion of proliferating CD4 cells was markedly higher in response to stimuli with HPA-1a antigen compared to the controls. When performing flow cytometric analysis, lymphocytes were gated using forward- and side scatter parameters. On day 6 $CD3^{+}CD8^{-}CD14^{-}$ cells were analysed for expression of CFSE in combination with CD4. On day 10, cells the surface TNF assay was tested in addition to performing the CFSE vs CD4 proliferation analysis. To avoid background TNF secretion from TCR-mediated stimulation, cells were not stained with anti-CD3. $CD8^{-}CD14^{-}$ lymphocytes were analyzed, and proliferating CD4 T cells were analyzed for TNF secretion as shown in Figure 3.11.

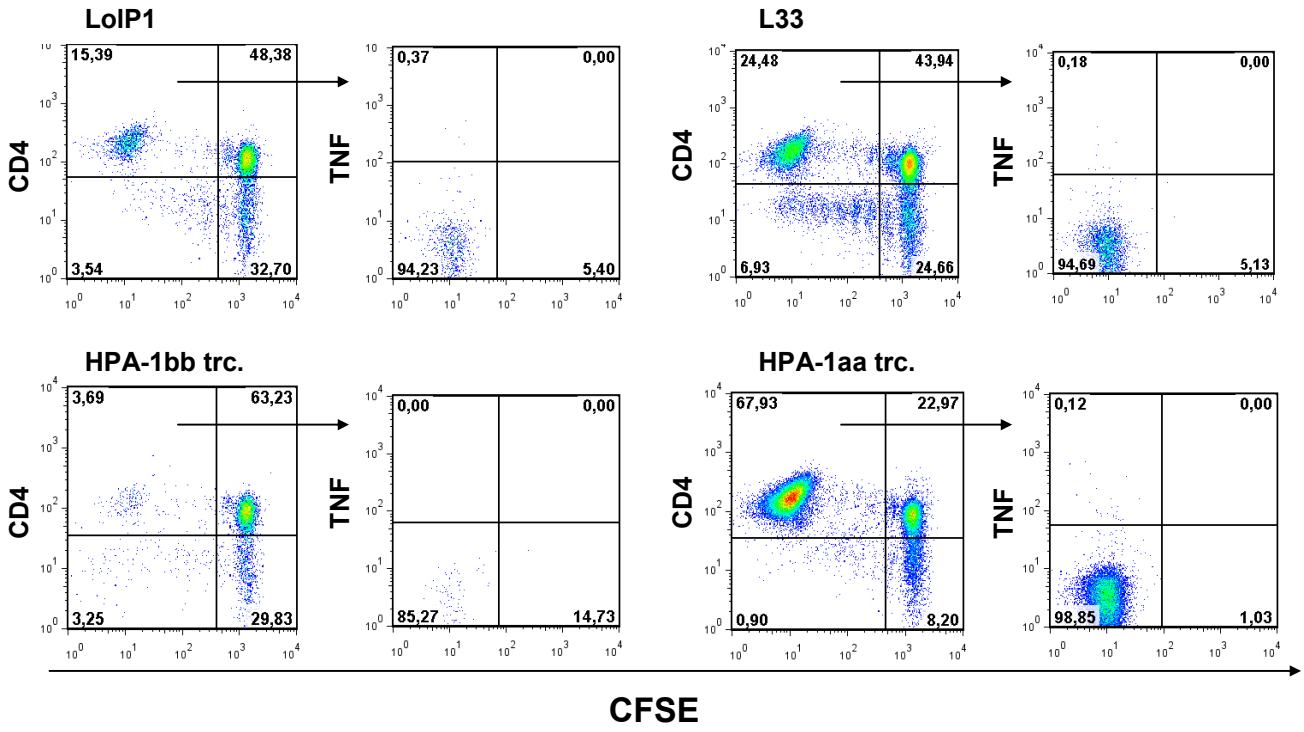

Figure 3.11 CFSE proliferation assay and surface TNF assay on Donor 8 PBMCs. On day 10 of proliferation after initial stimulation with antigen, the cells were stimulated again and assayed for TNF surface expression as detailed in the methods chapter. TNF expression is displayed for events in the upper left quadrant in the CFSE vs CD4 plots, as indicated (arrows).

These findings demonstrate that the enhanced culture conditions indeed resulted in more specific proliferation to stimulation with HPA-1a antigen, and that a small portion of the proliferating cells react with anti-TNF mAb following restimulation with specific antigen.

3.7 HPA-1a-specific T cells can be identified by a combination of the CFSE proliferation assay and surface TNF-expression

On day 11 of stimulation, PBMCs were stimulated with L33 peptide or HPA-1a platelets in the presence of TAPI-0 and PE-conjugated anti-TNF for 4.5 hours. Cells were washed in IMDM medium and labelled with 7-AAD (to gate and eliminate dead cells) and fluorescence-labelled mAbs. $CD8^-$ $CD14^-$ $7\text{-}AAD^-$ lymphocytes were analyzed (Figure 3.12) and single, proliferating CD4 T cells were sorted using FACSAria, and expanded with anti-CD3 (30 ng/mL) in round bottom 96 well plates using irradiated feeder cells (PBMCs and D4BL4 B-lymphoblasts). Recombinant IL-2 (50 U/mL) was added every four days, starting on day 1 of expansion.

Single cells were sorted from three different populations (Figure 3.12): one population of proliferating CD4 T cells (gate P6), one population of proliferating CD4 T cells with downregulated CD4 expression (gate P5) and one population of proliferating CD4 T cells producing TNF in response to HPA-1a antigen stimulation (Gate P4).

