UiT The Arctic University of Norway

Department of Medical Biology - Faculty of Health Sciences

Exploration of rabbit integrin β3 as a possible immune-tolerizing agent to prevent foetal and neonatal alloimmune thrombocytopenia.

Supervisors: Tor Brynjar Stuge and Maria Therese Ahlen

Lennart Maximilian van Ligtenberg

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Table of Contents

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Abbreviations

Ab	Antibody
APC	Antigen presenting cell
BCM	B-cell medium
BCR	B-cell receptor
B-LCL	B-lymphoblastoid cell line
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTLA4	Cytotoxic T lymphocyte-associated protein 4
CV	Column volumes
DC	Dendritic Cells
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FcRn	Neonatal Fc receptor
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
FSC	Forward scattered light
GALT	Gut associate lymphoid tissue
HLA	Human leukocyte antigen
HPA	Human platelet antigen
HTS	High-throughput screening

HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-15	Interleukin-15
IMDM	Iscove's modified Dulbecco's medium
Irr.	Irradiated
mAbs	Monoclonal antibodies
MHC II	Major histocompatibility complex class II
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA 0.2 %	Phosphate buffered saline + 0.2 % bovine serum albumin
PHA	Phytohemagglutinin
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SSC	Side scattered light
TACE	Tumor Necrosis factor-α-Converting Enzyme
TAPI-0	TNF-α Processing Inhibitor-0
TCM	T-cell medium
TCR	T-cell receptor
RBC	Red blood cells

Abstract

Foetal and neonatal alloimmune thrombocytopenia (FNAIT) is a disease caused by the production of antibodies by the mother to the platelets of the foetus. The alloimmune (from mother to unborn child) response is caused by a P33L substitution in the Human Platelet Antigen-1a (HPA-1a) in a homo- or heterozygous HPA-1a foetus, in a homozygous HPA-1b^{+} mother. Antigen presenting cells (APCs) of the mother recognize this substitution, often through the specific MHC II molecule called DRB3*01:01, and activate anti-HPA-1a^{+} platelet antibody production. Maternal platelets freely cross the placenta and induce platelet destruction in turn causing symptoms varying in severity like; purpura or cephalohematoma in the foetus. The production of maternal anti-platelet antibodies is assisted by HPA-1a specific T-cells able to bind the DRB3*01:01-HPA-1a antigen complex.

Studies have shown that DRB3*01:01 can also bind some other antigen peptides with hydrophobic residues amino acids at position 33 like valine, by which T-cells can be activated. Therefor it may be possible to find antigen from other (animal) sources that can also excite HPA-1a specific T-cells. Rabbit integrin β3 has homology with HPA-1a peptide recognized by HPA-1a specific T-cells except for a P33V substitution.

In this study 3 main aims will be followed, To determine if HPA-1a specific antibodies can bind rabbit platelets. 2) Examine whether rabbit platelets can activate HPA-1a specific T-cells. 3) To examine whether HPA-1a specific T cells can specifically recognize and be activated by rabbit integrin β3 protein, rabbit integrin β3 therefor needs to be isolated.. The rationale being to determine if rabbit integrin β3 is a viable antigen for oral tolerance induction therapy development in the long term.

A protein database was used for animal and human integrin β3 comparison for examination of theoretical binding capabilities of DRB3*01:01 and mAb 26.4 to various integrins and showed rabbit integrin β3 as compatible for both. TNF-α and proliferation assays were performed and showed rabbit integrin β3 is a viable activator of HPA-1a specific cells both for immediate and long term T-cell responses. integrin β3 isolation via affinity chromatography was attempted but shown to be unsuccessful via western blot. Thus, rabbit integrin β3 in platelet, protein or peptide form could potentially be used oral tolerance therapy development and should to be investigated further in the future.

1 Introduction

1.1 Fetal Neonatal Alloimmune Thrombocytopenia (FNAIT)

FNAIT is caused by the maternal formation of antibodies to human platelet antigen (HPA) phenotypically different from the HPA the mother carries, which then cross the placenta and destroy the fetal platelets (1). The maternal activation of anti-platelet antibody production by the immune system is induced by fetomaternal transfusions (or fetomaternal hemorrhage). This exposes the maternal antigen-presenting cells with the allogenic HPA (2, 3). In 1 in 800-1000 births, this mismatch in HPA occurs in pregnancies and these maternally produced anti-platelet alloantigen antibodies cross the placenta and induce platelet destruction. Causing an array of symptoms including: petechiae, purpura or cephalohaematoma at birth, associated with major risk of intracranial haemorrhage, which can lead to death or neurological sequelae in the child (2, 3). Recognition of allogenic HPA-1a, the most common HPA in the western world, is caused by a very specific difference in the integrin β3 amino acid chain: L33P. The change in amino acid causes the immune system of the HPA-1b/b individual to react to the HPA-1a/b or a/a individual (or also other genotypically different HPA variants) (1, 4-6). Additionally, many HPA-1a immunized women carry the MHC class II allele HLA-DRB3*01:01, indicating that anti-HPA-1a antibody production is assisted by T-cell responses restricted to this specific allele (6).

1.2 Human Platelet Antigens

Platelets present various platelet surface protein that have been found to be important in platelet specific auto- alloimmune diseases. They often exist in 2 or more phenotypic types that have single nucleotide polymorphisms (SNP's), which may be processed into antigens and are called human platelet antigens (HPA). These HPA can induce auto- or alloimmune responses when introduced to an individual through activation of the adaptive immune system (7). HPA-1 is the antigen associated with FNAIT as at least 3 phenotype HPA-1a, b and c (8, 9).

1.2.1 Integrin structure and function.

HPA-1 part of integrin β3, is a member of the integrin receptor family being adhesion molecules that facilitate interaction between cell and other cell, extracellular matrix or pathogen (10). Integrins regulate many biological functions including cell differentiation, wound healing and cell migration. Integrin consist generally of two non-covalently associated α and β subunit with long extracellular, single-spanning transmembrane domains and short cytoplasmic domains (10, 11). Integrins can relay signals bidirectionally across the plasma membrane, by ligand binding on the globular end of the extracellular domain and changing their conformation into higher or lower affinity positions (10, 12). The conformation of integrins is for example important in its active form for the adhesion to and exiting from blood vessels or in integrin β3 coagulation (7, 12).

1.2.2 Integrin β3 connection with FNAIT.

HPA-1, located on the membrane glycoprotein (GP) IIIa also called integrin β3, which mainly is found in three different variants, determined by a diallelic antigen system. HPA-1's three known phenotypes are, HPA-1a, 1b and 1c which respectively have a leucine, proline or valine at amino acid position 33 (8, 9). HPA-1c has been only reported once in the literature. This specific alloantigen is found with a 98% HPA-1a variant (a/a or a/b) ratio in Caucasians and in a 2% ratio HPA-1b (b/b), where the only difference in the amino acid chain is an L33P substitution (leucine to proline in the 33th position) (13). This SNP could in an HPA-1a^{+} fetus in pregnant HPA-1bb mothers cause HPA-1a specific antibodies to be made. Integrin β3 associates with 2 α variants; αIIb and αV (10). The αIIbβ3 receptor is found on platelets in quantities around 80.000 proteins per platelet, with the main function being platelet coagulation binding with fibrinogen, fibronectin, von Willebrand factor and vitronectin (7, 10, 14). The αVβ3 receptor can also be found on platelets at a quantity of a few thousand per platelet and are otherwise found on endothelium and it binds mainly fibronectin, fibrinogen and vitronectin (7, 15). The location of these integrin receptors explains the symptoms seen in fetuses in mothers with an FNAIT antibody response. HPA-1a specific antibodies bind and opsonize αIIbβ3 on platelets and αVβ3 on platelets and endothelium (16, 17). Which can

cause thrombocytopenia and brain hemorrhaging, due to endothelium apoptosis, dependent on the type of HPA-1a specific alloantibodies (16).

1.3 Major histocompatibility complex

The human major histocompatibility complex (MHC) or human leukocyte antigen (HLA) class II are the molecules on immunologic cells that present peptide antigens to T-cells. The MHC II consists of non-covalently bound α- and β-chains, which are highly genetically polymorphic, except the DR α-chain. The MHC II isotypes an individual will present on immune cells is dependent on the haplotype, or inherited versions of the different DQ, DR and DP α- and β-chains; the three loci encoding MHC class II molecules.

1.3.1 DRB3*01:01

DRB3*01:01 is highly associated with the immunization of HPA-1bb woman with HPA-1a (4, 18-20). Studies have shown that while DRB3*01:01 has a prevalence of ~28% in the normal population, the prevalence in alloimmunized women ~90-94% (4, 18-20). This link was further supported and examined by an article by Wu S. et al. where was found that L33 is one of three important anchoring motifs to HLA-DRB3*01:01 it. Where L33 binds significantly stronger than P33 (21). As well as an article by Parry C. S et al. where it was found that L33 binding to HLA-DRB3*01:01 is facilitated by a hydrophobic pocket in HLA-DRB3*01:01 (22), and in two articles by Ahlen M. T et al. finding a link between FNAIT and HLA-DRB3*01:01 showing binding via indirect allorecognition (4, 6).

1.3.2 Structure and Peptide Binding

The normally canonical motif for binding pocket in other alleles of MHC includes P6 but is silent in DRB3 thus having the distinctive P1-P4-P9 motif (22). Binding pocket P1 is a large and very hydrophobic, P4 has a high preference for negatively charged residues and P9 is a

also very hydrophobic (22). The characteristic of the specific pockets as well as the general morphology of the binding groove of the MHC class II molecules postulates that only certain peptides with the residues matching the different pockets will be able to bind there. The P9 binding pocket seems to have a determining role for the binding of HPA-1a peptide to the DRA/DRB3*01:01 MHC class II molecule. Peptides in which substitutions introduced in other positions than P9 did not influence binding significantly while the P33 substitution was not able to bind DRB3*01:01 (4). P33 was likely unable to bind due to steric hindrance of a cyclic residue as well as not being hydrophobic indicating MHC restriction.

1.4 T-cell Mediated Immune Responses against HPA-1a

In response to an alloantigen like HPA-1a, a large amount of signals and mechanisms are initiated for antigen uptake, differentiation of B-cell in B-lymphoblast for antigen production and T-cell activation and maturation in T-helper cells (12). T-cells are an important part of the humoral response seen as main effector mechanism in FNAITs. Naïve T-helper cells are stimulated by APCs presenting HPA-1a antigen. Activated T-helper cells start expressing and excreting a plethora of chemokines, surface proteins and cytokines. Once the activated T-helper cells have received sufficient stimulation by the MHC II-HPA-1a complex, B7 and cytokine they will start proliferation as well. Specific B-cells are meanwhile also presented with HPA-1a and starts to express various chemokines, surface proteins and cytokines as well. Activated B-cells travel from the follicle towards T-helper cells attracted to each other by chemokines. On interaction with the activated B-cell presents HPA-1a antigen to the activated T-helper cell and together with CD40 and cytokines induce B-cell proliferation and differentiation (12). B-lymphoblasts will then produce HPA-1a specific antibodies and undergo affinity maturation (12).

1.4.1 T-cell activation and proliferation study.

TNF-α secretion and proliferation are both import indicators of T-cell activation. Both have been used to measure the response of HPA-1a specific T-cells to HPA-1a and HPA-1b antigen. HPA-1a specific T-cells have shown to be specific to HPA-1a antigen but not HPA-

1b antigen both in PBMC from immunized women (23) and with clonal HPA-1a-specific $CD4^+$ T cells generated from PMBC isolated from immunized women (6, 24).

TNF-α is a pyrogen which is excreted on activated T-cells in its precursor membrane bound form. Tumor necrosis factor-α-converting enzyme (TACE) is a metalloproteinase that can cleave membrane bound TNF-α at the ala 76- val77 site (25). Only after TACE has cleaved the extracellular TNF-α section from its membrane-bound section is TNF-α truly extracellular excreted (25). TNF-α can bind either the TNFR1 non-immune cells or on immune cells, and activate its pyrogenic functions appropriate for each cell type. TNF-α Processing Inhibitor-0 (TAPI-0) is a metalloproteinase inhibitor specific for TACE (26) and TAPI-0 can prevent the precursor TNF-α from being released and solubilized. TNF-α will then remain, in its precursor form in the surface of the cell. TNF-α expression of a T-cell can then be stained for TNF-α and accurately measured by flow cytometry in an TNF-α excretion assay (Fig 1-1).

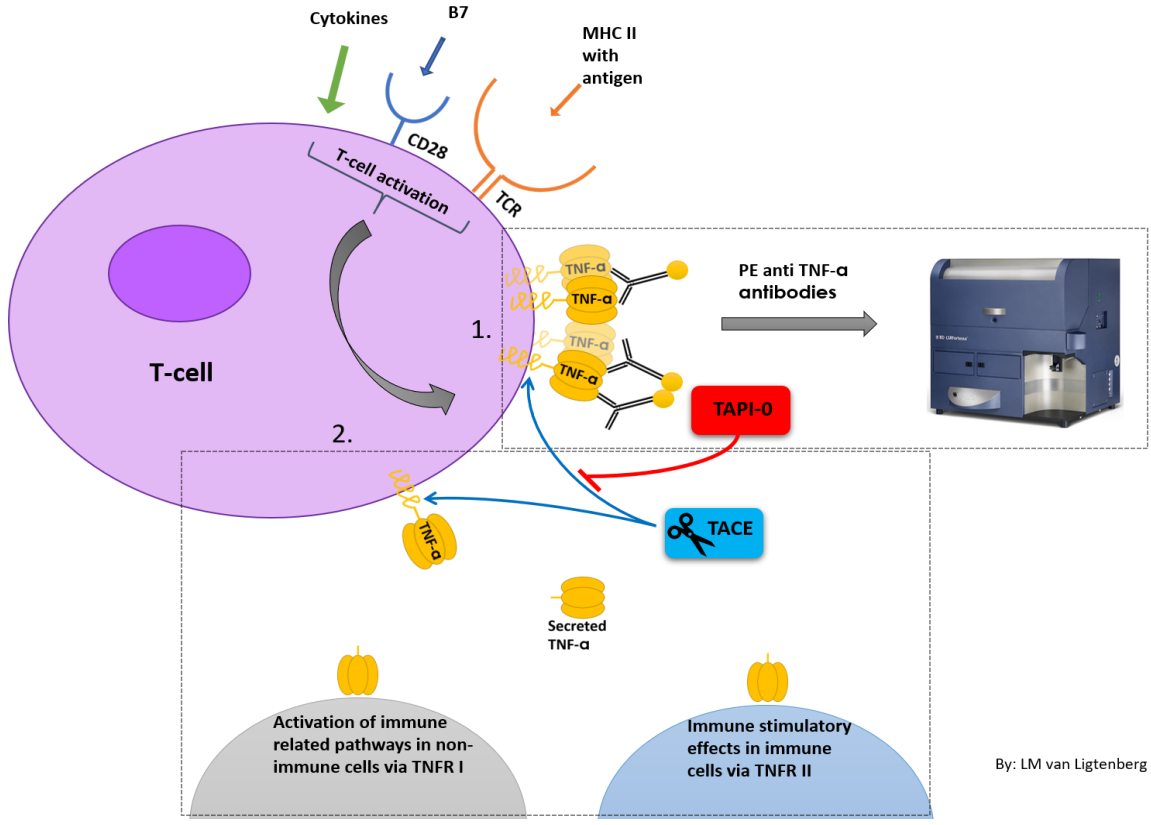

Figure 1-1 ***TNF-α excretion assay mechanism.***

Upon T-cell activation by the three main signaling routs, MHC II-peptide complex to TCR, B7 interaction with CD28 and cytokine stimulation, the T-cells starts excreting TNF-α. In 1. the TNF-α excretion assay is shown and in 2. the normal TNF-α excretion mechanism. Normally TNF-α will be cleaved from its transmembrane domain and do its effector function as a cytokine, in the assay however is TAPI-0 inhibiting TACE. TNF-α thus remains on the activated T-cell surface, can be bound by a fluorophore conjugated anti-TNF-α antibody and measured by flow cytometry.

Carboxyfluorescein succinimidyl ester (CFSE) staining was first used in immunological studies studying the migration of immune cells because of its ability the bind cellular protein stably over long periods of time (27). CFSE's stability and the fact that cellular proteins divide equally between the daughter cells made it an effective way of extracting information about the T-cells proliferation (28). CFSE staining is done with a compound called carboxyfluorescein diacetate succinimidyl ester which is not fluorescent in this state due to the two acetate groups present, but making the molecule highly permeable (27). The two acetate groups are rapidly removed by esterase's present in the cells cytoplasm making the compound able to be fluorescent (27). The succinimidyl group allows the CFSE to create highly stable bonds with cellular protein binding to lysine residues (27). Carboxylfluorescein bound to protein in the cells gives the fluorophore its stability for up to several months and is only diminished by division of the stained cells (27, 28). Up to 8 cell divisions can be made visible before the CFSE is too diluted to be detected reliably by flow cytometry (Fig 1-2).

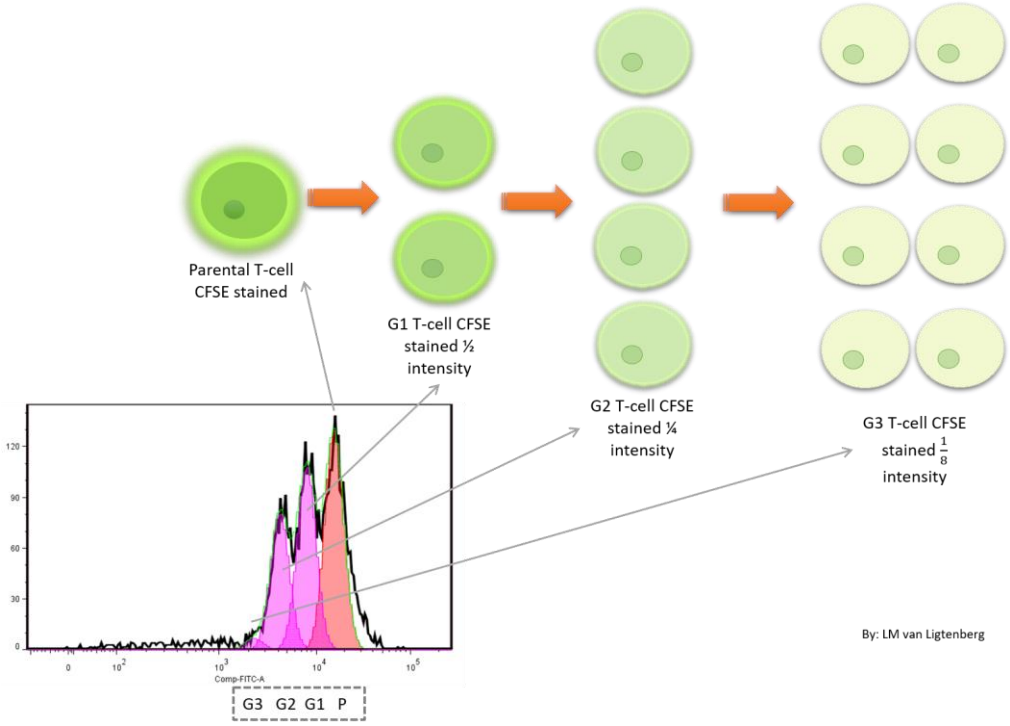

Figure 1-2 ***Proliferation assay mechanism.***

Activated T-cells will start proliferating and this can be measured with a proliferation assay. CFSE is fluorophore able to bind protein in the T-cells, is stable over long periods of time and is equally divided between daughter cells upon cell division. The CFSE mechanism causes the fluorescence intensity to half on every division as shown in the top. The parent population (P) is the population not activated, while activated T-cells will proliferate and decrease their fluorescence intensity by half in every subsequent generation (G1, 2, 3). CFSE fluorescence intensity can be measured by flow cytometry and thus the parent and daughter cell generation can be plotted on a histogram. The parent generation is shown in red while daughter generations are shown in pink shown in the bottom.