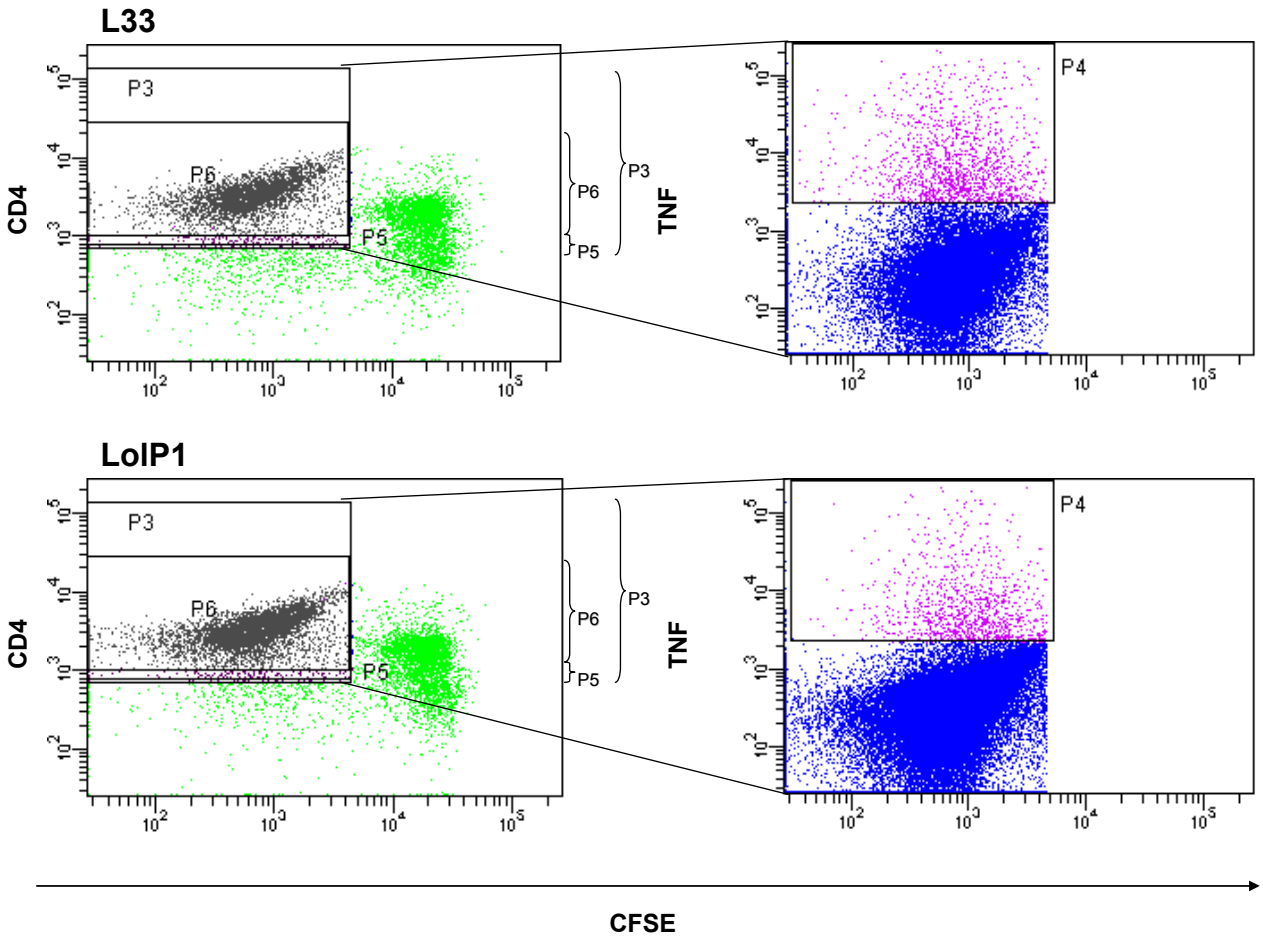

Figure 3.12 Single proliferating CD4 T cells were sorted from gates P4, P5 and P6 (60 cells from each region). The figure shows the sorting gates for peptide stimulated PBMCs. The same sorting gates were made for Donor 8 PBMCs stimulated with platelets. Cells were sorted from the P4 gates in L33 peptide-stimulated cultures and from gates P4, P5 and P6 in cultures stimulated with whole HPA-1a^{+} platelets. A compensation error is seen for CD4 due to missing settings for Horizon-V500 (conjugate on the used anti-CD4) on the FACSAria used for sorting. Settings for AmCyan were used.

After expansion of single sorted cells in culture with anti-CD3, proliferating clones were analyzed by IFNγ and IL-4 ELISPOT assay (Figure 3.13). 9 out of 28 proliferating clones from different populations (summarized in Table 3.9) were specific to HPA-1a antigen. TCRB gene analysis and sequencing was performed for five of these clones (summarized in Table 3.10). The remaining four HPA-1a-specific T cell clones were not expanded in time to a cell count that would yield both

sufficient amounts of RNA to perform *TCRB* sequence analysis *and* to establish these clones as long term cell lines. Expansion of these clones with the aim of establishing these as cell lines was regarded as more crucial and thus performed instead.

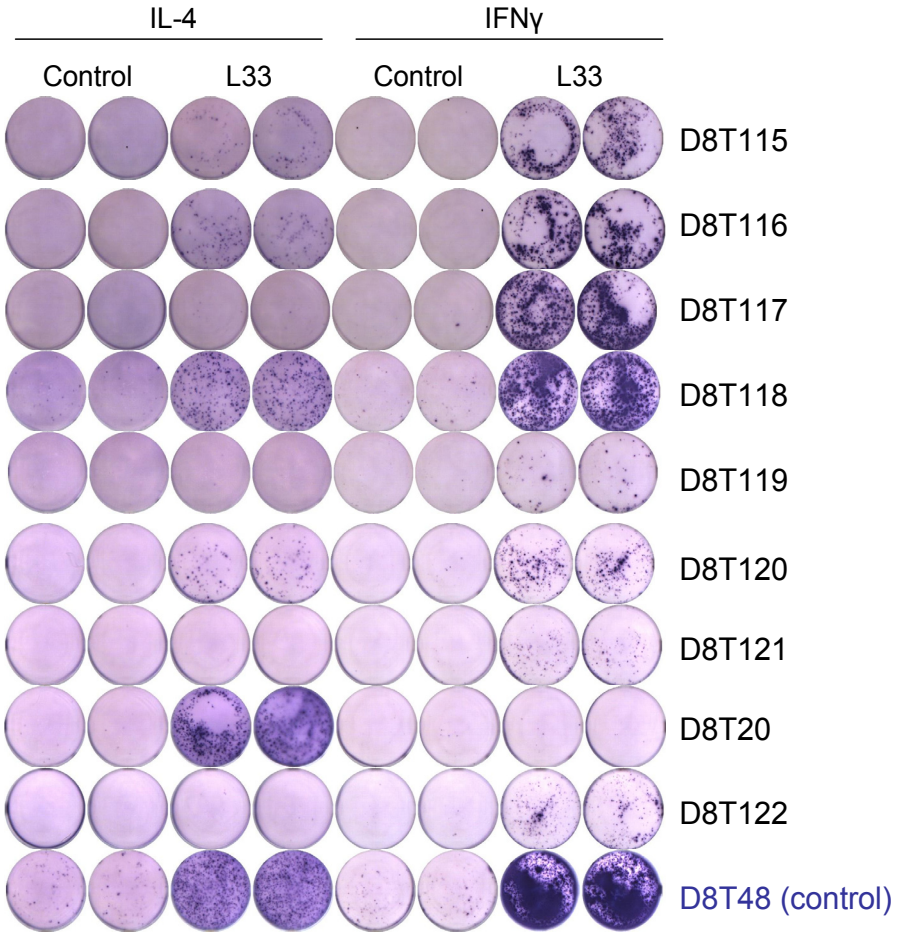

Figure 3.13 Of 28 proliferating Donor 8 clones, 9 were defined specific to HPA-1a by IFNγ/IL-4 ELISPOT. 6 of these were sorted based on TNF secretion after L33 restimulation, 1 was sorted from TNF-producing cells after restimulation with HPA-1a^{+} platelets and 1 was sorted from a population of cells co-cultured with HPA-1a^{+} platelets with down regulated CD4 expression, as summarized in table 3.9.

Table 3.9 Summary of proliferating clones from Donor 8 after sorting and expansion

Stimuli	Detection	Proliferating clones	HPA-1a specific clones
L33 peptide	TNF secretion	13 (of 60 sorted)	6 (46.2 %)
HPA-1a trc.	TNF secretion	6 (of 60 sorted)	2 (33.3 %)
HPA-1a trc.	CD4 down-regulation	3 (of 60 sorted)	1 (33.3 %)
HPA-1a trc.	CFSE low, CD4 high	6 (of 60 sorted)	0

Table 3.10 Summary of the *TCRB* rearrangements for 5 of the HPA-1a-specific Donor 8 T cell clones isolated after optimizing enrichment and detection of HPA-1a-specific CD4 T cells

	***TCRB* rearrangements**				**CDR3**	
Clone	***TRBV***	***TRBD***	***TRBJ***	***TRBC***	**Length**	**Amino acid sequence**
D8T115	11-1*01	2*01	2-1*01	2	13	ASSFRPRGVNEQF
D8T116	6-5*01	1*01	1-3*01	1	16	ASSPSPPVPGSGNTIY
D8T117	7-2*01 or 7-2*04	2*01	2-1*01	1 or 2	12	ASTLAGVTHEQF
D8T118	11-2*01	1*01	2-7*01	2	15	ASSLTTGDLTSYEQYF
D8T121	20-1*04 or 20-1*05 or 20-1*06	2*02	2-7*01	1 or 2	13	SARALAGGAYEQY

These findings demonstrate that the efficiency in identifying and isolating HPA-1a-specific T cell clones can be dramatically enhanced by combining CFSE proliferation and TNF expression assays.