1.5 Oral Tolerance

Oral tolerance is the suppression of systemic immune responses to a specific antigen following oral administration of the antigen. Induction of oral tolerance depends on activation of gut immune system such that subsequent immune responses to tolerogenic antigen are suppressed (29).

The main role of the gut associated lymphoid tissues (GALT) is maintaining tolerance towards the many food proteins, commensal microbiota and some other xenobiotics, while also protecting from the occasional pathogen (12, 29, 30). The GALT thus, has to choose and is able to react actively and passively to respectively harmful and benign foreign antigens. Generally the Peyer's patches are the initial site were antigens are detected and taken up by the immune system followed by presentation to multiple other types immune- or tolerogenic of T- and B-cells in the mesenteric lymph nodes (12, 29, 30).

1.6 Anti-human integrin β3 antibodies.

Four mono clonal antibodies (mAb) were used for the detection of HPA-1/integrin β3. Y2/51 and AP3 have both been shown to be specific to integrin β3 but not HPA-1a (31, 32). Mab SZ21 has been shown previously to be pseudo specific. SZ21 is mainly specific to HPA-1a but has some affinity in a dose dependent manner for HPA-1b as well (5, 33). The mAb 26.4 antibody has high affinity for HPA-1a but not for HPA-1b. The antibody was produced from an immortalized HPA-1a specific B-cell isolated from a HPA-1a immunized woman (5). Additionally is the epitope of mAb 26.4 at least in part specific for the same epitope recognized by the T-cell receptor (TCR) when the HPA-1a antigen is presented in DRA/DRB3*01:01. Mab 26.4 is known to be sensitive for amino acids in position 30, 32, 33, 39, 470 although many more amino acids may be indicated in mAb 26.4 binding (34).