4. Discussion

The work performed in this study showed that by optimizing culturing conditions during enrichment, as well as finding new strategies for identifying populations of proliferating antigen-specific T cells, the cloning efficiency of single HPA-1a specific CD4 T cells improved. HPA-1a-specific T cells can be isolated after a single stimuli compared previous isolations requiring several rounds of stimulation (47;48). Several observations during the course of this study lead to an improved strategy for enrichment, detection and isolation of HPA-1a-specific T cells from PBMCs of alloimmunized women:

4.1 CD4 T cells that proliferate in response to HPA-1a antigen stimuli exhibit down-regulation of surface CD4 expression

HPA-1a-specific T cells down-regulate CD4 in response to antigen-specific stimulation. This may be used as a hallmark of activation to distinguish these cells from proliferating cells with irrelevant specificities in the CFSE proliferation assay, as demonstrated in this study.

From the region of CD4 down-regulating T cells, three HPA-1a-specific T cell clones were isolated. TCRBV analysis revealed that these three single sorted cells originated from two distinct clones. However, these count as three identified and isolated antigen-specific T cells as they were detected and isolated separately. The separate isolation of several single cells from the same clonal lineage should be expected, as the cells are isolated from a population of T cells that have undergone a number of cell divisions.

After 14 days in culture, more proliferating CD4 T cells were present in HPA-1aa stimulated culture than in culture stimulated with HPA-1bb platelets, and a distinct population of proliferating T cells with down-regulated expression of CD4 on the surface was detected in the HPA-1a stimulated culture only. It has been demonstrated that internalization of the CD4 molecule occurs as a part of the regulation of the signalling events that mediate T cell activation. Modulation of surface CD4 expression in response to activation through the TCR complex has been reported, but the results are not consistent. Rapid down-regulation by internalization and subsequent lysosomal degradation of CD4 has been reported to occur within hours after activation by both monoclonal antibodies targeting the TCR complex, phorbol esters and specific antigen, followed by a recovery around 20 hours after stimulation (64-66). Another study reported a

marked down-regulation of surface CD4 expression 24 hours after stimulation of antigen-specific T cells with appropriate antigen without recovery after 48 hours (67). However, these studies have been performed on clonal T cell lines *in vitro*, and may not reflect the time course of CD4 expression during an immune response in PBMC tissue cultures *in vitro* where APCs, antigen and other immune cells are present or during *in vivo* immune responses.

The observation of CD4 down-regulation in a population of proliferating CD4 T cells 14 days after HPA-1a antigenic stimuli, and the presence of HPA-1a-specific T cells in this population (and not in the population of constitutive CD4 levels) suggests that the CD4 down-regulation that has been reported in connection with T cell activation may be a consistent physiological feature of HPA-1a-specific CD4 T cells. On the other hand, the cells are co-cultured with appropriate antigen for 14 days in the presence of antigen presenting cells, added IL-2 and other immune cells which secrete cytokines and other stimulatory molecules, suggesting that activated cells displaying down-regulated levels of surface-bound CD4 may have been re-stimulated several times during the enrichment.

A study published in 1997 showed that after immunizing Lewis rats with various antigens known to induce experimental allergic encephalomyelitis (EAE) in animal models, CD4 levels were down-regulated on CD4 T cells on days 4 and 10 after EAE induction *in vivo* and restored by day 21 (68). Although this observation may not be completely applicable to this present study, it is very interesting to see that CD4 modulation varies in both time-course and extent in different settings of T cell activation studied in different experimental models.

4.2 HPA-1a-specific T cell clones are likely to be the dominating T cells responding to foetal platelets in connection with NAIT

In this study, T cell clones isolated from cells proliferating in response to stimulation with HPA-1a-positive platelets were subsequently found to be specific for HPA-1a, by using peptide-pulsed APCs. The ability of the synthetic HPA-1a-derived L33 peptide to elicit an immune response in clones that were isolated after stimulation with whole platelets (Figure 3.2), meaning that they never previously encountered the synthetic L33 peptide, strongly suggests that the immune response to HPA-1a platelets *in vivo* is directed towards the L33/P33 polymorphism of the HPA-1 antigen. This observation fits well with the observation by Anani *et al*, who demonstrated that the

peptide fragments naturally processed and presented from the extracellular domain of β3-integrin by HLA-DRB3*0101 homozygous APCs were derived from HPA-1a (69).

However, further investigations are needed to examine the role of other alloantigens on foetal platelets, e.g. paternally inherited MHC class I molecules, which could also elicit allo-specific T cell responses (by indirect mechanisms). Because the polymorphism of MHC molecules, pending experiments will be conducted with the child's own platelets (or paternal platelets) to assay for recall responses to allogene MHC.

4.3 No distinct expression patterns of surface activation markers were observed in HPA-1a-specific T cells

The observation of CD4 down-regulation on activated HPA-1a specific CD4 T cells encouraged the investigation of other phenotypic properties that may distinguish cells proliferating in response to HPA-1a antigen from other cells proliferating with no obvious source of antigen-stimulation. The surface marker phenotyping was performed in order to see whether T cells activated specifically through the TCR were phenotypically distinguishable from other T cells proliferating in response to other stimuli. The expression pattern of various activation-, subset- and homing markers (Table 2.11) on HPA-1a specific T cells and the comparison of such patterns on various other cell populations (section 3.4) revealed no obvious phenotypical properties (expression pattern) that would allow the specific detection of HPA-1a specific T cells.

4.4 Proliferation of CD4 T cells with irrelevant specificities was reduced by replacing foetal bovine serum (FBS) with human serum in the culture medium during enrichment of antigen-specific T cells

One of the main challenges of the CFSE proliferation assay is that other T cells than those specific to the appropriate antigen will also proliferate. The potential sources of stimulation of these cells are not known, but they may be derived from various substances such as antigens present in the culture medium supplements, such as added serum. Other possible sources of stimulation may be contact with other cells, cytokines added to the cultures (or present in the serum supplement) or produced by the cultured cells or activation by antigen *in vivo* before or during blood sampling from the blood donor.

The CFSE proliferation assay performed on PBMCs from CMV positive donors (Table 3.4) on day 6 of proliferation at atmospheric O_2 content show reduced background proliferation when using culture medium supplemented with human serum only, compared to medium supplemented with 10 % FBS. The parallel assays enriched at 5 % O_2, thought to mimic physiological conditions, show no indications of improved enrichment of antigen-specific CD4 T cells compared to proliferation at atmospheric O_2 levels. The same was observed at day 14 after stimulation.

The improved enrichment of potential antigen-specific CD4 T cells observed when replacing FBS with human serum indicates that some of the background proliferation observed in previous assays may be derived from bovine antigens present in the FBS, or that growth factors known to be plentiful in FBS drives proliferation of cells that are not HPA-1a-specific. It is possible that background proliferation may be reduced further by using serum-free culture medium, but by removing serum completely, important growth factors, other nutrients and protecting protein will be lost, and may compromise the proliferation of the desired cells as well as the cells that proliferate "spontaneously".

The time course of the CD4 immune response monitored in these assays also indicate that the optimal time point of isolation of potential antigen-specific CD4 T cells is closer to 6 or 10 days than 14 days. The CFSE proliferation assay performed on day 14 with CMV peptides (Table 3.4) showed a massive proliferation of both potential antigen-specific cells as well as cells with irrelevant specificities, while at days 6 and 10 both low background proliferation as well as improved enrichment of potential antigen-specific cells was observed. This applies for the last proliferation assay performed with HPA-1a antigen on donor samples.

On day 10 of stimulation with HPA-1a antigen on Donor 8 PBMCs (Figure 3.11 and Table 3.8) the enrichment of antigen-specific cells seems to be much higher in platelet-stimulated cultures relative to peptide-stimulated cultures. This is an interesting observation, as stimulation with whole platelets will better resemble the activation of a T cell response to HPA-1a *in vivo*. One of the possible explanations for this, may be the generation of peptide fragments of different lengths when the HPA-1a antigen is processed by antigen presenting cells (69) and that a heterogeneous pool of peptide derived from the same antigen may engage a higher number of reactive T cells than a homogenous pool of identical, synthetic peptide. However, studies with the isolated HPA-1a-specific T cell clones show that they respond to both native antigen and synthetic peptide.

4.5 Combining the CFSE proliferation assay with surface-TNF detection dramatically improved the specific detection and isolation of HPA-1a-specific CD4 T cells

Capturing TNF produced in response to re-stimulation with L33 antigen on the cell surface improved the detection and isolation of HPA-1a-specific CD4 T cells. The complete elimination of CD4 T cells that proliferate without any obvious source of antigen stimuli in the CFSE proliferation assay, even with the most optimal enrichment conditions, is not likely. However, modifications of the culturing conditions during enrichment of CMV antigen-specific T cells from CMV-positive donors helped reduce the proliferation of CD4 T cells with irrelevant specificities when applying these modified conditions to the enrichment of HPA-1a specific CD4 T cells.

By applying the surface TNF assay to the isolation of single proliferating CD4 T cells in combination with optimized enrichment of antigen-specific CD4 T cells, the cloning efficiency improved markedly. The assay allowed the direct detection and viable isolation of proliferating CD4 T cells producing TNF in response to recent re-stimulation with HPA-1a antigen. Isolation of single proliferating, TNF secreting CD4 T cells yielded 13 clones from peptide stimulated culture and 6 clones from HPA-1a platelet stimulated cultures. Among these, 6 and 2 clones were HPA-1a specific, respectively. This finding demonstrates that a combination of the CFSE proliferation assay and TNF-surface detection after a subsequent stimulation with antigen dramatically improved detection of HPA-1a-specific T cells.

The surface TNF assay was not as efficient when applied to the platelet-stimulated cultures compared to the culture stimulated with peptide. The reason for this may be that the incubation time of only 4.5 hours did not allow the processing and presentation of sufficient amounts of antigen to stimulate the T cells at the same extent as peptide fragments pulsed directly to the HLA-DR52a molecules on the APC surface. In addition, peptide can bind directly to HLA-DR molecules expressed by the T cells (Table 3.5-3.7) allowing them to stimulate each other (Figure 3.9), in addition to the antigen presenting cells. Optimization of the assay applied to platelet stimuli was not performed, partially because such optimization was beyond the scope of this present study, and partially due to limited access to patient sample.

Single proliferating cells from a region of CD4 down-regulation and a region of constitutive CD4 levels were isolated from platelet-stimulated culture and gave rise to 3 and 6 proliferating clones,

respectively. One of these, originating from the region of CD4 down-regulation, was HPA-1a-specific.

The secretion of TNF in response to T cell activation is by no means restricted to apply to HPA-1a-specific T cells. There is reason to believe that this method for detecting and isolating antigen-specific T cells will be applicable to others who are working with T cell responses and wish to use clonal T cell lines for such studies.

The cloning efficiency with the assay proposed here is still not completely optimal. The HPA-1a specific T cells that are sorted and expanded, have divided several times in culture, and with a hypothetical cloning efficiency of 100 %, one would expect the same clone to be present in several wells after sorting and expansion. Since this is not the case with the sorting assays performed in this study, there is reason to believe that further optimisation measures can be made to increase the cloning efficiency additionally.

One must be aware that the different clones that are isolated here may, although all HPA-1a-specific, behave differently and display different features that might reflect diversity in T cell response associated with NAIT. The different clones use different TCRB genes, giving different CDR domains. Although specific to the same antigen, the different TCRs will recognize the same antigen in different ways, and these clones are valuable tools for mapping the common epitope recognized by different T cell receptors.

In addition, the clones display slightly different cytokine profiles in response to activation. As demonstrated by the ELISPOT assays performed in this study (Figures 3.2 and 3.13), some clones secrete only IFNγ, some secrete both IFNγ and IL-4 and one clone, D8T20, secrets IL-4 and no IFNγ in response to HPA-1a stimuli. If different clones have different patterns of cytokine production, and if these patterns change during the time course of an immune response, specific T cells may be overlooked when using detection strategies based on TNF production. This observation stresses the importance of optimizing different enrichment and detection strategies if one wants to isolate and study different T cell clones. In this study, two such strategies are optimized based on CD4 expression and TNF production. In addition, T cell clones can potentially be detected on the basis of other cytokines secreted in response to stimuli, i.e. by applying the Cytokine Secretion Assay detecting IFNγ- or IL-4 secretion to these protocols (70;71).

5. Future perspectives

The strong association between a specific MHC allele and immunization to HPA-1a, along with the identification of HPA-1a-specific T cells in the circulation of previously immunized women suggests that the activation of T cells may be the most critical event of the immune response that leads to the production of maternal HPA-1a-reactive antibodies. If this is the case, therapeutic strategies aimed to prevent activation of these T cells in HPA-1bb women will be the most rational therapy of choice.

For decades, immunotherapeutic strategies to enhance or suppress immune responses have been subject to intensive investigation. For a number of diseases including various cancers, rheumatoid disorders and autoimmune diseases, immunomodulatory strategies aiming to suppress T cell activation have revealed promising results.

HPA-1a-specific T cell clones may serve as useful tools to develop similar strategies for prevention of NAIT by preventing the activation of HPA-1a-specific T cells that may be present in HPA-1a-negative individuals. By identifying the common TCR epitope within the HLA-DR52a:HPA-1a peptide-complex, amino acid substitutions may be performed to develop synthetic peptide that may block activation of HPA-1a-specific T cells by binding to HLA-DR52a with higher affinity than HPA-1a peptide without activating these T cells. Another strategy may be to prevent HPA-1a mediated activation of specific T cell activation by inducing tolerance to HPA-1a in these T cells, i.e. through anergy, a strategy proven successful in other disease models, such as allergies, as reviewed by Akdis and Akdis (72).

The most potentially specific way of detecting HPA-1a-specific T cells in PBMC cultures in controlled *in vitro* experiments, is by using multimeric recombinant MHC:peptide (pMHC) complexes. Recombinant HLA-DR52a molecules with HPA-1a-derived L33 peptide in the peptide binding groove can be multimerized by biotinylating the tails of the MHC class II molecules and joining these together to a tetrameric complex via a streptavidin core with four binding sites for biotin. By using a streptavidin core conjucated to a fluorochrome, T cells that have bound the pMHC-complex can be visualized directly by flow cytometry and isolated by FACS sorting. These tetramers can have many other applications in investigating the immune response, i.e. by tracking T cells in *in vivo* NAIT animal models to investiagate the pathogenesis of NAIT at the cellular level.