1.7 Rabbit integrin β3.

The human integrin β3 gene ITGB3 on chromosome 17 and rabbit ITGB3 on chromosome 19 show 81.79 % homology (35). Rabbit integrin β3 has similar functions in rabbits as it has in human. Protein alignment of the rabbit and human integrin β3 shows up to 95% homology with 750 identical amino acid position and 29 that are similar (36). The epitope associated with FNAIT in rabbit shows homology with the rare HPA-1c epitope. Which has a valine at amino acid position 33 instead of a leucine. (8).

```
P05106 ITB3_HUMAN           1   MRARPRPRPLWATVLALGALAGVGVGGPNICTTRGVSSCQQCLAVSPMCAWCSDEALPLG        60
Q9TUN3 Q9TUN3_RABIT         1   MRARARPRPLWAAVLALGALAGVGVGGPNICTTRGVSSCQLCLAVSPMCAWCSDEALPVG        60
                                **** ********:*************************** ******************:*

P05106 ITB3_HUMAN          61   SPRCDLKENLLKDNCAPESIEFPVSEARVLEDRPLSDKGSGDSSQVTQVSPQRIALRLRP       120
Q9TUN3 Q9TUN3_RABIT        61   SPRCDLRENLLQDNCAPESIEFPVSEAQILEARPLSDKGSGDSSQVIQVSPQRIALRLRP       120
                                ******:****:***************::** ************** *************

P05106 ITB3_HUMAN         121   DDSKNFSIQVRQVEDYPVDIYYLMDLSYSMKDDLWSIQNLGTKLATQMRKLTSNLRIGFG       180
Q9TUN3 Q9TUN3_RABIT       121   DDSKTFSIQVRQVEDYPVDIYYLMDLSYSMKDDLRSIQNLGTKLASQMRKLTSNLRIGFG       180
                                ****.****************************** **********:**************

P05106 ITB3_HUMAN         181   AFVDKPVSPYMYISPPEALENPCYDMKTTCLPMFGYKHVLTLTDQVTRFNEEVKKQSVSR       240
Q9TUN3 Q9TUN3_RABIT       181   AFVDKPVSPYMYISPPEALRNPCYDMKTTCLPMFGYKHVLTLTDQVTRFNEEVKKQNVSR       240
                                ******************.*************************************.***

P05106 ITB3_HUMAN         241   NRDAPEGGFDAIMQATVCDEKIGWRNDASHLLVFTTDAKTHIALDGRLAGIVQPNDGQCH       300
Q9TUN3 Q9TUN3_RABIT       241   NRDAPEGGFDAIMQATVCDEKIGWRNDASHLLVFTTDAKTHIALDGRLAGIVRPNDGQCH       300
                                ************************************************:*******

P05106 ITB3_HUMAN         301   VGSDNHYSASTTMDYPSLGLMTEKLSQKNINLIFAVTENVVNLYQNYSELIPGTTVGVLS       360
Q9TUN3 Q9TUN3_RABIT       301   IGGDNHYSASTTMDYPSLGLMTEKLSQKNINLIFAVTENVFNLYQNYSELIPGTTVGVLS       360
                                :*.*************************************.******************

P05106 ITB3_HUMAN         361   MDSSNVLQLIVDAYGKIRSKVELEVRDLPEELSLSFNATCLNNEVIPGLKSCMGLKIGDT       420
Q9TUN3 Q9TUN3_RABIT       361   TNSSNILQLIVDAYGKIRSKVELEVRDLPEELSLSFNATCLNNEVIPGLKSCVGLKIGDT       420
                                 :***:************************************************:*******

P05106 ITB3_HUMAN         421   VSFSIEAKVRGCPQEKEKSFTIKPVGFKDSLIVQVTFDCDCACQAQAEPNSHRCNNGNGT       480
Q9TUN3 Q9TUN3_RABIT       421   VSFSIEAKVRGCPQEKEKSFTIKPVGFKDSLTVQVTFDCDCACQAHAEPHSHRCNNGNGT       480
                                ****************************** *************:***:***********

P05106 ITB3_HUMAN         481   FECGVCRCGPGWLGSQCECSEEDYRPSQQDECSPREGQPVCSQRGECLCGQCVCHSSDFG       540
Q9TUN3 Q9TUN3_RABIT       481   FECGVCRCGPGWLGSQCECSEEDYSPSQQDQCSPKEGQPICSQRGECLCGQCVCHSSDFG       540
                                ********************** *****:***:****:********************

P05106 ITB3_HUMAN         541   KITGKYCECDDFSCVRYKGEMCSGHGQCSCGDCLCDSDWTGYYCNCTTRTDTCMSSNGLL       600
Q9TUN3 Q9TUN3_RABIT       541   KITGKYCECDDFSCVRYKGEMCSGHGQCSCGDCLCDSDWTGYYCNCTTRTDTCMSSNGLL       600
                                ************************************************************

P05106 ITB3_HUMAN         601   CSGRGKCECGSCVCIQPGSYGDTCEKCPTCPDACTFKKECVECKKFDRGALHDENTCNRY       660
Q9TUN3 Q9TUN3_RABIT       601   CSGRGKCECGSCVCIQPGSYGDTCEKCPTCPDACTFKKECVECKKFERGVLHEENTCSRY       660
                                ***********************************************:**.**:****.**

P05106 ITB3_HUMAN         661   CRDEIESVKELKDTGKDAVNCTYKNEDDCVVRFQYYEDSSGKSILYVVEEPECPKGPDIL       720
Q9TUN3 Q9TUN3_RABIT       661   CRDEMESVKELRDTGKDAVNCTYKNEDDCVVRFQYYEDSSGKSILYVVEEPECPKGPDIL       720
                                ****:******:************************************************

P05106 ITB3_HUMAN         721   VVLLSVMGAILLIGLAALLIWKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTF       780
Q9TUN3 Q9TUN3_RABIT       721   VVLLSVMGAILLIGFALLLIWKLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTF       780
                                **************:* ******************************************

P05106 ITB3_HUMAN         781   TNITYRGT                                                    788
Q9TUN3 Q9TUN3_RABIT       781   TNITYRGT                                                    788
                                ********
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Figure 1-3 ***Alignment of human and rabbit integrin β3.***

*Here the amino acid alignment of human and rabbit integrin β3 or shown. * represent the amino acids in that position being identical, while : means a similar type of amino acid is found in that position. An empty space signifies an amino acid substitution in rabbit integrin β3 of an amino acid not similar to human integrin β3.The human HPA-1a T cell epitope is highlighted. The alignment was made with the proteins with identifier P05106 (human integrin β3) and Q9TUN3 (rabbit integrin β3) on https://www.uniprot.org/align/A20200806E5A08BB0B2D1C45B0C7BC3B55FD265560A1FABN.*

1.8 Hypothesis and Aims of this Study.

Recently found data from Immunology group at UiT showed that a similar immunological response might be produced by substitution of the Leucine with another small hydrophobic amino acid, like Valine in the HPA-1a T-cell epitope (4, 6, 21, 22). This specific amino acid sequence is found in the rabbit Integrin β3 and might thus be able to elicit similar responses from a human immune system (4). Many new discoveries have been made recently in oral tolerance and its uses in allergy reduction and application for autoimmune diseases. Oral tolerance may be induced by digested antigen presentation to the gut-associated lymphoid tissue. HPA-1a or rabbit Integrin β3 could following this line of thinking be used to induce oral tolerance in individuals at risk for FNAIT in the future (29).

During this master the following objectives will be followed. 1) To determine if HPA-1a specific antibodies can bind rabbit platelets. 2) Examine whether rabbit platelets can activate HPA-1a specific T-cells. 3) To examine whether HPA-1a specific T cells can specifically recognize and be activated by rabbit integrin β3 protein, rabbit integrin β3 therefor needs to be isolated. The goals presented in this thesis have the long-term aim in mind; to prevent the occurrence or reduce symptoms of FNAIT by induction of oral tolerance against human platelet alloantigen (HPA)-1a, in pregnant women.

2 Materials and Methods

2.1 Materials and Reagents

All reagents, buffers and media used are mentioned in Table 1 Appendix. And a list of all used antigens of different types are listed in Table 1.

Table 1 Complete list of antigens used for T-cell stimulation during this master thesis.

Name	Antigen Type	Amino acid Sequence of Interest*
HPA-1a	Platelet	AWCSDEALPLGS
	Protein	AWCSDEALPLGS
L33	Peptide	AWCSDEALPLGS
HPA-1b	Platelet	AWCSDEALPPGS
Rabbit integrin β3	Platelet	AWCSDEALPVGS
Rabbit integrin β3	Protein	AWCSDEALPVGS
lolP1	Peptide	VWRIDTPDKLTG

B-cell medium (BCM) is a mixture of Iscove's Modified Dulbecco's Medium (IMDM; Lonza BioWhittaker, Verviers, Belgium) with 10 % Fetal Bovine Serum (FBS; Gibco by Life Technologies), 1 % PenicillinStreptomycin (Sigma-Aldrich).

And T-cell medium (TCM) consists of Iscove's Modified Dulbecco's Medium (IMDM; Lonza BioWhittaker, Verviers, Belgium) with 10 % Fetal Bovine Serum (FBS; Gibco by Life Technologies), 4 % human HPA-1bb serum and 1 % PenicillinStreptomycin (Sigma-Aldrich). All media used were sterile filtrated using Nalgene – RapidFlow filter bottles and then stored at 4 °C. Human serum used in TCM was prepared from plasma from HPA-1a$^-$ donors obtained from the blood bank at UNN Tromsø. The anticoagulant $CaCl_2$ was added to the plasma at 1:100 ratio and incubated overnight at 37 °C. The following day all fluid with clumping was centrifuged for 15 min at 3000 g and the serum supernatant was transferred to a new tube. To inactivate the complement system in the serum a heat inactivation treatment was performed leaving the tubes in a water bath for 25 min at 56 °C. The heat-inactivated serum is stored at -70 °C and thawed when needed for medium preparation.

Peptides used were all obtained from Eurogentec (Liege, Belgium). Platelets were obtained from donors and are further discussed in the Cells and Platelets section. All antigens used for determining their ability to activate T-cell were of HPA-1a, HPA-1b or integrin of animal

origin. lolP1 peptide was used in the T-cell activation assay as a negative control exploiting its ability to stably bind in the HLA-DRB3*01:01 binding pocket without activating the HPA-1a specific T-cells (21, 37). The peptides were dissolved in 40% water and 60% ethanol stored at –20°C

2.2 Cells and Platelets

The current study was performed without need of approval of the Regional Committee for Medical Research Ethics, North-Norway. Buffy coats obtained from the blood bank at UNN were from consenting anonymous donors and only used for research purposes, thus not in need of REK approval.

2.2.1 HPA-1a specific T-cell and B-LCL clones

HPA-1a specific T-cell clones that were used during the study were previously isolated and stored in nitrogen storage at the Immunology Research group at UiT as described by Ahlen M.T et.al. (38, 39).

HLA-DRB3*01:01^{+} B-LCL (D4BL4 and D48BL6) used during the study were previously isolated and stored in nitrogen storage at the Immunology Research group at UiT as described by Ahlen M.T et.al. (38, 39). All B-cell and T-cell clones Epstein-Barr virus (EBV) transformed were isolated from HPA-1bb, HLA-DRB3*01:01-positive donors.

2.2.2 Platelets

Platelets used were isolated from HPA-1a/a and HPA-1b/b anonymous individuals donating blood at the Diagnostic Clinic at the University Hospital of North Norway (. All blood samples obtained for this purpose were from consenting anonymous donors and only used for research purposes. Additionally animal platelets used in this study were bought from Charles River, France /the European rabbit breed (Oryctolagus Cuniculus).

2.3 Cryopreservation

Cells in cryopreservation can be stored for up to several years without the need to keep the cells in culture. Cell are suspended in a medium that protects the cell from the rapid freezing process, cooling the samples to -196 °C. Samples are pelleted and resuspended in freeze medium consisting of 90 % FBS and 10 % DMSO. Freezing cells at high cell concentration is recommended and were usually between 1-10*10^6 cells/ 1,5 mL. Cryotubes with 1,5 mL of cells suspension are pre-cooled -1°C/minute to -70 °C and within a week transferred to nitrogen storage at -196 °C. Samples were thawed quickly placing the cryotubes in a 50 °C water bath until only a fraction of the sample is frozen. The last frozen fraction was thawed with body heat and quickly transferred and resuspended to a 15 mL tube containing 10 mL 4 °C BCM or TCM.

2.4 Cell Culturing

All cells cultured were kept 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere and regularly split and/or washed depending on future experiments, cell viability and confluence. B-LCL are EBV-transformed and grew without external stimulation, the HPA-1a specific T-cells however need to be stimulated to proliferate done by T-cell expansion of the culture. Cells were kept in culture flasks or culture plates fitting to the culture volume needed for specific goals. Cells were split when the pH drops below 7.2 pH (yellow culture medium), generally every 2 to 7 days. B-cells were cultured in BCM while T-cells were cultured in TCM, generally with added IL-2 (50U/mL) and if when T-cells looked less viable IL-15 (5ng/mL). Cultures was washed after every 2-3 rounds of splitting to avoid build-up of cellular waist decreasing viability of the cells. Extreme care was taken to adhere to a high standard of sterility during culturing and experiments as most or long term cultures.

2.5 Expansion of Antigen-Specific T-cell lines

To enumate clonal HPA-1a specific T-cells they have to be expanded by stimulation with appropriate antigen. Antigens that were used as stimulants for expansion were mainly PHA and anti-CD3 Ab but HPA-1a antigen could also be used. To prepare feeder cells, B-LCL cultures were started 1 week before and feeder cells were isolated by density gradient the day before and rested. B-LCL, T-cell and feeder cells all were counted on a haemocytometer and feeder cells were resuspended in TCM. Both feeder cells (B-LCL and PBMC's) were radiated (16.000 rad). On a 24 wells plate the following was added per well; $2*10^5$-$5*10^5$ T-cells, B.LCL-cells $7{,}5*10^5$ cells, PBMC $3*10^6$ cells and TCM containing cnti-CD3 Ab (30 ng/mL of expansion culture) to a total volume of 2 mL. Generally between 1,5-$3*10^6$ T-cells can be obtained per well after expansion and an appropriate amount of well were expanded for future experiments. The expansion was incubated 37°C, in a 7.5% CO2 humidified atmosphere for 7-14 days depending on T-cells confluency following normal T-cell culture protocol. On the second day IL-2 (50 U/mL) and IL-15 (5ng/mL) to each expansion well. After expansion T-cells were washed or lymphopreped to remove the stimulant and dead cell debris.

2.6 Density gradients

2.6.1 PBMC and Red Blood Cell isolation from Buffy Coats

For the isolation of PBMC's and red blood cell (RBC) and also in the monocyte enrichment protocol the density gradient lymphoprep is used to separate cells depending on their density. With lymphoprep, 3 fractions can be obtained; from bottom to top RBC/polymorphonuclear cells, PBMC and plasma. Depending what is need one or both PBMC's and RBC were isolated following this protocol.

Here, mainly buffy coats (50 mL) were used and for monocyte enrichment smaller sample sizes were used. The samples were placed in an appropriate size tube (15,50 mL) and the samples were diluted with the same amount of PBS. The diluted sample was mixed by pipetting with a pipetboy and layer carefully on 2-3 cm (3 mL in 15 mL tube and 12,5 mL in

50 mL tube) The tubes are centrifuged for 20-30 minutes at 800 g with no brakes. The interphase (PBMC's) is transferred to a new 15 mL tube. If RBC are isolated they can be pipetted in a similar manner to a separate tube after the interphase has been isolated. The isolated sample tubes are centrifuged at 250 g for 6 minutes washed with 10 mL 0,2% PBSA and supernatant decanted 3 times. The samples are resuspended in BCM after the last wash. The cells are then kept in the incubator until used at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere.

2.6.2 Preparation of Platelet Rich Plasma