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Appendix

Appendix I. CFSE proliferation assays of PBMCs from CMV positive blood donors (Presented in section 3.3)

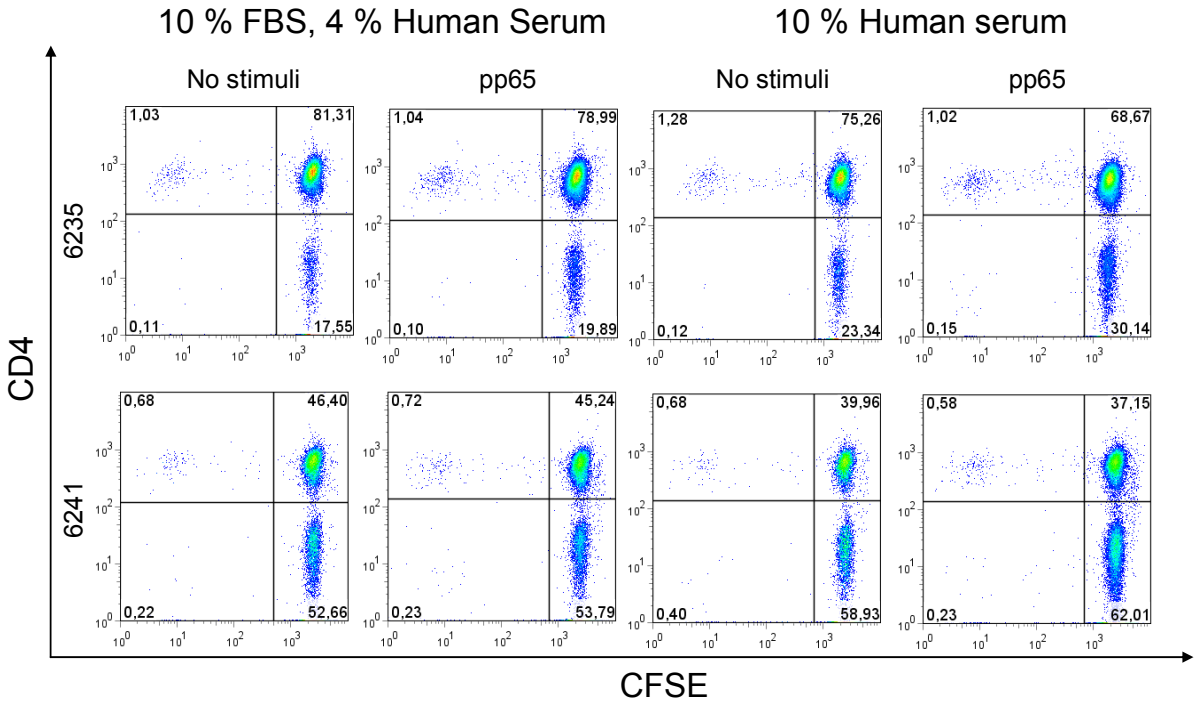

Figure A.1 – CFSE proliferation assay performed in atmospheric O_2 content of PBMCs from two CMV-positive donors on day 3 of stimulation.

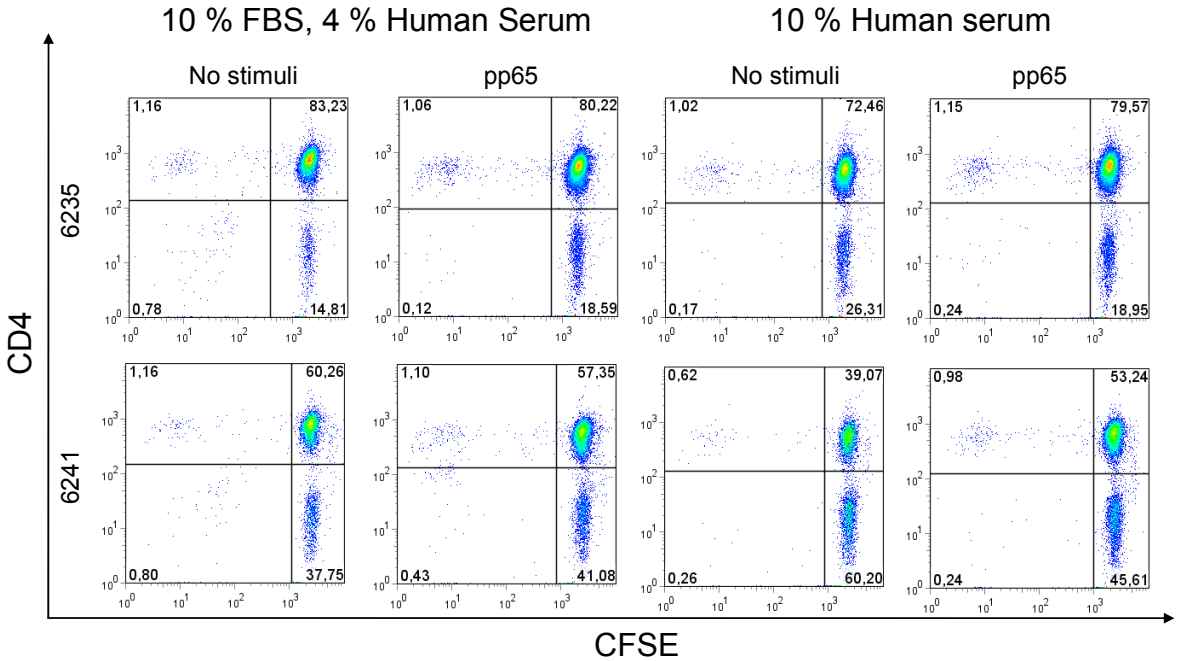

Figure A.2 – CFSE proliferation assay performed in 5 % O_2 content of PBMCs from two CMV-positive donors on day 3 of stimulation.

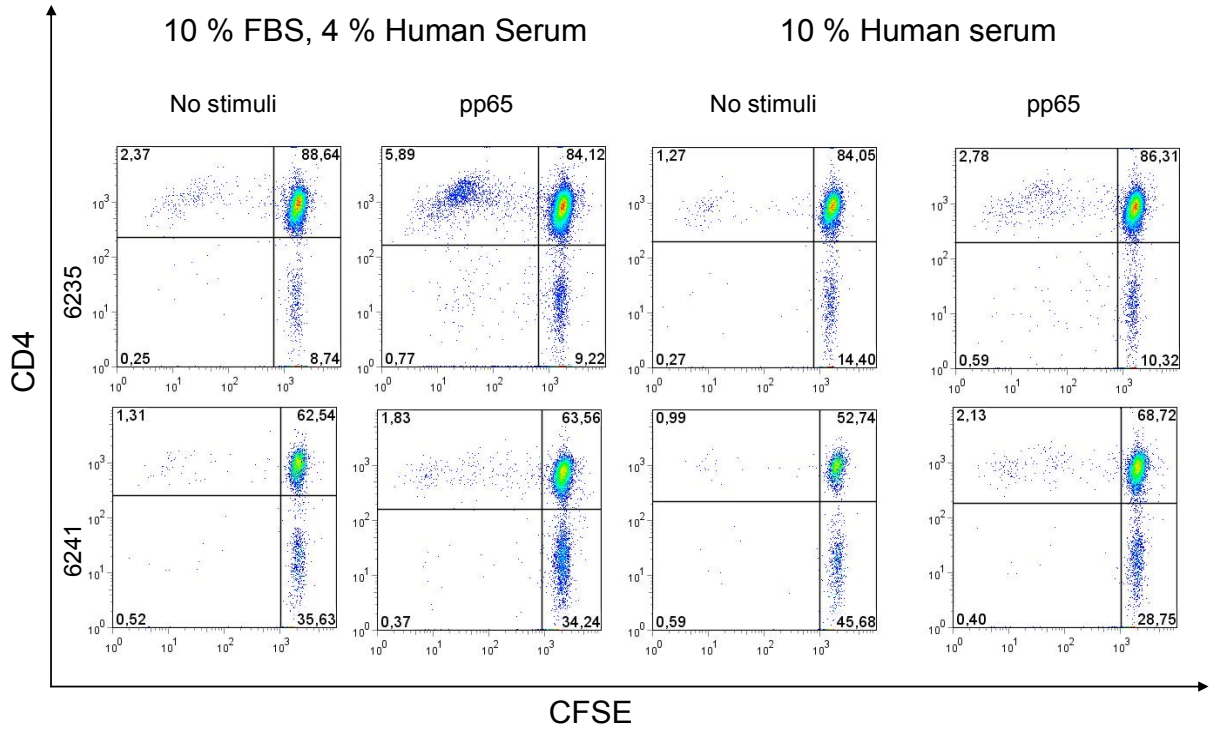

Figure A.3 – CFSE proliferation assay performed in atmospheric O_2 content of PBMCs from two CMV-positive donors on day 6 of stimulation.

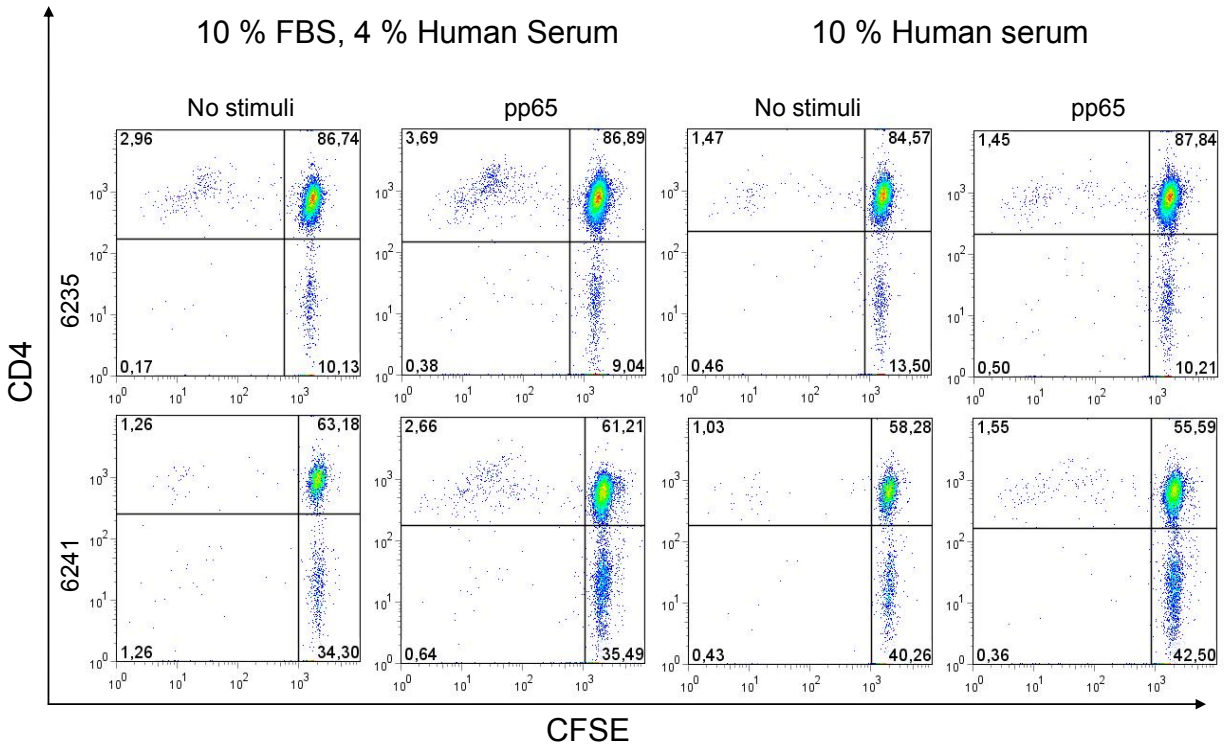

Figure A.4 – CFSE proliferation assay performed in 5 % O_2 content of PBMCs from two CMV-positive donors on day 6 of stimulation.

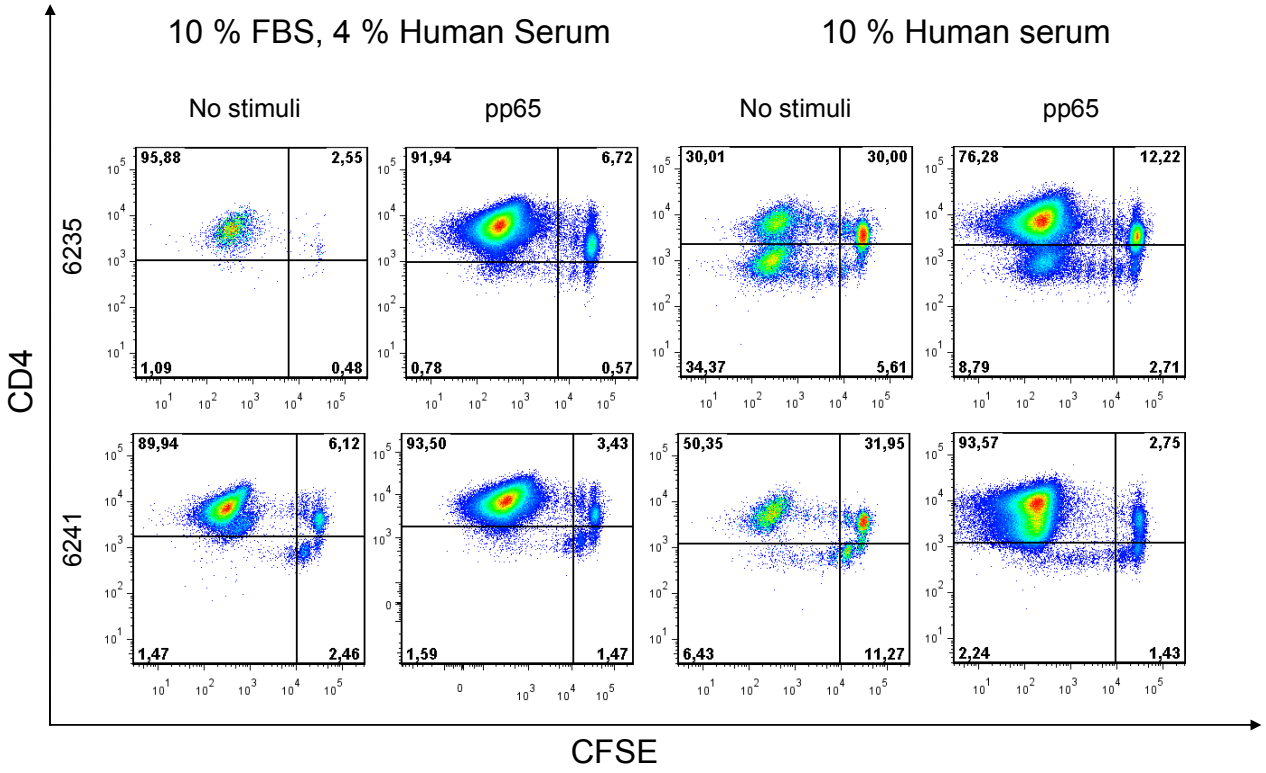

Figure A.5 – CFSE proliferation assay performed in atmospheric O_2 content of PBMCs from two CMV-positive donors on day 14 of stimulation.