Preparation of platelet rich plasma (PRP) is performed on either whole blood or buffy coats from an appropriate donor. Either Heparin or EDTA should be added to the culture medium to avoid coagulation of the platelets. If no anticoagulant can be used (the following protocol may not work with an anticoagulant) extreme care should be taken not to disturb or move the platelets too harshly or platelet activation may occur. A soft spin is performed on the sample for 15 minutes at 150 g. The yellow supernatant (top layer) is collected from the falcon tube and pipetted into a new 15 or 50 mL tube. The tube is centrifuged for 2 minutes a 200 g and again the supernatant is removed and place in a new tube. Then, a final spin is performed for 10 minutes at 2000 g to pellet the platelets and the supernatant is decanted. The platelet pallet is resuspended in BCM or TCM depending on what it is used for in following protocols. Platelet rich plasma will from now on be written as platelets.

2.6.3 Monocyte Enrichment

Monocyte enrichment was performed to obtain monocytes used as APC's in T-cells activation assays. For the enrichment of monocytes from PBMC's the RosetteSep™ Human monocyte enrichment cocktail (Stemcell technologies) was used. The protocol provided by the manufacturer was followed with slight modifications. In short, 800 µL of RBC were isolated with lymphoprep (see PBMC and Red Blood Cell isolation from Buffy Coats). PBMC containing HLA-DRA/DRB3*01:01 MHC class II monocytes were thawed and rested the day before to rest the PBMC's. PBMC's were then resuspended in 1,2 mL FBS and

mixed with the 0,8 mL of RBC as well as 100 µl ResetteSep Monocyte Enrichement cocktail. The mixture was incubated for 20 minutes at room temperature and then diluted with 2 mL of BCM before the 4 mL mixture was layered, carefully, on top of the lymphoprep. From here lymphoprep protocol was followed again and monocytes were washed 3 times with 0,2% PBSA (see PBMC and Red Blood Cell isolation from Buffy Coats). Enriched monocytes were used for antigen pulsing.

2.7 Flow cytometry and Fluorescent Staining of Cells and Platelets

For this project, the BD Fortessa at UiT Core Facilities was used either by manual loading of flow tubes or with a High-Throughput Screening (HTS) system in 96w format. PMT voltages were routinely calibrated with BD CS&T Beads, and the compensation set-up was calculated with single stained samples. Various protocols made use of flow cytometry analysis which were described in section in detail 2.8, 2.10.2, 2.11, 2.12 and 2.13. Fluorophore conjugated antibodies and their functions are shown in Table 3.

Table 2 Complete list of the fluorophore conjugated antibodies used during this master thesis used in flow cytometry and CFSE.

Target	Name	Produced by	Product number	Amount used (µL)	Function
HPA-1a	26.4 – Alexafluor 488	In house	\	0,5	HPA antibodies specificity assay
HPA-1a	SZ-21 - FITC	Bechman Coulter	IM1758	1,6	HPA antibodies specificity assay
HPA (human)	Y2/51 - FITC	Dako	F0803	2,5	HPA antibodies specificity assay
HPA (human)	AP-3 – Alexafluor 488	?	?	0,25	HPA antibodies specificity assay
TNF-α	PE-mouse anti-human TNF-α	BD Pharmingen	554513	0,3	TNF-α assay
	CFSE Celltrace	Thermo Fisher	C34554	5 µL /500 µL T-cells	Proliferation assay
CD3	CD3-PE (compensation)	Biolegend	300408	2	Proliferation assay
CD14	CD14-PE	Biolegend	301806	1	Proliferation assay
CD4	CD4-PE-AlexaFluor 610	Invitrogen	MHCD0422	0,5	Proliferation assay

Information about similarities and differences with regards to epitope reactivity in HPA-1a, HPA-1b and Rabbit Integrin was gathered by flow cytometry Here the following antibodies were used; AP3 (Alexa Fluor 488) and Y2/51 (FITC; Fluorescein isothiocyanate) are integrin β3-specific, 26.4 (Alexa Fluor 488) is HPA-1a-specific and Sz21 (FITC) is, in low concentration, shown to be HPA-1a-specific (33). The samples were gated for platelets on a double logarithmic scale and the Alexa Fluor 488 and FITC (emission maximum at 519 nm) fluorophores intensity were measured using the B detector (530/30 nm filter) on the blue (488 nm) laser.

To analyse the ability of B-LCL to load the L33 and lolP1 peptides a peptide-biotin + streptavidin-PE (emission maximum at 578 nm) setup was used and detected with the E detector (585/15 nm filter) on the YellowGreen (561 nm) laser. The samples were gated for B-LCL.

To determine if and how much HPA-1a specific T-cell were activated by measure of proliferation, a TNF secretion and CFSE proliferation assay were performed. The TNF-α assay was performed using PE-mouse anti-human TNF-α (emission maximum at 578 nm) was used measuring intensity using the E detector (585/15 nm filter) on the YellowGreen (561 nm) laser. The samples were gated for $CFSE^+$ T-cells and shown in a histogram with CFSE intensity on the x-axis and TNF-α (PE) expression of the y-axis. In the proliferation assay CFSE, CD3-PE (compensation), CD14-PE and CD4-PE-AlexaFluor 610 mAb conjugates were used. PE was detected as previously described and PE-AlexaFluor 610 (emission maximum at 628 nm) intensity was measured using the YellowGreen (561 nm) laser on the D filter. First T-cells were gated in a FSC/SSC plot, this population was again gated for $CD4^+$ $CD14^-$ T-cells and this population was shown in a histogram for CFSE intensity.

2.8 Flowtest of platelet opsonization by integrin β3 specific antibodies.

Human HPA-1aa, HPA-1bb and rabbit platelets were reacted with four different antibodies The examined antibodies will be; the anti-HPA-1a mAbs 26.4 (0.6 mg/mL, AF-488) and SZ21 (0.15 mg/mL, FITC), the anti-β3-integrin mAbs Y2/51 (0.1 mg/mL, FITC) and AP-3 (1 mg/mL, AF-488).

The antibodies were diluted in 0,2 % PBSA containing 2 mM EDTA at a concentration of 10 µg/mL and. 25 µL per well in a 96 wells round bottom plate. 25 µL of platelet suspension was added to mixed by pipetting and incubated for 30 minutes on ice in the dark. TCM was added to the plate was centrifuged at 1900g for 5 minutes, fluid decanted and washed twice with 200 µL 0,2% PBSA/EDTA per well. After a final centrifugation and decanting of wash buffer a 100 µL of 0,2% PBSA/EDTA was added, and kept on ice until analysis. The samples were run on the flowcytometer and analyzed with FlowJo^{TM} v7/8 (Becton Dickinson and Company, California).

2.9 Carboxyfluorescein N-succinimidyl Ester (CFSE) Staining

CFSE staining is the process where the non-fluorescent highly permeable carboxyfluorescein diacetate succinimidyl ester is enzymatically altered by the removal of two acetate groups (27). Hereafter the molecule can fluoresce and binds stably with cellular protein lysine residues (27). This technique was mainly used to stain T-cell over long periods of time for flow cytometry analysis and proliferation assays. CFSE is explained in more detail in the proliferation assay section.

To be stained cell are spun down cells washed twice with PBS and the medium is discarded. The cell pellet is resuspended in 500 µl PBS. 10 µl 250 µg/ml CFSE dye is transferred to the resuspended cells (5 µM). The suspension mixed thoroughly and immediately transferred to 37˚C water bath for 10 min. After the of incubation 5 mL of ice cold IMDM 10% FBS 1% Pen/Strep is added and the cells are transferred to ice for 5 minutes. The cells are centrifuged 4 min at 350 x g and washed with PBSA 3 times. 1-2 ml in IMDM + 10% FBS + 4% human serum 1% Pen/Strep (TCM) is added to the T-cells and placed in the incubator at 37°C, in a

7.5% CO_2, 6,3% O_2 in a humidified atmosphere to rest until used for experimentation. If the stained T-cells are kept for more than a few hours IL-2 is added in a concentration of 50 U/mL.

2.10 Antigen Pulsing of APC and T-cells

The pulsing of APC including B-LCL and enriched monocytes from HLA-DRB3*01:01 positive donors was used in several different assays. Therefore the method used for pulsing various cells with various compounds is described here separately. And will in the experiments where they were used be referred to as for example "platelet derived HPA-1a pulsed monocytes". All pulsing protocols below for APC are per well and yield enough cells to stimulate 1 well of T-cells on a 96 wells round bottom plate. An appropriate amount of wells should thus be prepared for the respective T-cell stimulation experiments.

2.10.1 Peptide Pulsing of B-LCL

Various peptide stock dilutions are used so the peptides were diluted to a concentration of 5 µM peptide for appropriate loading. B-LCL were counted and resuspended $4*10^6$/8 mL TCM, meaning every 100 µL contains 50.000 cells. An appropriate amount of wells with B-LCL is prepared for the experiment in question (TNF-α, peptide loading assay or proliferation assay) and for each peptide to be loaded. B-LCL with L33, lolP1 and no peptide were prepared.

Adamantane ethanol mix was made by mixing 0,25 µL [100mM] AdEtOH + 0,75 µL DMSO + 9 µL TCM multiplied by the amount of wells (200 µL volume) + some extra. 10 µL of Adamantane mix was added to each well and acted as an MHC II loading enhancer. Each well thus contains 100 µL B-cells were added + 10 µL AdEtOH mix + 90 µL peptide. The round bottom plates were incubated for 4 hours at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere. All well were centrifuged in RotinaFuge 420R at 2100 rpm for 4 min, supernatant decanted and resuspended in TCM. The pulsed B-LCL can now be used in the various assays.

To determine if the B-LCL used were viable and able peptide loaders a peptide loading assay can be performed as described below. This method uses the biotin – streptavidin binding to measure how much and if at all B-LCL can bind peptide in their MHC II molecules. Streptavidin is bound to a fluorophore and can be analysed by flow cytometry.

The pulsing of the B-LCL is performed as described above with similar peptides, as there may be interpeptide differences. The peptides used during the assay will have conjugated biotin. 5 µM of peptide was added to each sample. After 4 hour incubation the plate was centrifuged on the RotinaFuge 420R at 2100 rpm for 4 min. The supernatant was decanted and 200 µL PBSA/Azide to each well, centrifuged and supernatant decanted. A 10 µL Streptavidin – PE + 390 µL PBSA/Azide (0,5 + 19,5) was made and 20 µL Streptavidin – PE dilution was added to each well. The plate was tapped to mix thoroughly and incubated for 15 minutes on ice in the dark. 160 µL PBSA/Azide was added and the plate was thereafter centrifuged and supernatant decanted. 200 µL PBSA/Azide was added and all samples were resuspended. All samples were transferred to FACS tubes or inserted plate into the plate reader and analysed in the flowcytometer.

2.10.2 Protein Pulsing of Monocytes with 26.4 linked Dynabeads

26.4 linked Dynabeads were fashioned using the Dynabeads antibody coupling kit (Novex, life technologies), Dynabeads M-270 Epoxy (Novex, life technologies) and the 26.4 monoclonal antibody from in house production. The protocol was adapted from Dynabeads® Antibody Coupling Kit Catalog number 14311D Revision Date June 2012 (Rev. 001) from Novex. The recommended amount of antibody was used for optimal coupling (5-10 µg/mg of 26.4) and not optimal the amount for optimal binding capacity (20-30 µg/mg). 5 mg of beads ($6,7*10^7$ beads/mL) was coupled with 26.4 following the appropriate steps in the protocol for that amount. The antibody coupling takes two days.

On the same day the 26.4 linked Dynabeads were incubated with ligand, platelet rich plasma was prepared (Section 2.6.2) or previously stored platelets from donors were thawed from cryo-storage. Extreme care was taken not to cause aggregation of platelets during this process as it is unknown what effect this has on HPA-1a molecules in this assay. HPA-$1a^+$ platelets and rabbit platelets were lysed with 0,1% TRITON X-100 and an protease inhibitor

incubating for 1 hour at room temperature. Then, the lysates were centrifuged at 15000-17000 g (highest available) for 45 minutes after which the supernatants were obtained and added to ~1 mg of 26.4 linked Dynabeads for each lysate type and incubated overnight at 4 °C in a rotator.

On day 3 the 26.4 linked Dynabeads with either rabbit integrin or HPA-1a bound or empty 26.4 Dynabeads were incubated with the previously enriched monocytes. The overnight incubated beads were washed twice with help of a magnet using the washing buffer from the Dynabeads antibody coupling kit (Novex, life technologies). 0,1-1x10^6 beads bound with either integrin variant are pipetted from the respective tubes and resuspended in TCM (100 µL) and 5x10^4 monocytes in TCM (100 µL) are mixed together and incubated overnight 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere.

On day 4 the Dynabeads pulsed monocytes are centrifuged at 400 g for 4 minutes, 100 µL TCM was removed and the remaining 100 µL can be used in TNF- α or proliferation assay for T-cell activation.

To assess the amount of 26.4 and integrin β3 protein (HPA-1a and rabbit integrin) bound to the beads, antibodies with conjugated reporters can be used to detect the integrin bound in the 26.4. The SZ21 mAb (1:1000 from a 0,2 mg/mL stock) was used as the detecting antibody with Goat anti-rabbit IgG-HRP as secondary Ab for western blot. Of all variant of beads 2x10^6 beads were taken and standard western blot protocol was followed (see section 2.13.6) with the above mentioned antibodies. The protocol was adapted from a protocol by Gøril Heide from an currently unpublished article.

2.10.3 Platelet Derived Antigen Pulsing of Monocytes

HPA-1a$^+$ platelets, HPA-1b$^+$ platelets and rabbit platelets were washed and counted on a haemocytometer and checked for viability on the microscope. Each platelets variant was incubated with the previously enriched monocytes. 0,1-1x10^6 platelets of the respective variants are pipetted from the respective tubes and resuspended in TCM (100 µL) and 5x10^4 monocytes in TCM (100 µL) are mixed together and incubated in a 96 wells round bottom plate well overnight 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere.

2.11 TNF-α Secretion Assay

To measure the of activation of T-cells a TNF-α secretion assay done. T-cells were activated using either antigens derived from peptide, protein antigens bound on beads or whole platelet (see Table 1). lolP1 peptide was used as a control utilizing lolP1's ability to stably bind in the HLA-DRB3*01:01 binding pocket without activating the HPA-1a specific T-cells (21, 37).

APC cells (HLA-DRB3*01:01$^+$ B-LCL or HLA-DRB3*01:01$^+$ monocytes) were incubated with antigen (see 2.10) for 4 hours with adamantane ethanol (AdEtOH) if HLA-DRB3*01:01$^+$ B-LCL were pulsed or overnight if the pulsed APC's were HLA-DRB3*01:01$^+$ monocytes. Previously expanded HPA-1a specific T-cells are washed or lymphopreped and CFSE stained (see 2.9) and rested overnight with IL-2 in an incubator at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere. The following day the CFSE stained T-cells were counted and washed once with 0,2% PBSA. CFSE stained T-cells were resuspended in TCM without IL-2 or IL-15 at a 5-10*10^5 T-cells/ml concentration. The ratio of APC to T-cells was around 1:2-5. All B-LCL (D4BL4) and monocytes pulsed with various platelets protein or peptide types are counted, washed and resuspended at a concentration of 1-5*10^6 APC/ml in TCM. 50 µL of CFSE HPA-1a specific CFSE stained T-cells and 50 µL are added per well on a 96 wells round bottom plate. A anti-TNF- α /TAPI-0 mix was made containing 0,2 µL TAPI-0/well (Enzo 1mM, cat.no: BML-PI133-0001), 0,3 µL/well PE mouse anti-human TNF- α (BD bioscience 0,2 mg/mL cat.no: 554513) and 4,5 µL/well TCM for a total of 5 µL/well. 5 µL of anti-TNF- α /TAPI-0 mix was added per well to all samples and controls. The plates were incubated for 4 hours at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere. After the 4 hours of incubation the samples were resuspended, washed with 2mL of 0,2% PBSA and flow cytometry was performed.

2.12 Proliferation Assay

The proliferation assay is the second assay type used in this study as a way to examine the T-cells stimulating ability of certain peptides, proteins and platelet types. As before mentioned they can not only be used to determine if activation of T-cells is present but also the amount of activation and the percentage of population that has been activated.

T-cells clone (D8T104) were examined and the response was assessed in a proliferation assay performed as discribed by M.T. Ahlen et.al. (38). T-cells were stimulated by means of peptide antigens, protein antigens bound on beads or whole platelet derived antigens (see Table 1).

The preparation for the proliferation assay started one day before the assay start APC cells (HLA-DRB3*01:01$^+$ B-LCL or HLA-DRB3*01:01$^+$ monocytes) were incubated with antigen (see 2.10) for 4 hours with adamantane ethanol (AdEtOH) if HLA-DRB3*01:01$^+$ B-LCL were pulsed or overnight if the pulsed APC's were HLA-DRB3*01:01$^+$ monocytes. Ready expanded HPA-1a specific T-cells, washed or lymphopreped to stop the expansion of the cells, were CFSE stained (see 2.9) and rested overnight with IL-2 in an incubator at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere. The CFSE stained T-cells and pulsed B-LCL (D4BL4) and monocytes were prepared and placed in their respective wells at the same ratios as in the TNF-α secretion assay. The round bottom plates were incubated for 6-7 days at 37°C, in a 7.5% CO_2, 6,3% O_2 in a humidified atmosphere. The next day (day 1) 100 µL/well of new medium containing 100 U/mL IL-2 and optionally 10 ng/mL IL-15 was added so that the concentration in each well will be 50 U/mL IL-2 and if used 5 ng/mL IL-15. New medium containing 50 U/mL IL-2 and if used 5 ng/mL IL-15 was provided to the samples as necessary when medium started turning orange. When T-cell confluency was determined to be appropriately high, the samples were resuspended, incubated for 20 minutes on ice with anti CD4/anti CD14 in the dark. After incubated samples were washed twice with 2mL of 0,2% PBSA and flow cytometry was performed.

2.13 Protein Isolation

2.13.1 Size-Exclusion column preparation (Sephadex G50)

Sephadex G-50 Superfine is a well-established gel filtration resin for desalting and buffer exchange of biomolecules >30 kDa. Here we use the resin as a prefilter to separate all molecules over 30 kDa from all molecules that are below that molecular weight. Hereby being able to use more pure samples affinity column.

The current protocol was based on the Size Exclusion Chromatography Principles and Methods from GE healthcare and instructions disposable plastic columns by Thermo scientific and in short the following was performed. The swelling process was accelerated by using a water bath at 90°C for 1 hour serving simultaneously to degas the suspension. The rehydrated suspension was allowed to cool before use washed with 5-10 column volumes (CV) on a sintered glass filter and resuspended in 1 CV of milliQ water. The resin was washed with 20% ethanol storage solution and moved to a column with porous disc in the bottom, sealing the column with a porous disc over the resin. The pre-filtration column was filled with ~10 mL or 10 cm of resin and ran using gravity. Fractions were collected in 0,5-1 mL samples and analysed for protein concentration using nanodrop.

2.13.2 Affinity filtration column preparation (Sepharose 4B CNBr-activated)

CNBr- activated Sepharose 4B is a resin that can be chemically linked to molecules containing an amine. The molecules used for the preparation of the affinity column used in this study was mAb 26.4, which is highly specific for HPA-1a and presumably rabbit integrin.

The current protocol was based on and adjusted from; Affinity Chromatography Vol. 3: Specific Groups of Biomolecules, GE Healthcare, 2014 and Methods from GE healthcare and instructions disposable plastic columns by Thermo scientific and , in short, performed as follows. An appropriate amount of freeze dried sepharose 4b CNBr-activated (1 gram for 2,5-3 ml of resin) was weighed and the following buffers were prepared; 1 mM HCl (washing

and rehydration buffer), 0,2 M $NaHCO^3$ 0,5 M NaCl - pH 9.1 (coupling buffer), 0,1 M Tris-HCl – pH 8 (blocking buffer), 0,1 M acetate buffer 0,5 M NaCl – pH 4 (washing buffer low pH), 0,1 M Tris-HCl 0,5M NaCl – pH 8 (washing buffer high pH), 0,02% Sodium Azide (storage solution). The sepharose powder was rehydrated and washed with 200 mL washing buffer in small aliquots on a sintered glass filter. The rehydrated resin is collected and resuspended in coupling buffer + 26.4 mAb (2-5 mg mAb per mL resin) was added in 1:2 (v/v) ratio and incubated for 1-2 hours and room temperature. The mAb solution was kept for nanodrop analysis of 26.4 binding to the resin beads. The resin was washed with 5 resin volumes of washing buffer and then incubated for 1 hour in blocking buffer, blocking any remaining active groups. The final washing step is 3 cycles of high and low pH washing buffer after which the resin is place into a fitting column as done in the sephadex G50 protocol and can be store in storage buffer at 4 °C.

2.13.3 Purification of human HPA-1a or Rabbit Integrin β3 antigen.

The protocol for purification of HPA-1a or rabbit integrin was based on the method used by T. Bakchoul, O. Meyer et.al. and adjusted to fit the current study (40). A prefiltration size exclusion column with Sephadex G50 for removal of all molecules under 30 kDa and sequentially an 26.4 mAb linked affinity column to purify and isolate the integrin in question from the pre-filtered fractions.

The following buffers were made. Lysis buffer consists of 0,1% Triton X-100 20 mmol/L Tris 150 mmol/L NaCl 100 µL/L protease inhibitor pH 7.4 (Roche, cOmplete, Mini, EDTA-free). Three washing buffers were made for washing the column in high and low salt concentration once the protein solution has been run through the affinity column. Washing buffer 1 made from 50 mmol/L Tris 5 mmol/L NaCl 0,1% Triton X-100 - pH 8, washing buffer 2 consisting of 100 mmol/L Tris 500 mmol/L NaCl 0,1% Triton X-100 - pH 8 and washing buffer 3 is a solution containing 50 mmol/L Tris 5 mmol/L NaCl - pH 8. Elution buffer contains 100 mmol/L trisodium citrate dehydrate – pH 2. And finally the catching buffer which consists of 1 mol/L Tris-HCl – pH 9,5 to neutralize the elution buffer and prevent protein loss after elution. All buffers are degassed before use in the columns to maintain high efficiency of the columns. Hereafter these buffers will only be mentioned in this method by their respective names.

HPA-1a or rabbit integrin platelets were washed three times and pelleted, being cautious not to activate the platelets in the process (Section 2.6.2). The obtained platelet pallet was resuspended in lysis buffer and incubated for 1 hour at 4 °C. The platelet lysate was centrifuged at 17.000 g (or the highest possible) for 45 minutes at 4 °C. The pre-filter column was made ready by washing the column with degassed MilliQ water (20% ethanol maybe added) and equilibrating the column with the lysis buffer. The lysis supernatant was then run through the pre-filter column and collected in fractions from the moment platelet lysate was added until the total volume run volume is sample volume +1 or 2 extra column volumes with lysis buffer (0,5-1ml per fraction). The pre-filtration column was washed with MilliQ water with 0,02% NaN^3. The collected fractions were analysed on the nanodrop to determine the fraction with protein of interest. The affinity column was washed with each 2 column volumes of washing buffer 1,2 and 3 after which the column was equilibrated with 4-5 column volumes of lysis buffer without TRITON X-100. The fractions containing the proteins of sizes 30 kDa and above were run on the affinity column + 1-2 column volumes of lysis buffer without TRITON X-100 to keep the column wet while the last of the sample volume runs on the column. The column was washed sequentially with 10 ml of washing buffer 1,2 and 3. The sample and wash run through were both collected in 1ml fraction. The column is eluted with 5 mL elution buffer (~0,5 mL/fraction) which is collected in fraction tubes containing each 0,5 mL of catching buffer for suspension neutralization. These fractions were then analysed by nanodrop, SDS-PAGE or western blot depending on what sensitivity level was required. Once the fraction with target protein have been determined, the target fraction were dialysed and concentrated using the Amicon ultracel 50k 15 ml. The complete setup is visually described in appendix II-I.

2.13.4 Nanodrop

The protocol for nanodrop analysis was done as instructed by the manufacturer using the A280 absorption measurement option on the Nanodrop One/One (NanoDrop Micro-UV/Vis Spectrophotometers NanoDrop One User Guide 269-309102 Revision A May 2017).

2.13.5 SDS-PAGE

SDS-PAGE was used to verify the purified fractions from the affinity column under reducing conditions. Reagents for SDS-PAGE were purchased from Invitrogen by Life Technologies. 10 µL samples were taken from affinity column fractions to a Eppendorf tube containing 3.8 µL NuPAGE 4x LDS Sample Buffer and 1.5 µL NuPAGE 10x Sample Reducing Agent. The samples were incubated on a heat block at 70 °C for 10 minutes. 600 mL of 1x NuPAGE Running buffer was made by diluting 30 mL of 20x NuPAGE Running Buffer in 570 mL MilliQ water. A NuPAGE® Novex® 4-12% Bis-Tris Gels, 1.5 mm 15 well gel was rinsed with water thoroughly on the outside and in the wells with a syringe. The gel was inserted in the inner chamber, the chamber was filled with 1x Running buffer. The outer chamber was filled with the remaining 1x Running buffer and 500 µL antioxidant was added to the inner chamber. 7 µL of each ladder, Novex Sharp Pre-stained and Unstained Protein Standard as well as 12 µL of each prepared sample was loaded to the gel. The gel is run for 35 minutes on 200 V using the programmed settings on XCell SureLock Mini-Cell by Invitrogen, Life Technologies.

The gel was washed with deionized water heated close to boiling and placing the gel on an orbital shaker for 1 minute, discarding the water afterwards. This washing step was repeated 3 times and there after stained for 20 minutes on a shaker with 20 mL SimplyBlue SafeStain also heated close to boiling. The gel was then washed with 100 mL of deionized water on a shaker for 10 minutes before 20 mL of 20% NaCl was supplied. The gel left on the shaker overnight and analyzed the following day.

2.13.6 Western Blot

Western blot is a method for the detection of specific protein in a sample by the binding of protein-specific antibodies. The western blotting is performed on the developed SDS-PAGE gel from which the proteins are transferred to a nitrocellulose membrane by electroblotting. The detection of protein is done by specific antibodies conjugated with colorimetric, chemiluminescent or fluorescent reporter molecules (41). Here the chemiluminescent method with horseradish peroxidase (HRP)

SDS-PAGE under reduced condition was performed as described in section 2.13.5, however the unstained ladder was changed to MagicMark XP Western Protein Standard (Invitrogen by Life Technologies) and of both ladders only 1 µL was loaded into the respective wells.

A membrane, Invitrolon PVDF Filter Paper Sandwich 0.45 um Pore Size (Invitrogen by Life Technologies) was prepared by saturating the membrane first in 100 % methanol for 3 seconds, secondly in deionized water for 10 seconds and finally 5 minutes in 1x Transfer Buffer diluted from NuPAGE 20x Transfer Buffer (Invitrogen by Life Technologies). The plastic shell and wells from the developed SDS-PAGE gel were removed and the membrane was place on top of the gel. Filter papers were placed on top and bottom of the membrane and gel construction and then sandwiched between 1x Transfer Buffer moistened sponges. This sandwich was then positioned in the blotting apparatus with 1x Transfer Buffer filling the inner chamber and water in the outer chamber. The gel was electroblotted for 1 hour at 25 V using the pre-programmed settings on XCell SureLock Mini-Cell by Invitrogen, Life Technologies.

The membrane was washed three times in PBS 0,05% Tween 20 for 10-15 minutes. While the membrane is incubated for 1 hour in blocking reagent (0.2 % goat IgG; Thermo Scientific, Waltham, Massachusetts to SuperBlock (PBS) Blocking Buffer (Thermo Scientific) and 0.05 % Tween 20) human anti-ITGB3 antibody (26.4) was diluted 1:1000 in Blocking Buffer supplied with 0.05 % Tween 20 to 0,2 µg/mL. Once blocking had finished the membrane was incubated overnight in primary antibody solution at 4 ° C with minimal rotation.

The next day, the membrane was washed three times in PBS 0.05% Tween 20 for 10-15 minutes with gentle shaking. Polyclonal goat anti-mouse IgG (HRP) (Sigma-Aldrich) was diluted 1/10000 in blocking buffer with 0.05 % Tween 20, 1 µl to 10 ml and incubate for 1 hour gently shaking. Again, the membrane was washed three times after which the substrate solution was made by mixing SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) components 1: 1 and pipetting 2 mL onto the membrane. The membrane was incubated for 5 minutes in darkness and then photographed using ImageQuant LAS 4000 (GE Healthcare Life Sciences) at the Bioimaging Core facility, UiT.

3 Results

3.1 Rabbit and human integrin β3 amino acid sequences are similar at residues known to form the HPA-1a epitope except for valine at residue 33.

To examine the differences and similarities between the 25-33, 39 and 470 amino acid positions in Integrin β3 in various animals, a search was performed on ensemble.org. The current list includes only a few animals, but care was taken to include animals which are more available for research purposes and for collection of sufficient quantities of blood for potential therapy. The comparison is made to show what animals could be potential sources of integrin β3 protein similar enough to HPA-1a Integrin β3 to potentially activate the HPA-1a specific T-cells.

Our T-cell clones specific for HPA-1a integrin β3 are specific for the 25-33(L33) amino acid sequence of the integrin β3. This 25-33 amino acid sequence is presented in the DRA/DRB3*01:01 MHC II on APC like monocytes or B-LCL to which the T-cell clones can bind and get potentially activated. The DRB3*01:01 has 3 main anchor pockets P1, P4 and P9 and one less important anchor pocket P6 (22, 37). P9 is of specific importance to our research as it is the binding site of the amino acid at position 33, where the L33P mutation from HPA-1a to HPA-1b is found. The amino acid sequence 25-33 of the integrin β3 with the relevant anchor pockets is shown in table 1. Specifically human HPA-1c and rabbit integrin are identical. Also the L33V difference from HPA-1a to HPA-1c/rabbit integrin β3 is important as Leucine and Valine are both small hydrophobic amino acid and may bind equally strong in the hydrophobic anchor pocket P9 (4, 8, 34). Multiple other animals are shown, having 1 or more mutations with non- similar amino acids.