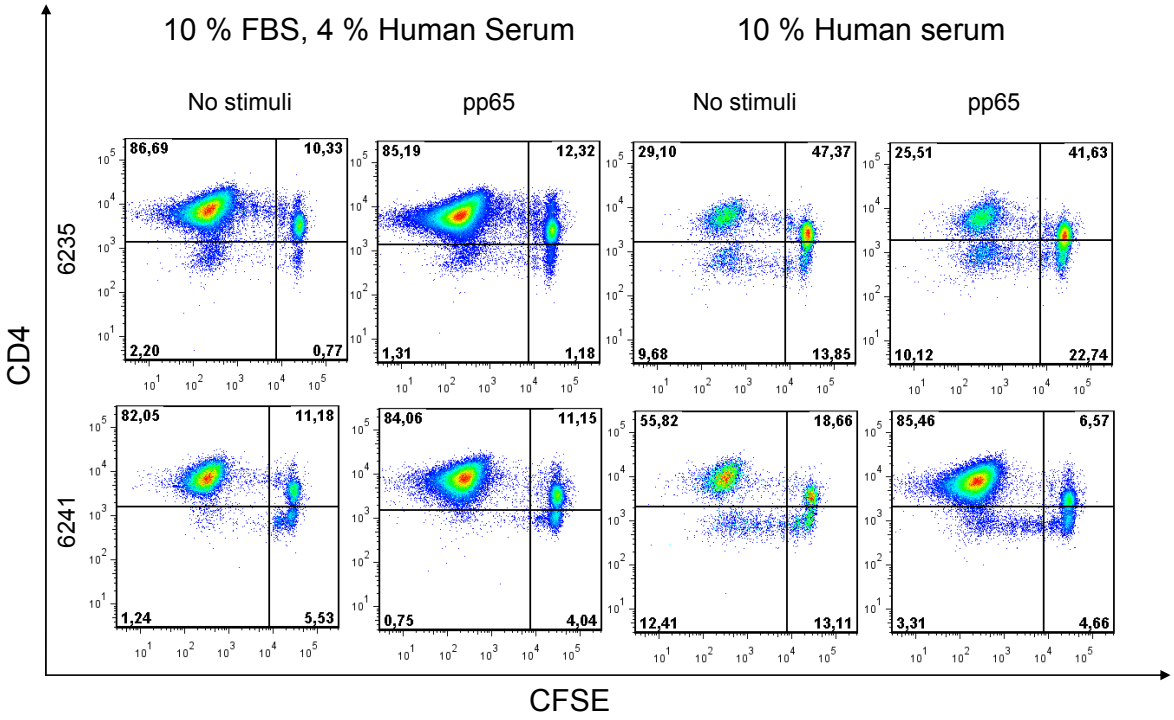

Figure A.6 – CFSE proliferation assay performed in 5 % O_2 content of PBMCs from two CMV-positive donors on day 14 of stimulation.

Appendix II. CFSE proliferation assays of PBMCs from an HPA-1a immunized HPA-1bb female (Section 3.6)

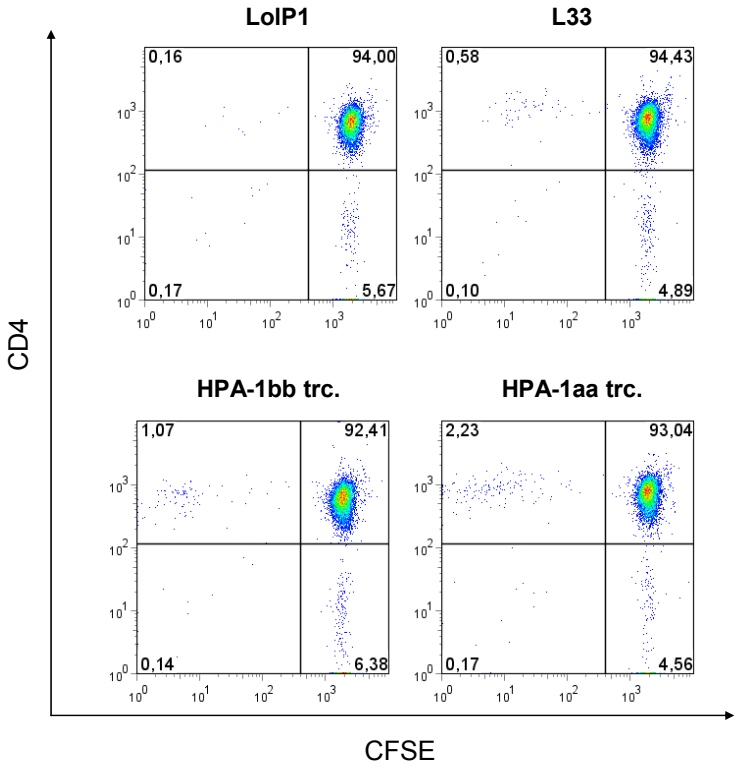

Figure A.7. CFSE proliferation assay of Donor 8 PBMCs on day 3 of stimulation

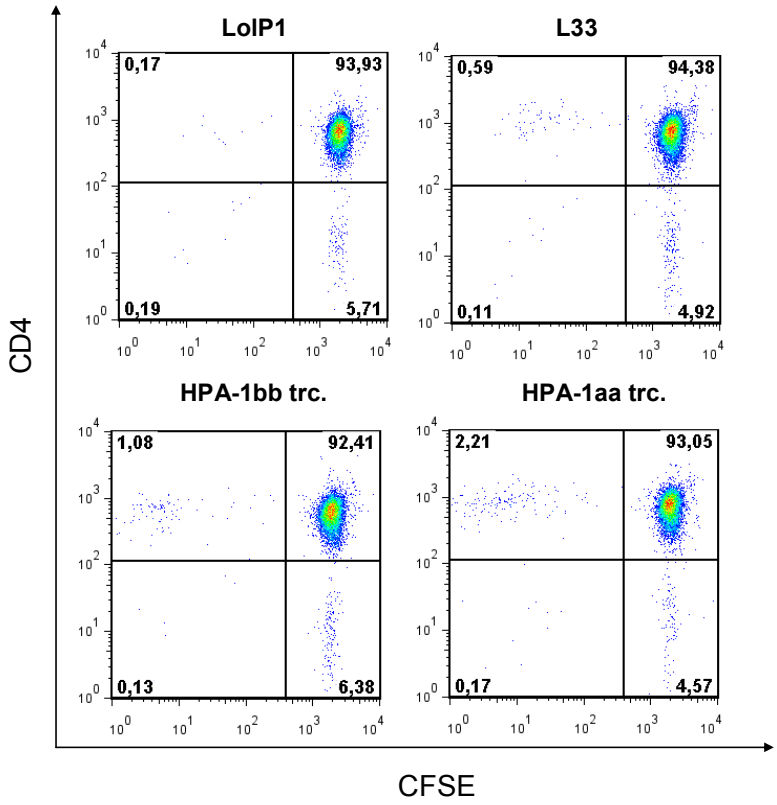

Figure A.7. CFSE proliferation assay of Donor 8 PBMCs on day 6 of stimulation

Appendix III. TCRB sequence analysis of HPA-1a-specific T cell clones isolated with optimized enrichment- and detection strategies