Additionally is the binding site of 26.4 mAb shown in table 1, being amino acid position 30, 32, 33, 39, 470 also called APLDQ (from the HPA-1a integrin β3 amino acid sequence), however other amino acid in between positions 33 and 470 may also be important in 26.4 binding (34). Again here the characteristics of the amino acids play an important role and similar amino acids can be found in HPA-1a, HPA-1c and rabbit integrin (APLDQ or APVDQ) but not in the other animals shown. Although several species of wild animals (e.g. primates, bears, dogs and cats) have a sequence similar to the HPA-1a epitope, these animals

would for practical or esthetical reasons arguably not be suitable as donors of blood for therapeutic purposes. Among common household animals, only the rabbit has a sequence that could potentially mimic the HPA-1a epitope.

Table 3 ***Ensemble.org amino acid sequence data from various organisms compared at the 25-33, 39 and 470 positions of the Integrin β3 protein.***

	Amino Acid Position in Integrin β3											
Animal	Position 25-33									Position 39	Position 470	Comments
	P1*			P4*		**P6***			**P9***			
HPA-1a	W	C	S	D	E	A	L	P	L	D	Q	Position 25-33 also: Primates, Bears, Panda, Fox, Dog, Cat, Sea otter, Seals, Walrus
HPA-1b	W	C	S	D	E	A	L	P	P	D	Q	
HPA-1c †	W	C	S	D	E	A	L	P	V	D	Q	
Rabbit (Oryctolagus cuniculus)	W	C	S	D	E	A	L	P	V	D	Q	
Bovine (Bos taurus)	W	C	S	D	E	A	L	P	P	N	Q	Position 25-33 also: Goat, Sheep, Whales and Dolphins
Horse (Equus caballus)	W	C	S	D	A	A	L	P	L	N	Q	
Wild Boar (Sus scrofa)	W	C	S	D	E	D	L	P	L	N	Q	
Hedgehog (Erinaceus europaeus)	W	C	S	D	E	A	L	P	R	D	Q	
Guinea pig (Cavia porcellus)	W	C	S	D	K	N	L	P	L	N	Q	
Hamster (Mesocricetus auratus)	W	C	S	D	E	A	L	P	Q	N	M	
Rat (Rattus norvegicus)	W	C	S	D	E	S	L	P	Q	N	M	
Mouse (Mus musculus)	W	C	S	D	E	T	L	S	Q	N	M	

[i] *) Show binding pocket of the DRB3*01:01 MHC II

[ii] In Orange; the APLDQ amino acids that form the 26.4 epitope as hypothized and tested by Huiying Zhi et.al. In; *High-resolution mapping of the polyclonal immune response to the human platelet alloantigen HPA-1a (PlA1)*

[iii] Other possible aa for P9 could be A,I,L,M,F,W,Y and V all hydrophobic in characteristic. This is because the P9 pocket is only fitting for hydrophobic anchor residues.

[iiii] Amino acids underlined are different from the human HPA-1aa variant of integrin β3

[iiiii] †) Human HPA-1cc has only been described once before by Sentot Santoso et.al. In; *A naturally occurring Leu33Val mutation in b3-integrin impairs the HPA-1a epitope: the third allele of HPA-1*

3.2 Anti-HPA-1a antibodies bind rabbit platelets

Various types of platelets from HPA-1aa+, HPA-1bb+ human and rabbit donors were analysed to specify their binding capacity for Y2/51, SZ21, 26.4 and AP3 mAb's.

The cryostored samples were taken at earlier point in time then fresh or preserved platelet samples as well as at different FITC/AlexaFluor 488 detector voltages. Negative will be classified as all MFI lower than 50, slightly positive for MFI between 51-1000 and all above 1001 MFI will be positive. The cryopreserved samples showed the following results (Fig 4-1). HPA-1a platelets where positive for SZ21, 26.4, AP3 and Y2/51 and negative for the no mAB control. HPA-1b platelets where positive for AP3 and Y2/51, only slightly positive for SZ21 and negative for the 26.4 mAb and no mAB control. Rabbit platelets where positive for SZ21, 26.4 and negative for no mAb, Y2/51 and AP3. Fresh and preserved platelets controls showed similar results to their respective partners (Fig 4-1). Thus, rabbit platelets are bound by HPA-1a-specific monoclonal antibodies.

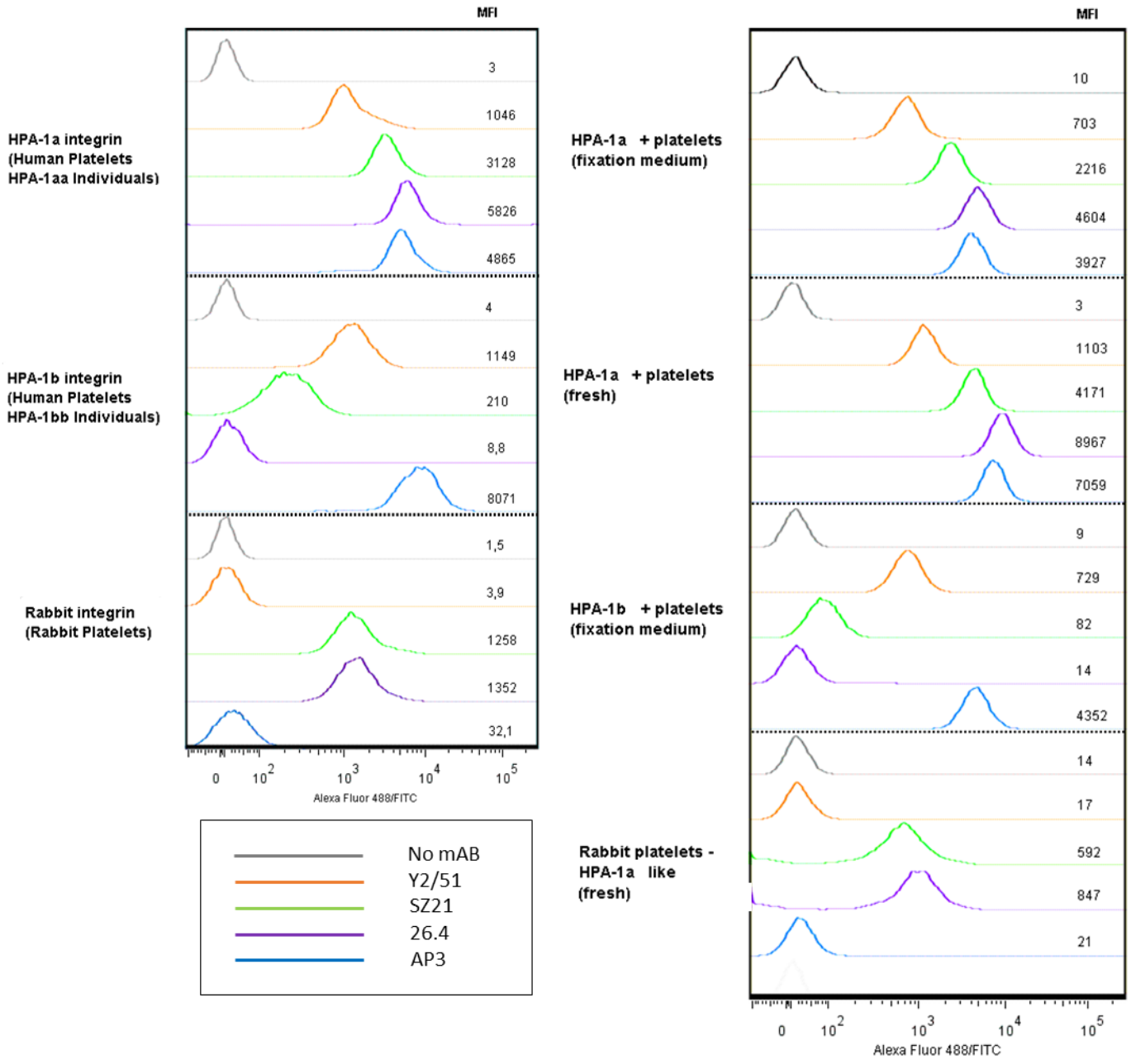

Figure 3-1 ***Binding of various anti-human integrin β3 antibodies to HPA-1a, HPA-1b and rabbit platelets fresh, preserved or cryopreserved.***

The various platelets were incubated with Y2/51, SZ21, 26.4, AP3 or without conjugated with FITC or AlexaFluor 488. human β3 integrin can be bound by AP3 and Y2/51, while 26.4 and SZ21 are specific for the HPA-1a variant of β3 integrin. The mean fluorescence intensity (MFI) is shown for each sample. On the left the cryopreserved samples are shown while on the right preserved or fresh samples are shown.

3.3 Monocytes, PBMC's and B-LCL's were analysed and prepared for proliferation assay.

Various control steps were taken and performed to ascertain the significance of the final results. Adamantane ethanol (AdEtOH), a known enhancer of peptide-binding to MHC class II molecules, was assessed for improved antigen-presentation to T cells by peptide-pulsed B-LCL (See Appendix II). Also density gradient monocyte enrichment was analysed and T-cell CFSE staining quantified. Finally, the binding of 26.4 mAb on Dynabeads and consequently the binding of either HPA-1a or rabbit integrin was quantified by western blot.

3.3.1 B-LCL D4BL4's ability to present L33 and LolP1 peptides was verified.

To analyse whether AdEtOH could enhance the capability of our B-LCL clone D4BL4 to present L33 and LolP1 peptides, binding of biotin-labelled peptides (L33-biotin and LolP1-biotin) to D4BL4 cells was assess using streptavidin conjugated with PE. This method allows for a quantification of the surface presentation of the respective peptides. Both L33 and LolP1 were tested with or without AdEtOH as an MHC II loading enhancer, as well as control with no peptide and only biotin without peptide bound also with and without AdEtOH (control of AdEtOH improved binding in appendix II-II). Quantification of peptide binding in MHC II was measured in MFI by flow cytometry.

Results showed that L33-biotin binding to B-LCL with AdEtOH was highest with a MFI of 508, likely binding the DRB3*01:01-peptide complex. LolP1-biotin binding was measured at a MFI of 370,5 with AdEtOH. L33-biotin and LolP1-biotin without added AdEtOH had a measured MFI of respectively 190,7 and 135,3. The no peptide and only biotin control with added AdEtOH showed a MFI of respectively 120,1 and 138,3 of unspecific streptavidin or biotin binding in MHC II. Without AdEtOH the no peptide and biotin control showed a unspecific binding quantification of 74,3 MFI for the no peptide control and 94,6 MFI for only biotin binding (Fig 4-2 A) In Fig 4-2 B the results can be seen summarized in a bar graph showing more accurately the individual differences of the peptide MHC II binding stability and quantitation. Our B-LCL cell line was thus shown to take up and present L33 and lolP1, likely through DRB3*01:01.

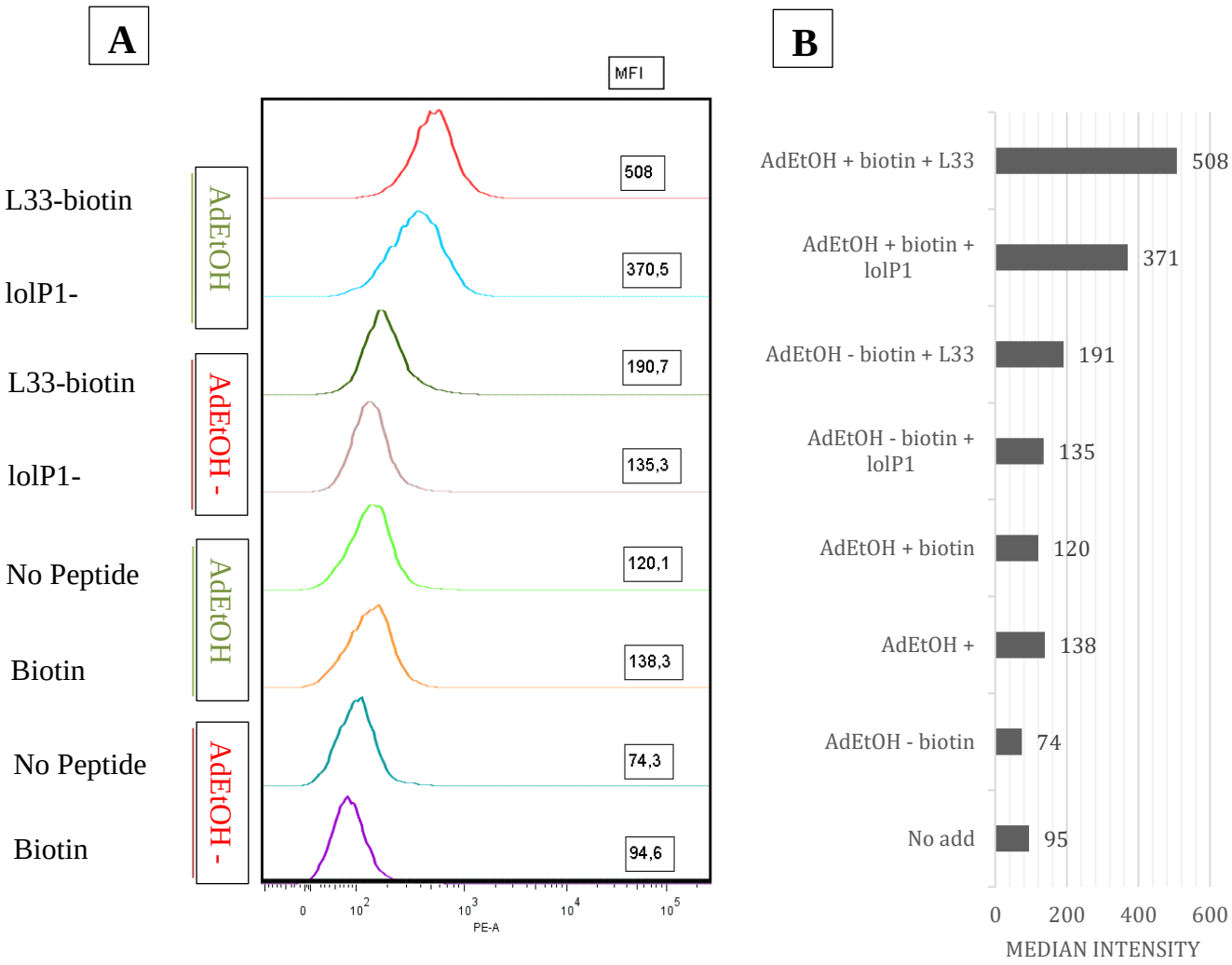

Figure 3-2 ***quantification of B-LCL clone D4BL4's MHC II binding ability to biotin-L33 and biotin-lolP1 using Adamantane Ethanol as a loading enhancer.***

The binding of L33-biotin and lolP1-biotin was quantified in the presence and absence of AdEtOH to determine if the clone was viable as an APC for proliferation studies. The B-LCLs were first incubated with the biotin-"peptide" or with just biotin or no peptide and variably adamantane ethanol. Then streptavidin-PE was added and the samples were measured by flow cytometry, shown in A with on the right the MFI. In B the MFI values are summarized to show the differences in MFI per sample more accurate. Biotin was linked to peptides by glycine using biotin-labeled glycine linkers

3.3.2 Monocytes could be significantly enriched in a PBMC population by the density gradient method.

For the presentation of antigen via protein or whole platelets, monocytes were used as suitable APC for their ability to phagocytose and process larger antigens. PBMC's were isolated from buffy coats, evaluated for viability under the microscope (results not shown).

Thereafter, they were treated with RosetteSep monocyte enrichment cocktail and again treated with a density gradient.

Gating for live cells and suspected monocytes is shown on the left, the live cells are then further analysed on PE intensity shown on the right (Fig 4-4). Non enriched PBMC generally had around 10,6% monocytes within the PBMC population. Monocytes enriched PBMC showed in large population in the suspected monocytes in the scatter plot on the left. Also in the PE intensity histogram increased monocyte count could be detected at 64,7%. Monocytes enrichment controls were performed before all proliferation experiments and figure 4-4 is a representation of the general result (other monocytes enrichments controls not shown).

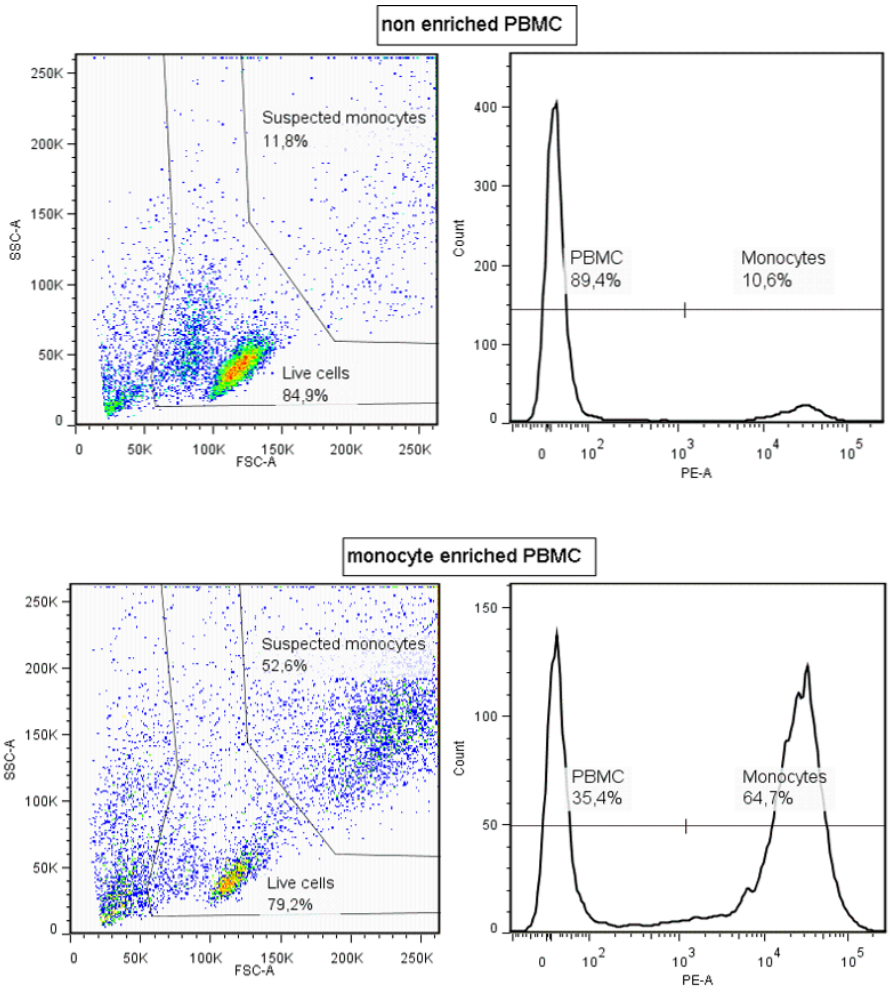

Figure 3-3 ***Monocyte enrichment control on non-enriched and enriched PBMC populations.***

On the left the scatter plot is represented and on the right a histogram of PE intensity from the mAb anti-CD14-PE binding monocytes is shown. The upper two figures represent the non-enriched PBMC population and the lower two figures show a monocyte enriched PBMC population. A suspected monocyte gate is shown in the scatter plots to visualize were monocytes are expected to appear. A live cell gate is shown excluding dead material, and is further analyzed in the histogram. Both histograms show the PBMC population without monocytes and the monocytes bound with anti-CD14-PE.

3.3.3 CFSE staining of HPA-1a specific T-cells was quantified.

The CFSE staining is an integral part of the proliferation assay and was therefore assessed by flow cytometry analysis. 1 day after staining D8T104 cells were validated for CFSE staining and the intensity of CFSE staining was monitored to determine where the parent population is expected to be in the proliferation experiments for a specific green channel detector voltage.

Both non-stained and CFSE stained D8T104 cells were analysed before every proliferation assay to respectively determine the autofluorescence and CFSE staining intensity. The T-cell population was gated as shown in figure 4-5 for both stained and unstained D8T104. No significant changes in the T-cell population before and after staining could be detected. The non-CFSE stained D8T104 showed 98,2% T-cell population at autofluorescence intensities of around $2*10^2$. A smaller T-cell population of 1,56% could be detected at an intensity close to 10^5. The CFSE stained T-cells showed a population of 99,9% of the total population at a CFSE intensity of $\sim 7*10^4$ with a negligible 0,101% of the total population at auto fluorescent CFSE intensity (Fig 4-5). CFSE staining was thus successful and could reliably be used for further analysis in T-cell activation assays.

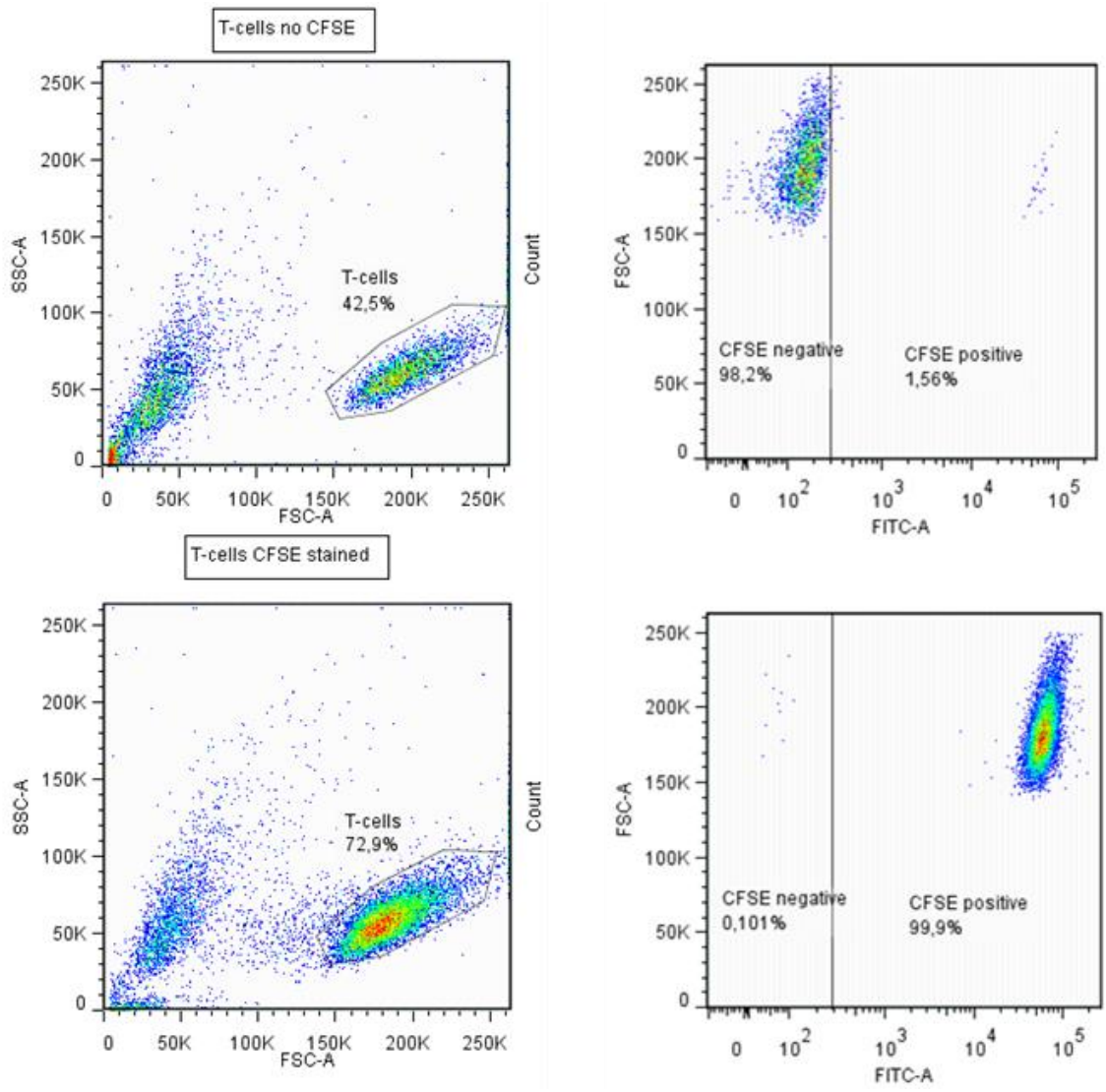

Figure 3-4 ***CFSE staining control of T-cell clone D8T104.***

D8T104 was CFSE stained and then analyzed by flow cytometry measuring in the green FITC channel. CFSE stained T-cells (bottom) were compared to non-stained T-cells (top). On the left scatter plots show the gating for the T-cell population, which were in turn further analyzed in a histogram for CFSE intensity. The histogram shows a CFSE negative region where non-stained T-cells are suspected to be and a CFSE positive region were CFSE stained cell can be expected to appear.

3.3.4 Dynabeads conjugated mAb 26.4 can bind HPA-1a and Rabbit integrin.

A western blot analysis of the beads was performed firstly to determine if the Dynabeads had been conjugated with the in-house 26.4 mAb and secondly if in turn the 26.4 was able to bind rabbit or HPA-1a integrin. 1 µL and 10 µL of Dynabeads with only 26.4 mAb or 26.4 mAb conjugated Dynabeads incubated with rabbit or HPA-1a integrin were examined. The 26.4 mAb was detected with anti-human IgG and the integrins were detected with SZ21 (Fig 4-1).

Several strong bands at 57 kDa, and weaker bands at 120 kDa, 80 kDa, 55 kDa, 48 kDa, 33 kDa and 27 kDa were seen for the samples in western blot A (26.4 dynabeads with HPA-1a or rabbit integrin) (Fig 4-1A). The western blot B presented with faint bands at 50 kDa and 25 kDa only in the beads 26.4 10 µL sample (Fig 4-1B), showing that mAb 26.4 was coupled to the beads. Thus, mAb 26.4-coupled beads precipitated several molecule from rabbit platelet lysate detectable by mAb SZ21.

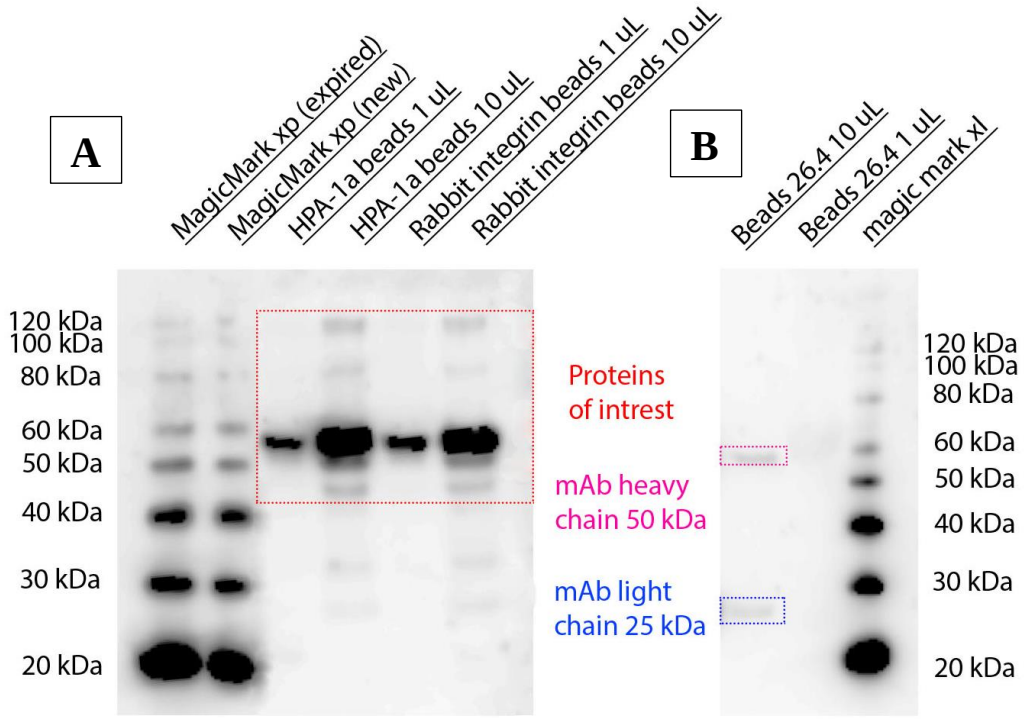

Figure 3-5 ***Western blot of Dynabeads conjugated with 26.4 and incubated with either rabbit or HPA-1a integrin showed significant binding of the respective integrin types.***

Rabbit and HPA-1a integrin from respectively rabbit and human HPA-1aa+ donors platelets lysate was captured on 26.4 conjugated Dynabeads. The beads were analyzed under reducing conditions on a western blot. The western blot showed several strong bands at 57 kDa, and weaker bands at 120 kDa, 80 kDa, 55 kDa, 48 kDa, 33 kDa and 27 kDa for the samples in blot A. The western blot B showed one band at 50 kDa and 25 kDa only in the beads 26.4 10 µL sample. For both western blot MagicMark XP western protein standard were used.

3.4 T-cell activation assays show HPA-1a specific T-cell activation by rabbit integrin β antigen.

Shown below are the results from the TNF-α and proliferation assays. The T-cells were incubated with B-LCL or monocytes presenting HPA-1a, HPA-1bs, LolP1 or rabbit integrin antigenic peptides derived from peptides, protein or whole platelets. The activation was then

measured by the excretion of TNF-α in the TNF-α assay and by proliferation percentage of the total T-cell population in the proliferation assay. The TNF-α assay measured the activation after 4 hours and the proliferation after 5-7 days.

3.4.1 TNF-α assays

The TNF-α assays were done with one T-cell clone, D8T104, specific to HPA-1a. The TNF-α expression was measured by the inhibition of TACE by TAPI 0 after which the membrane bound TNF-α was bound by an anti-TNF-α PE mAb.

A control for TNF-α expression was performed by measuring the T-cell clone for TNF-α expression without incubation of any APC. This blank control showed no TNF-α expression with 99,9% of T-cells being in the TNF-α^- gate (Fig 4-7). The rest of the experiment is divided into 3 parts; platelet-pulsed monocytes, integrin β3-pulsed monocytes and peptide-pulsed B-LCL. As a negative control for the platelet-pulsed monocyte T-cell clone incubation proliferation assay, the D8T104 presented with HPA-1b platelet antigen showed only a small population of 1,69% being TNF-α^+. Rabbit platelet-pulsed monocytes activated 89,5% of the D8T104 population and HPA-1a$^+$ platelet pulsed monocytes activated 90,5% of the D8T104 population. In the protein-pulsed monocytes proliferation assay the proteins were bound on Dynabeads before-hand. An extra control here was incubating the D8T104 T-cells with Dynabeads pulsed monocytes, where 2,43% of the T-cell population was in the TNF-α^+ gate. The rabbit integrin activated 4,75% of the D8T104 population and HPA-1a integrin activated 29,8% of the D8T104 population. The final part was B-LCL pulsing with LolP1 and L33 peptide as well as a B-LCL only control. The B-LCL control showed 0,92% of the D8T104 population being activated, LolP1 activated 0,97% in the TNF-α^+ gate and L33 had 58,1% of the D8T104 population being activated (Fig 4-7). The X-axis shows CFSE and the Y-axis showed TNF-α expression by PE. The TNF-α assay shows that rabbit integrin β3 antigen, in platelet and protein form, can stimulate HPA-1a specific T-cells to excrete TNF-α after 4 hours.

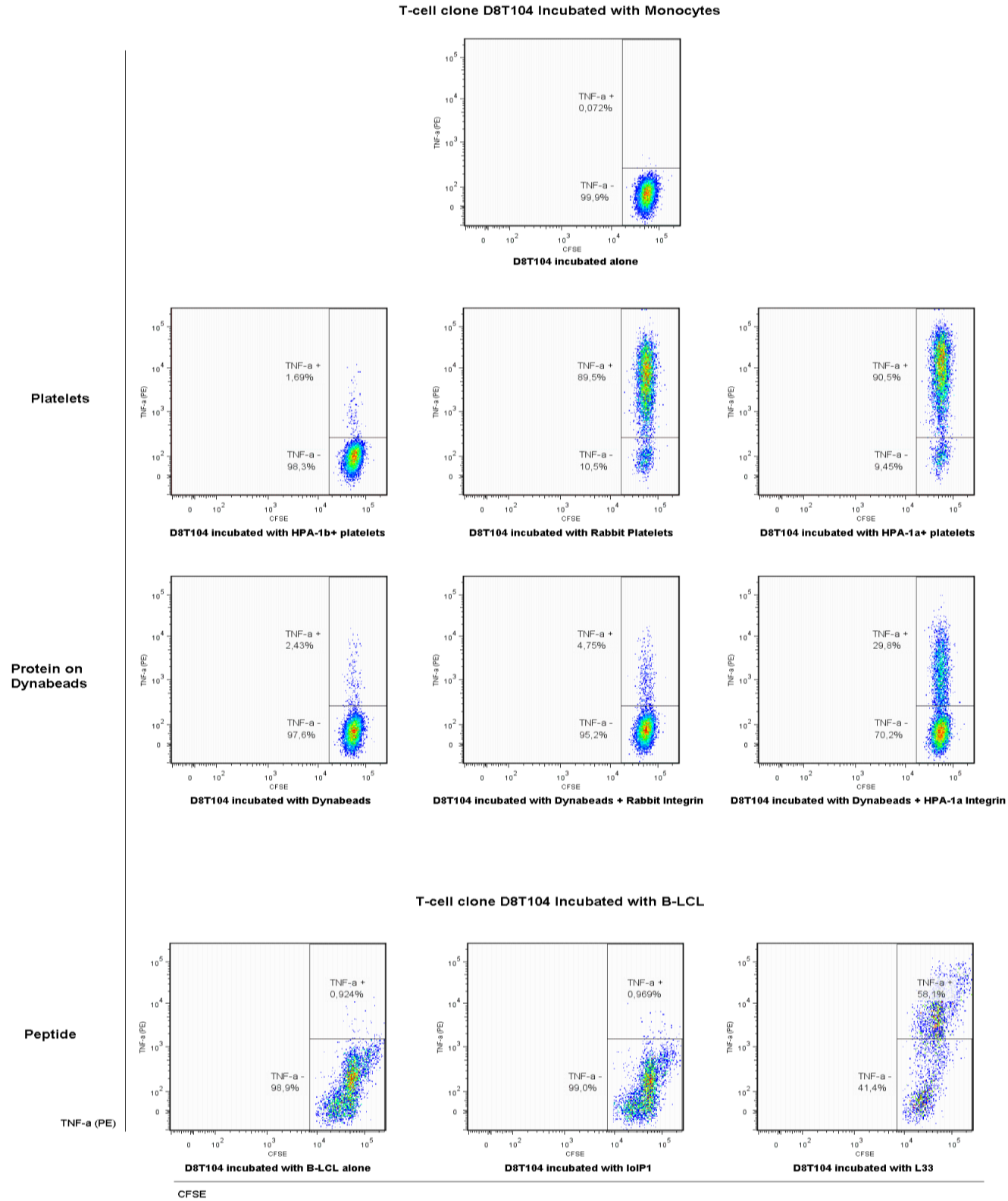

Figure 3-6 ***TNF-α expression in D8T104 T-cell clones incubated with peptide, protein or whole platelet-pulsed APC's.***

A TNF-α assay was performed on D8T104 T-cells incubated with various antigen pulsed APC. The following APC pulsed with antigen were used (top to bottom); A control with no APC. A platelet series with monocytes pulsed with HPA-1b$^+$, HPA-1a$^+$ and rabbit platelets. A protein (bound on Dynabeads) series with monocytes pulsed with only Dynabeads, Dynabeads bound rabbit integrin and Dynabeads bound HPA-1a integrin. And finally a peptide series with B-LCL non-pulsed, pulsed with LolP1 (negative control) and pulsed with L33 (HPA-1a-like peptide). On the X-axis CFSE intensity is shown with D8T104 CFSE stained, they end up on the right of the dot plots. On the Y-axis the PE intensity (TNF-α expression) is presented. A gate for TNF-α$^+$ T-cells is shown in top right corners and in the lower right corner a TNF-α$^-$ gate. The percentages shown in the dot plots represent the ratios of the D8T104 populations expressing TNF-α or not. APC's were not stained with CFSE and were gated out by being FITC negative (data not shown).

This assay was done in duplicate and for the orderliness of the results only one of the repetitions is shown for the flow cytometry results. The complete results are compiled in figure 4-8 where the percentage of the D8T104 population that has been activated can be found

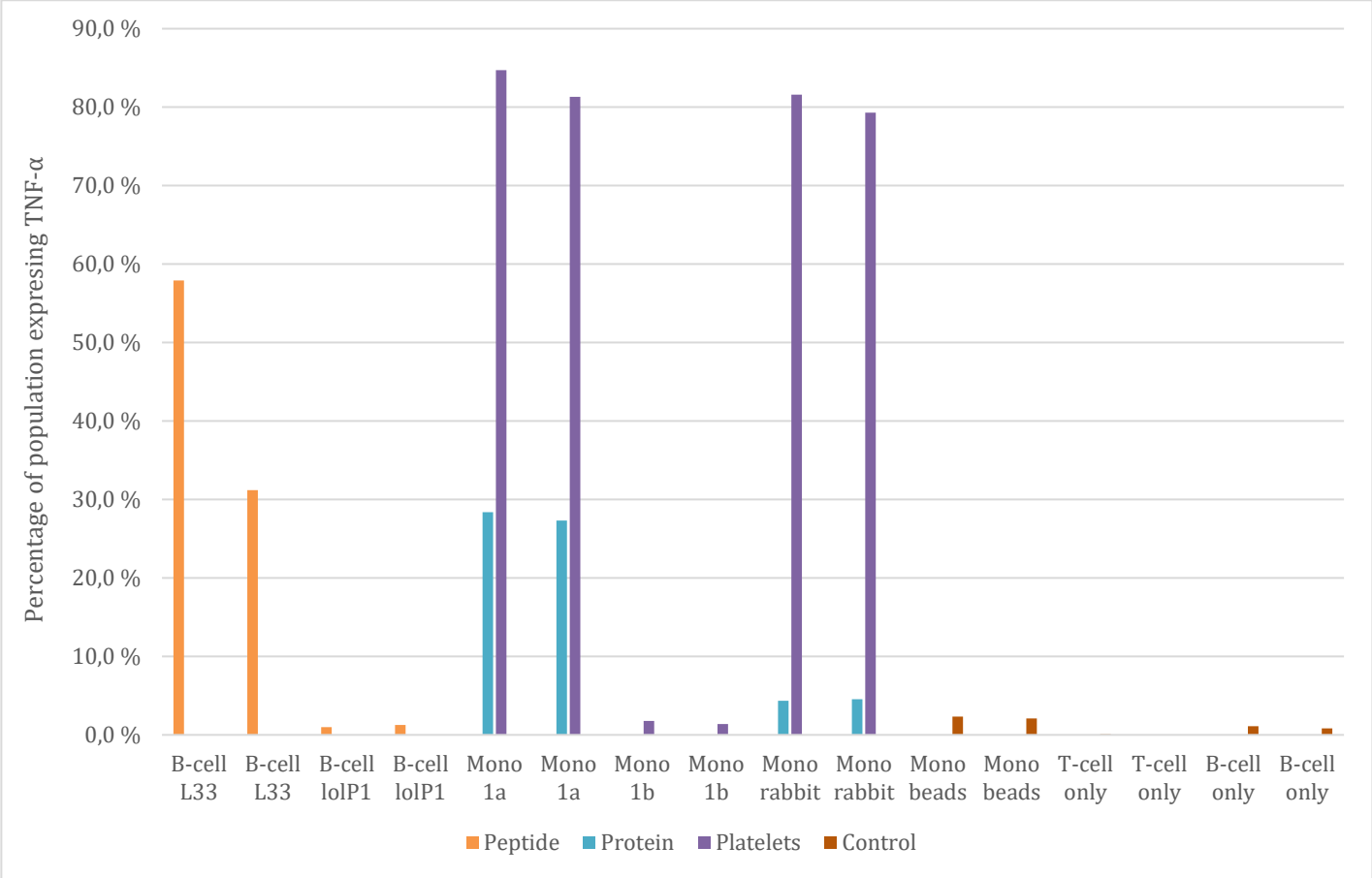

Figure 3-7 ***D8T104 T-cell clone activation measured in the percentage of the population excreting TNF-α measured by flow cytometry.***

The complete result from the TNF-α assay is compiled here in a graph with sample types on the X-axis and the percentage of the population that has started expressing TNF-α on the Y-axis. In orange the peptide series is shown, blue is for the protein series, purple for the platelet series and red for the controls. No mono(cyte) 1b protein stimulation was performed in this panel.

3.4.2 Proliferation assays

A second method of measuring the activation of specific T-cells is the proliferation assay. The HPA-1a specific T-cell clones were presented via APC's with various antigens and then measured for the percentage of the population having proliferated. The assay is described in detail in section 2.12, but in short; the T-cells were CFSE stained and if activated will start proliferation causing the CFSE intensity to half every division. This decrease can be made visible and measured by flow cytometry.

As a preliminary test of the proliferation assay the setup was run with only platelets. A proliferation assay had been performed before by M.T. Ahlen et.al and was used to determine if it could be replicated in a similar sense (39). Here, the T-cell clone D8T104 was presented with platelet derived antigens from HPA-1a, HPA-1b and rabbit platelets as well as a control with no platelets. Monocytes were used for the presentation of platelet derived antigens. The results showed that 36,7% of the D8T104 population had proliferated by HPA-1a platelet derived antigen presentation. 10,8%, 38,5% and 15,1% activation of the D8T104 cells could be measured in respectively HPA-1b, rabbit and no platelet derived antigen presentation (Fig 4-9). These results indicated that the assay is functional and ready to be used for a full antigen panel.

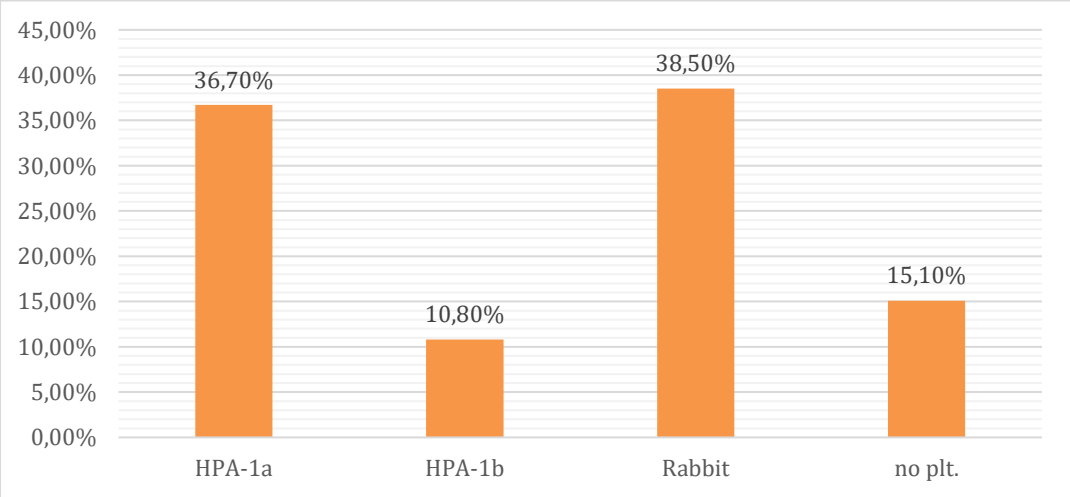

Figure 3-8 ***D8T104 T-cell clone stimulated by various platelet derived antigens measured in the percentage of T-cell population that proliferated.***

The results from the preliminary proliferation assay are compiled here in a graph with sample types on the X-axis and the percentage of the population that has proliferated on the Y-axis. The samples shown her are HPA-1a$^+$platelet-derived antigen presentation (HPA-1a), HPA-1b$^+$platelet-derived antigen presentation (HPA-1b), rabbit platelet-derived antigen presentation (rabbit) and a control were non-pulsed monocytes were incubated with D8T104.

The complete setup for the proliferation assay is shown below in figure 4-10, similarly divided as done in the TNF-α assay into a peptide, a protein on Dynabeads and platelet series. All proliferation histograms have been analysed with FlowJo software using the proliferation function providing an overlay showing in red the parent population and in pink the daughter populations (1 daughter population per peak). Two controls were performed, a non-CFSE stained T-cell control at a 105 CFSE (FITC) intensity signifying the T-cell population after having lost all fluorescence (about 8 divisions) (Fig 4-10 top left). The second control shows non-stimulated CFSE-stained D8T104, to show where the population is at full CFSE intensity as well as the amount of non-specific activation to be expected (Fig 4-10 top right). Here, the CFSE intensity was at 50.000 were about 1,9% of the population had started proliferating. The peptide series shows D8T104 responding to LolP1 (negative control), L33 and a control with non-pulsed B-LCL's. The no peptide (non-pulsed B-LCL) control showed a 0,1% portion of the original HPA-1a specific T-cell population having proliferated. The LolP1 and L33 stimulated D8T104 population respectively show a 0,4% and 37,8% of the CFSE stained D8T104 population proliferating. In the L33 peptide-pulsed B-LCL incubated with D8T104 a histogram with at least 3 individual peaks can be seen representing a non-dividing parent and two dividing daughter populations. The protein of Dynabeads series had one control with only 26.4 Dynabeads -pulsed monocytes (no protein), a rabbit integrin bound on 26.4 Dynabeads and HPA-1a integrin bound on 26.4 Dynabeads, all incubated with monocytes as APC before culture with D8T104 cells. The no protein control showed 9,4% of the T-cell population proliferating and the rabbit integrin and HPA-1a integrin samples 8,0 and 16% show of the T-cell population proliferating. Individual peaks are harder to recognize in these samples, but all three have up to 6 visible peaks. The final series were samples where monocytes pulsed with HPA-1b, rabbit or HPA-$1a^+$ platelets were incubated with D8T104. The D8T104 T-cells showed respectively 5,9%, 94,6% and 99,5% of the D8T104 population proliferating (Fig 4-10). Proliferation results showed that rabbit platelet antigen was able to stimulate HPA-1a specific T-cell to be activated and proliferate, but it could not conclusively be shown that rabbit integrin β3 could.

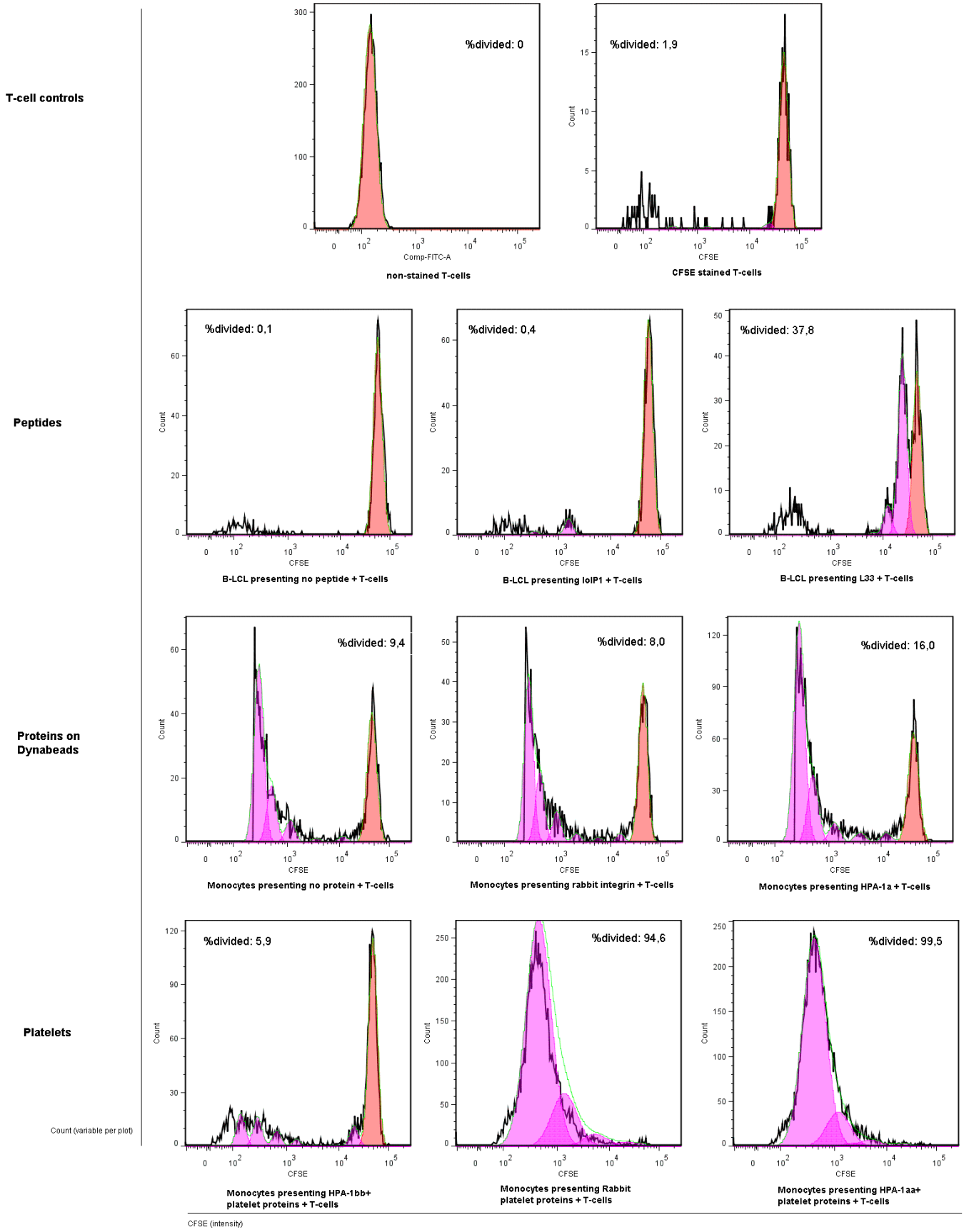

Figure 3-9 ***Proliferation measure as a percentage of the parent population of D8T104 T-cell clone incubated with peptide, protein or whole platelet-pulsed APC's.***

A proliferation assay was performed on D8T104 T-cells incubated with various antigen pulsed APC. The following APC pulsed with antigen were used (top to bottom); A control with unstained and CFSE stained D8T104, both without APC. A peptide series with B-LCL non-pulsed, pulsed with LolP1 (negative control) and pulsed with L33 (HPA-1a-like peptide). A protein (bound on Dynabeads) series with monocytes pulsed with only Dynabeads, Dynabeads bound rabbit integrin and Dynabeads bound HPA-1a integrin. And finally a platelet series with monocytes pulsed with HPA-1b$^+$, HPA-1a$^+$ and rabbit platelets. On the X-axis CFSE intensity is shown with D8T104 CFSE stained showing on the right of the dot plots and moving to the left when dividing a halving CFSE intensity. On the Y-axis the count is presented. The histograms have an overlay showing the parent population (non-dividing T-cells) in red and daughter populations (dividing T-cells) in pink peaks. Also, the %divided shows the percentage of the parent population having divided in each histogram.

Also this proliferation assay was done in duplicate and to represent the results in an understandable manner the results of only one of the repetitions is shown for the flow cytometry results. The complete results are compiled in figure 4-11 where the percentage of the D8T104 population that has proliferated is shown.

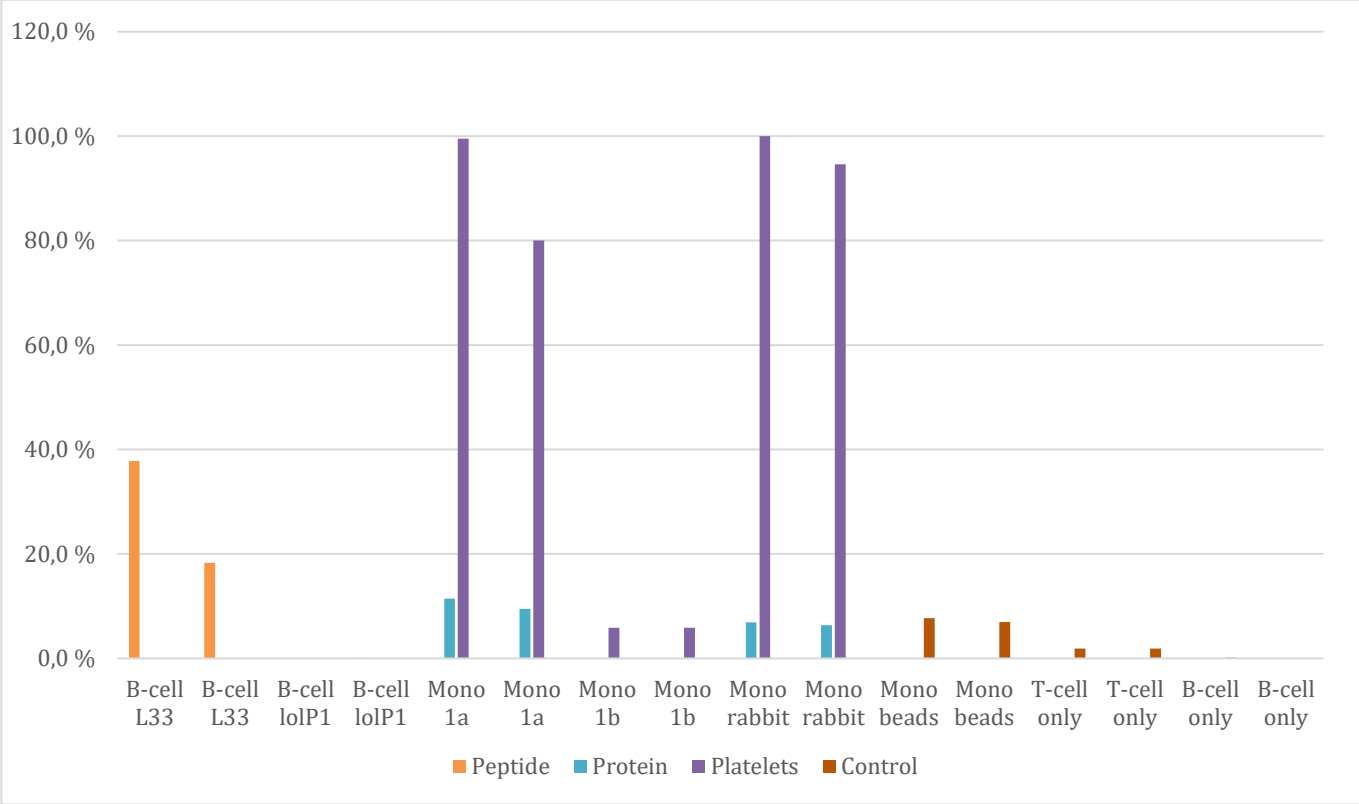

Figure 3-10 ***D8T104 T-cell clone activation measured in the percentage of the population having proliferated measured by flow cytometry.***

The complete results from the proliferation assay is compiled here in a graph with sample types on the X-axis and the percentage of the population that has proliferated compared to the original population on the Y-axis. In orange the peptide series is shown, blue is for the protein bound on Dynabeads series, purple for the platelet series and red for the controls. No mono(cyte) 1b protein stimulation was performed in this panel.

3.5 HPA-1a and rabbit integrin protein purification results

In this result section the isolation and purification results on rabbit and HPA-1a integrin β3 are presented. Two controls where performed respectively measuring that the effectivity and void volume of the Sephadex G50 (pre-filter) column and the affinity resin had bound the

mAb 26.4. Finally, a preliminary test of the complete purification setup (pre-filter and affinity column) was tested with a small sample of rabbit platelet lysate.

3.5.1 Molecules > 30 kDa could be isolated with the prefiltration column.

A sephadex G50 prefiltration column was made to filtrate out all molecules smaller than 30 kDa from the rabbit and human HPA-1a+ platelet lysate. The prefiltration of the platelet lysates was performed to add a more refined sample on the valuable 26.4 mAb affinity column.

The column was tested with bovine albumin (65 kDa) to determine the void volume and in which fractions the integrin molecules could be expected to be. Protein concentration was measured by A280 absorption by Nanodrop One/One. The albumin showed an increased concentration of protein in fractions 2,5 to 4 (0,5 mL per fraction) and two lesser increase in protein concentration in fraction 10 and 11,5. The filtrated rabbit platelet lysate showed increase in concentration from fractions 2 to 5 (1 mL per fraction) and a second increase from fraction 6 to 8 with a slow decrease in concentration towards fraction 13 (Fig 4-12). It could be concluded that larger proteins exited the column after 2 mL (2 fractions) of void volume up to 6 mL (fraction 6), as albumin coincides with this top and therefor it may be expected that integrin β3 resides in homologous fractions.

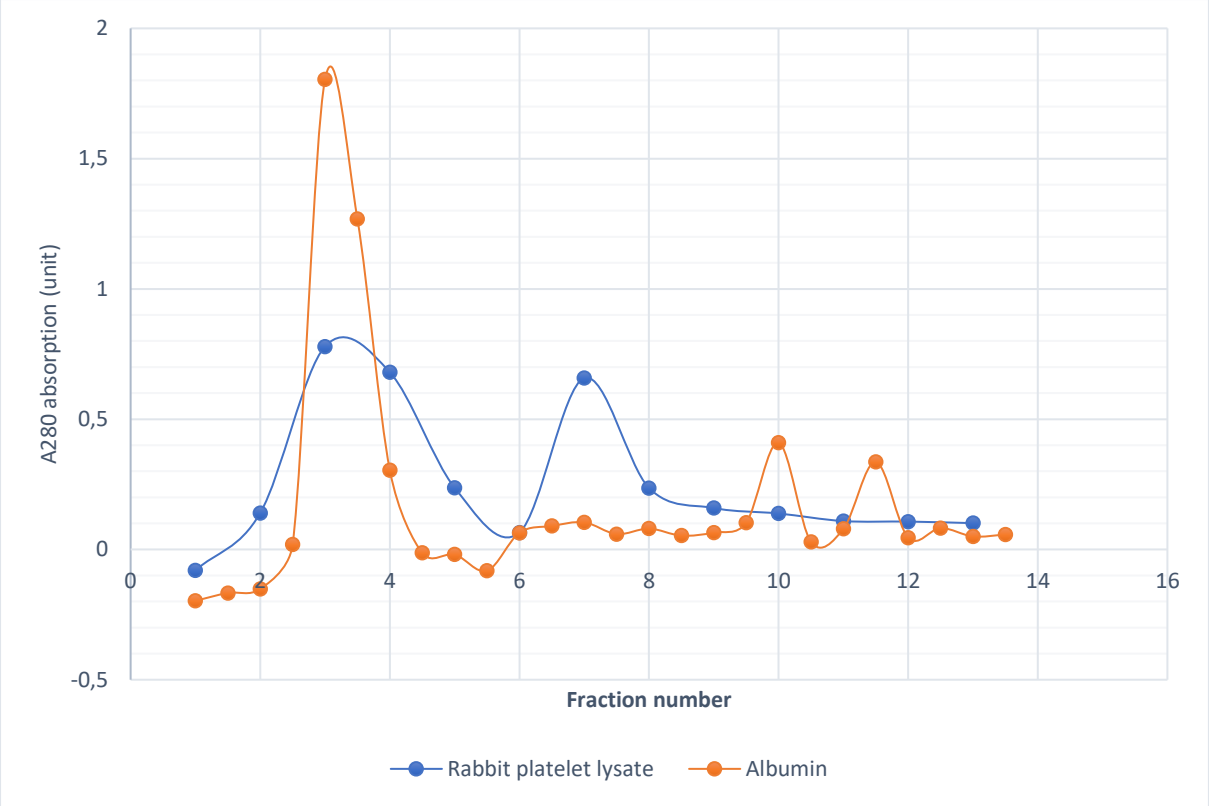

Figure 3-11 ***A280 absorption measurement of filtered fraction from pre-filter column by Nanodrop of Albumin and rabbit platelet lysate.***

The control protein albumin was filtered through the pre-filter column and plotted for A280 absorption per 0,5 mL fraction. Rabbit platelets lysate was filtered and measured by A280 absorption per 1 mL fraction. Both measurements were plotted in a graph with A280 absorption on Y-axis and fraction number on the X-axis, visualizing protein concentration per fraction for the respective samples.

3.5.2 26.4 mAb was found in the supernatant of boiled 26.4 mAb-linked CNBr Sepharose resin.

A western blot was also performed on a small sample of 26.4 mAb-linked CNBr Sepharose resin. The resin was boiled to detach the 26.4 from the resin, centrifuge the sample and then determine if 26.4 mAb could be found in the supernatant.

From left to right the following lanes can be seen; the magic mark XP molecular ladder, a 10 µL sample volume of 26.4 mAb resin and a 1 µL of 26.4 mAb bound resin. Both sample lanes showed a band at about 58 kDa and 25 kDa, where the 10 µL sample volume of 26.4 mAb resin showed slightly darker bands (Fig 4-13) mAb 26.4 was thus bound on the CNBr-activated resin.

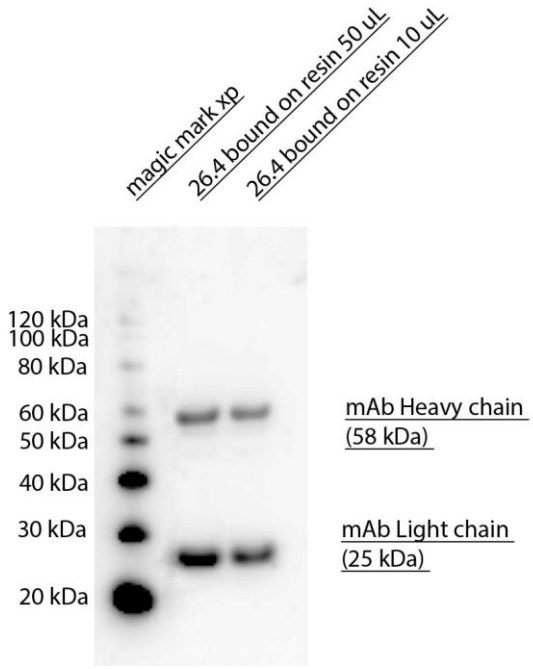

Figure 3-12 ***Western blot of boiled 26.4 mAb linked CNBr Sepharose resin.***

26.4 mAb linked CNBr sepharose resin was boiled to detach the mAb from the resin. 1 µL and 10 µL samples were taken from the supernatant and used for western blot sample preparation and run for analysis. The supernatant were analyzed under reducing conditions on a western blot. The western blot showed 2 strong bands at 58 kDa, and 2 strong bands at 25 kDa for the samples. For both western blot MagicMark XP western protein standard were used. The primary antibody used was rabbit anti-human IgG and the secondary was anti-rabbit IgG HRP.

3.5.3 No Rabbit and HPA-1a integrin could found in the elution fraction for HPA-1a integrin isolation.

A final western blot was done on human and filtrated rabbit platelet lysate. Human platelets lysate at no dilution, 100x dilution and 10.000x dilution. Pre-filtrate fractions F2 to F4, the wash fraction (whole wash was collected in 1 fraction) and Elution fraction E8 to E10 and E14 + E15 were analysed. Human platelet lysate samples showed a continues band for the undiluted sample, while the 100x diluted sample showed several band at 75, 70, 58, 43, 25 and 19 kDa. The 10.000x diluted human platelet lysate did not show any noticeable bands. Moving on to the pre-filtrated and affinity isolated fractions, several bands were found in the pre-filtrate fraction 3 and 4 at 125, 82, 75, 58 and 42 kDa. The darkest band is at 75 kDa. Also the Wash fraction showed a vague band at 75 kDa. No bands could be detected in the elution samples of the rabbit platelet lysate. The magic mark XP molecular weight ladder was

used for the size determination of the bands. Rabbit integrin β3 could be detected in the fraction obtained from the pre filtration column but not in the fraction from the affinity column.

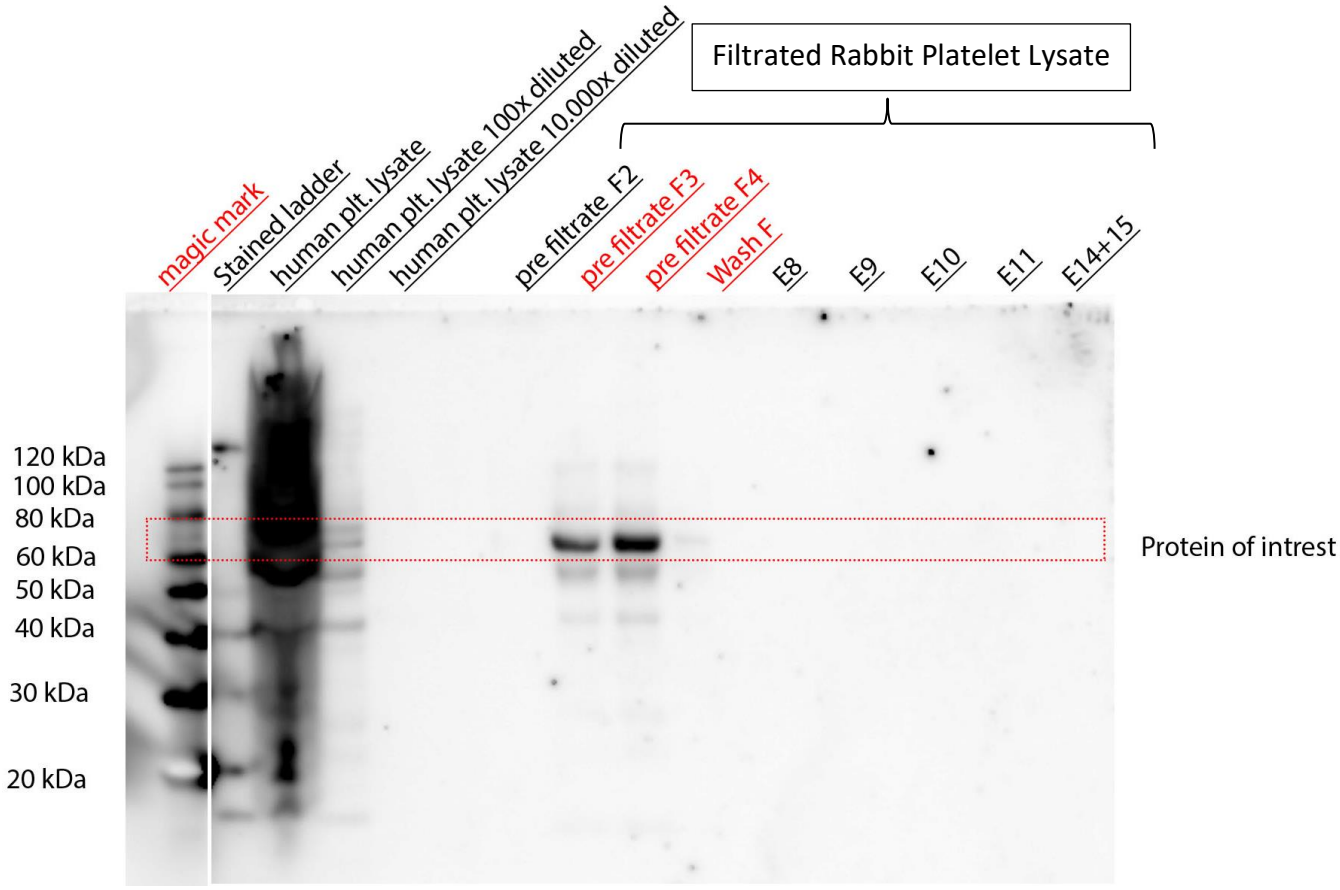

Figure 3-13 ***Western blot of human platelet lysate at various dilution states and various fraction of pre-filtrated or affinity purified rabbit platelet lysate.***

12 µL samples were taken from human and rabbit platelet lysate as depicted above. The lysate and purified fractions were analyzed under reducing conditions on a western blot. The western blot showed 1 continuous band for the human platelet lysate amd several fainter bands for the 100x diluted human platelet lysate. Strong bands at 75 kDa, and lighter bands at 75, 70, 58, 43, 25 and 19 kDa for the pre-filtrate F3 and F4 samples were detected. 1 Faint band at 75 kDa was also found in the wash fraction, while no band could be found in the elution fractions. For both western blot MagicMark XP western protein standard were used.

4 Discussion

During this study the long term goal is to prevent the occurrence of FNAIT by the induction of oral tolerance against human platelet antigen, HPA-1a, in pregnant HPA-1bb women. To achieve this long term goal 3 primary objectives were set for this study. Firstly, to determine if HPA-1a specific antibodies can bind rabbit platelets. Secondly, examine whether rabbit platelets can activate HPA-1a specific T-cells. Finally, to examine whether HPA-1a specific T cells can specifically recognize and be activated by rabbit integrin β3 protein, rabbit integrin β3 therefor needs to be isolated.

4.1 Rabbit integrin is, in theory, an activating antigen for HPA-1a specific T-cells.

In previous studies, it was proven that certain peptides with 1 amino acid substitution in the HPA-1a antigen L33 could still induce activation in HPA-1a specific T-cells (4). Specifically the V33 (AWCSDEALPVGS) and I33 (AWCSDEALPIGS) peptides with respectively Valine and Isoleucine substituted at position 33 of the HPA-1a integrin β3 protein, were identified as equally effective activators (4). As previously explained, the DRB3*01:01 consists of 3 main binding pockets P1, P4 and P9 with different characteristics determined by the make-up of the binding pockets amino acids (22). The recognition of rabbit platelet antigen by HPA-1a-specific T cells is likely due to efficient anchoring of rabbit V33 peptide, present in rabbit integrin β3, to the HLA-DRA/DRB3*01:01 MHC class II molecule (22); the valine residue will then be buried in the P9 pocket and the remaining residues within the T cell epitope will be similar to that of HPA-1a. Thus, the valine is likely not contacted directly by the TCR, but serves to anchor the peptide to the MHC molecule.