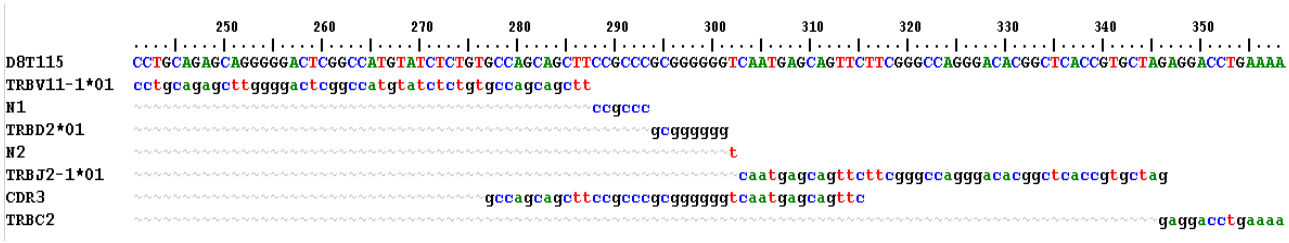

Figure A.8. Sequence alignment for HPA-1a-specific T cell clone D8T115

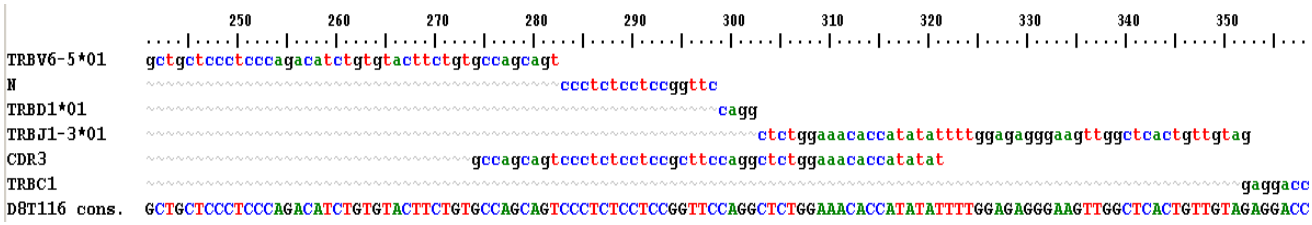

Figure A.9. Sequence alignment for HPA-1a-specific T cell clone D8T116

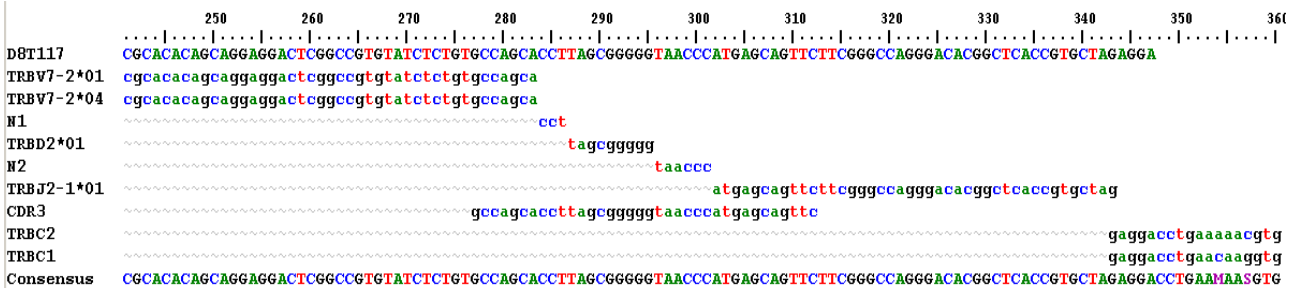

Figure A.10. Sequence alignment for HPA-1a-specific T cell clone D8T117

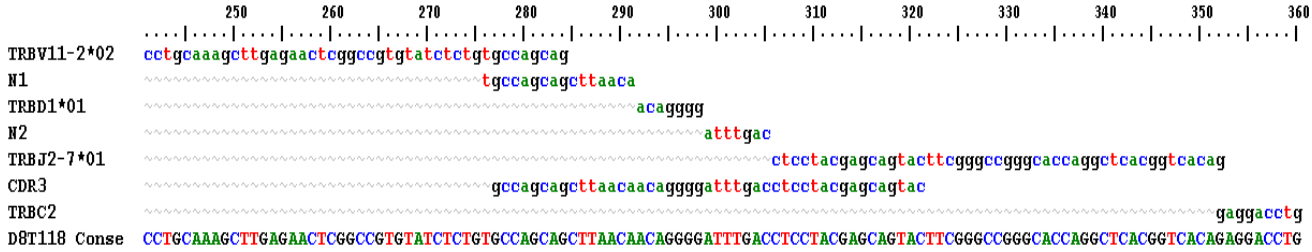

Figure A.11. Sequence alignment for HPA-1a-specific T cell clone D8T118

```
                  250       260       270       280       290       300       310       320       330       340       350
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
D8T121            ACCAGTGCCCATCCTGAAGACAGCAGCTTCTACATCTGCAGTGCTAGGGCACTAGCGGGAGGCGCCTACGAGCAGTACTTCGGGCCGGGCACCAGGCTCACGGTCACAGAGGACCTGA
TRBV20-1*04        accagtgcccatcctgaagacagcagcttctacatctgcagtgctag
TRBV20-1*05        accagtgcccatcctgaagacagcagcttctacatctgcagtgctag
TRBV20-1*06        accagtgcccatcctgaagacagcagcttctacatctgcagtgct
N1                                                          ctagggc
TRBD2*02                                                       actagcgggagg
N2                                                                cg
TRBJ2-7*01                                                        cctacgagcagtacttcgggccgggcaccaggctcacggtcacag
CDR3                                                        agtgctagggcactagcgggaggcgcctacgagcagtac
TRBC1                                                                                                          gaggacctga
TRBC2                                                                                                          gaggacctga
Consensus         ACCAGTGCCCATCCTGAAGACAGCAGCTTCTACATCTGCAGTGCTAGGGCACTAGCGGGAGGCGCCTACGAGCAGTACTTCGGGCCGGGCACCAGGCTCACGGTCACAGAGGACCTGA
```

Figure A.12. Sequence alignment for HPA-1a-specific T cell clone D8T121

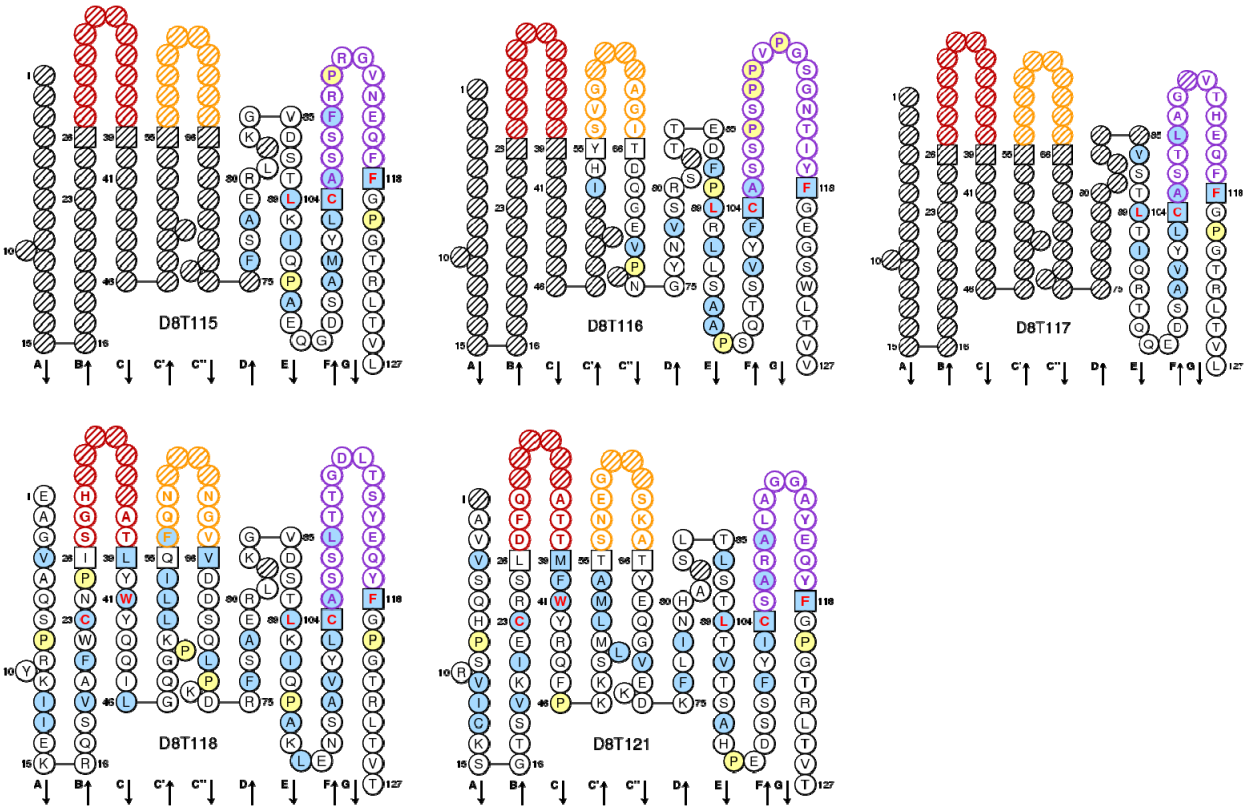

Figure A.13. Schematic visualization of the proposed amino acid sequences of the T cell receptor CDR3 domains (purple) of T cell clones D8T115, D8T116, D8T117, D8T118 and D8T121. This analysis was performed using the IMGT/Collier-de-Perles tool available at http://www.imgt.org. IMGT unique numbering for V-domains determines the lengths of CDR-IMGT (62).