Using rabbit blood products for treatment by oral tolerance induction would be more esthetical and culturally acceptable compared to using human or even animals such as apes, cats, dogs and bears, which all carry the HPA-1a T cell epitope. Also, some of these animals would probably be challenging to draw blood from. Obtaining HPA-1a like antigens from other sources, like rabbits, is be more ethical and manageable. Additionally, rabbit could be assumed to be generally safe in an oral therapy as rabbit is used as a source of food.

4.2 Binding of the 26.4 antibody to the rabbit platelets indicates HPA-1a specific T-cell may be specific to rabbit integrin β3.

The antibody binding panel performed on human HPA-1a$^+$, HPA-1b$^+$ or rabbit$^+$ platelets showed binding of human platelets as expected from previous studies (5, 31, 32, 34). Rabbit platelets were not bound by AP3 and Y2/51 as they are specific to human integrin β3 (31, 32). Mabs 26.4 and SZ21 however were both able to bind the integrin β3 on rabbit platelets since they are both specific for HPA-1a defining epitope of integrin β3 (5, 34). When the binding of mAb 26.4 to rabbit and HPA-1a integrin are compared, the binding of 26.4 does show to be less in rabbit platelets, which is could be due to the the leucine to valine substitution at aa position 33. Here, however, mAb 26.4 and SZ21 show the same reduced binding. An important note is that for mAb 26.4 to bind with high affinity it needs a aspartic acid (D) at aa position 39 and a glutamine (Q) at aa position 470 and possibly others (5). Both positions are homologous is rabbit integrin β3. An alternative explanation could be simply that less platelets were in the rabbit platelet samples or that they were of lower viability reducing the overall integrin β3 HPA-1 antigen quantity. Nevertheless, the binding of mAb 26.4 to rabbit platelets and presumably integrin β3, which has some overlap with the HPA-1 epitope, gives an indication HPA-1a specific T-cells may be activated by rabbit platelets and/or integrin.

4.3 Is the antigen on rabbit platelets that activates HPA-1a-specific T cells integrin β3?

Our data suggests that HPA-1a may be activated by rabbit platelets/integrin β3 T-cell activation was measured. Here the main aim is to determine if HPA-1a specific T-cells are able to be activated by rabbit platelets and more interestingly if rabbit integrin β3 is the cause. T-cells activation assays were used to determine if DRB3-peptide (rabbit integrin)-HPA-1a specific TCR interaction yields equal results in stimulation of HPA-1a antigen. Also, since

HPA-1a specific antibodies bind rabbit platelets, one could argue that if antibodies and T cells are binding the same antigen, the antigen captured by these antibodies in rabbit platelet lysates should be the same antigen that activates HPA-1a specific T cells. This is discussed below.

4.3.1 HPA-1a specific T-cell activation with 26.4 mAb linked Dynabeads bound HPA-1a or rabbit integrin presented by monocytes showed inconclusive results.

The human HPA-1a^{+} and rabbit integrin β3 proteins bound on mAb 26.4-conjugated Dynabeads yielded inconsistent and unexpected results as both respective antigens caused little to no significant activation in T-cells. Both from theory as explained above, and from assay results of T-cell stimulation with peptide and with whole platelets from HPA-1a^{+} donors and rabbits , it may be assumed something has failed to work in Dynabeads protein binding or processing and presentation of antigen on beads by APCs. The method of using Dynabeads M-270 Epoxy as platform for antigen take up by monocytes T-cell activation in in viro cell culture assays has been successfully examined and used before (42). In theory the antigen bound to the 26.4 mAb-linked Dynabeads would be internalized by the monocytes, which would process the antigen into smaller peptides loaded into MHC II (DRB3*01:01). This means that even if the antigen (rabbit or HPA-1a integrin β3) is bound by 26.4 at the same epitope as our APCs MHC II would need to bind it, it wouldn't interfere since the whole mAb-antigen complex is processed and dissociated. The problem thus likely lies elsewhere. The qualitative control of 26.4-linked Dynabeads did additionally show that Dynabeads were able to be conjugated with mAb 26.4 and subsequently with HPA-1a or rabbit integrin β3. This qualitative method of examining the conjugation of the antibody and binding of the antigen to Dynabeads is not able to quantify the amount of antigen bound to the beads with certainty. The problem may thus be that to little antigen was bound to the beads for monocytes to take up and significantly activate HPA-1a specific T-cells. This is also further supported by the fact HPA-1a antigen was able to stimulate D8T104 to a small degree. A possible factor for the low quantities of antigen bound on the Dynabeads could be the use of Triton X-100 instead of RIPA buffer previously used for lysing of platelets in this

protocol. The different lysis method may have affected or degraded the target proteins to a certain degree for example. In the future RIPA buffer should be used and possibly further protocol fine tuning could be performed to investigate the ability of rabbit or HPA-1a integrin β3 protein to stimulate HPA-1a specific T-cells.

4.3.2 Rabbit integrin β3 antigen possibly has an equal ability to activate and cause proliferation in HPA-1a specific T-cells as HPA-1a antigen.

To evaluate the reactiveness of HPA-1a specific T-cells to APC presenting rabbit integrin antigen ("peptide", protein and whole platelet), TNF-α and proliferation assays were performed. The TNF-α assay measures immediate T-cell responses starting right after TCR activation by an appropriate antigen-MHC complex. Our results showed all blanks and negative controls without TNF-α expression, additionally stimulation with L33 peptide showed a response as expect from previous assays performed in our group (4). Also, we found that antigen from rabbit platelet lysate captured with anti-HPA-1a mAb 26.4 was able to stimulate a relatively weak TNF-α expression on HPA-1a specific T-cells. Furthermore, the captured antigen was also detectable with a second anti-HPA-1a mAb SZ21. This suggests that the isolated antigen recognized by HPA-1a- specific T-cells is rabbit integrin β3. Previously the peptide V33, being the L33 equivalent for rabbit integrin β3, been shown by M. T. Ahlen et.al, to induce TNF-α secretion with similar intensity to L33 (4). Since TNF-α secretion in T-cell is an immediate response but not solely defining for a full T-cell response (43), to further support the hypothesis that rabbit integrin β3 can stimulate HPA-1a specific T-cells the proliferation was measured. The results show close to equal D8T104 activation and proliferation by both monocyte presented human HPA-1a^{+} and rabbit platelets. Rabbit integrin β3 protein was not able to cause proliferation in D8T104 and HPA-1a only had a small population of proliferating T-cells, possibly due to low antigen amount captured on beads.

It can be concluded that rabbit integrin β3 can cause activation of HPA-1a specific T-cells, as shown most conclusively from platelet derived antigens. And additionally, can show stimulation of T-cell both in short term (TNF-α excretion) and also in the long term (proliferation) indicating that the T-cell are being immunologically activated (12, 43). As of

now, to our knowledge, no other studies have been done activating HPA-1a specific T-cells with rabbit or more general animal antigen. Except, where a whole range of 12 aa long peptides with various aa substitutions were examined for their ability to activate HPA-1a specific T-cells as discussed above (4). Since rabbit integrin β3 antigen can be used to activate HPA-1a specific T-cells, the respective antigen may possibly also be used to induce tolerance in HPA-1a specific T-cells. Several tolerogenic immunological environments have been identified in the human body, like thymus, MALT, GALT and also certain mechanisms which can function throughout the body (12). Specifically the GALT is an interesting and also practical environment to examine tolerance induction for FNAIT, as the antigen only needs to be eaten, preferably in larger doses and possibly with adjuvants (29, 44-46). Tolerance induction via oral tolerance is mainly practical since rabbit is already commonly eaten and has thus been proven not to be a health hazard. However many different factors play a role in tolerance induction and it would need to be tested in the future in for example an animal model, to be come closer to a certain answer. Future perspectives on immunotherapies and in vivo testing, as well as a possible animal modal will be discussed later.

4.4 Integrin β3 isolation by affinity filtration was not successful.

As a measure of obtaining integrin β3 of HPA-1a or rabbit type a protocol was constructed using a size-exclusion pre-filtration column and an affinity isolation column with 26.4 linked CNBr activated sepharose resin. The obtained integrin β3 was then to be used in the TNF-α and proliferation assay, but we were unable to isolate enough target protein for detection and thus likely not enough for T-cell stimulation. The size-exclusion pre-filtration column was used to filter out salts and proteins under 30 kDa. The nanodrop results of the pre-filter fractions showed that molecules over 30 kDa exited the column first, as the bovine serum albumin (65 Da) peak overlapped the first peak of our platelet lysate sample. Protein smaller in size than 30 kDa are assumed to be the second peak and subsequent peaks are small molecules like salts as state by manufacturer (47). A western blot performed on the fractions taken after the void volume (first three fractions) showed that the target protein integrin β3 was present in the filtered fraction . By calculation it was determined that the 26.4 linked

affinity column has a capacity of ~7,3 mg of rabbit of HPA-1a integrin β3 (see appendix II – I), which is more than enough for T-cell stimulation. Additional calculation showed that approximately 22 µg of integrin β3 can be obtained/5 mL of rabbit or human blood (see appendix II – I)(14, 48). This showed that the affinity column has a high enough capacity for our purposes during this study, but that amount of integrin β3 present is low and only 3 factors higher than the western blot detection limit (WB detection limit is 0,1 ng). Since, during this study, the main reason for starting protocol testing of integrin β3 was concept development the experiment was done anyway. Integrin β3 is estimated at a size of 90-110 kDa from the GPIIIa subunit and the GPIIa with a heavy chain at 120 kDa and light chain at 22 kDa linked by disulfide bonds. Dependent on a large number of variables are these subunit seen together or apart and can therefore vary widely in weight, anywhere from ~90 kDa to 255 kDa (49). Rabbit integrin β3 is very similar in weight and subunit build-up although thought be 2-10 kDa heavier in general (50). A note to the comparison between western blot figures 4-5 and 4-13 where the integrin β3 sizes vary slightly. This was likely due to the beads with integrin bound being warmed to 70°C seen in figure 4-6 while the heavier integrin β3 in the isolate was not warmed at all. Western blot results of the fractions captured after the void fractions did not show any detectable levels of integrin β3, some integrin β3 however was detected in the wash fractions and pre-filtrate fractions (Fig 4-13). Probable reasons for losing a factor 3 or more of target protein are likely to be dilation during the process of isolation, loss of target protein during the wash, target protein retention in the column during elution and/or varying platelet quality. The manufacturer of the CNBr activated sepharose resin also states larger quantities of protein are preferred as protein may be lost in the process due various reasons (51). For future application of this method, significantly larger volumes of platelet lysate should be used to negate losses during the filtration process.

4.5 Future perspectives

4.5.1 Immune therapy through oral tolerance and it's opportunities for optimization.

As mentioned before in the introduction is there to this point still no treatment for FNAIT, prophylactic or therapeutic. A method that could potentially be used both as a prophylaxis before the onset of FNAIT and to lesser degree as therapeutic treatment during or for subsequent pregnancies could be of great interest. mAb 26.4 has and is being researched as a treatment for FNAIT in the form of blocking antibodies or antibody mediated immune suppression (5, 52). Rabbit integrin β3 however, has potential to be used as an antigen for oral tolerance induction in platelet, possible protein and also peptide forms as seen in this thesis and previous studies within our group (4). The rabbit platelets could for example be ingested by ingesting rabbit, its blood in some form or isolated rabbit integrin β3 protein, which is low impact for the patient. To be sure how effective this would be more study would need to be done, for example, if cooked rabbit would still have the intact L33 region, how much digestion affects the protein or if the dose of antigen would high enough. An interesting study that could be performed could also be to statistically analyse if communities regularly ingesting rabbit have lower occurrence of FNAIT than communities that do not normally eat rabbit.

The results from this thesis can potentially be moulded into a plethora of different ways to adjust and make it more effective as an inducer of oral tolerance. A next step may be to research the effect of oral ingestion of rabbit β3 platelets or protein on HPA-1a tolerance in an animal

5 Conclusion

During the course of this 1 year master thesis aims were set as preparation and research into the potential of oral tolerance therapy (and other immunological therapies) using the HPA-1a antigen as means of tolerizing the patient to the respective antigen. The first aim was to ascertain if HPA-1a antibodies were specific to rabbit integrin β3 in protein, molecule (and peptide) form. This was done to determine if rabbit integrin β3 is similar enough in primary and tertiary protein structure to human HPA-1a^{+} integrin β3. Both analysis from protein databases and previous studies show promising similarity of the rabbit integrin β3 to HPA-1a, especially the 25-33 aa region. This finding was further confirmed by the antibody antigen binding assay, where HPA-1a-specific mAbs SZ21 and 26.4 bound significantly to rabbit integrin β3 on rabbit platelets. Since 26.4 mAb is specific, in part, for the same epitope as are HPA-1a specific T-cell, stimulation of HPA-1a-specific T cells with antigen captured by mAb 26.4 from rabbit platelet lysate lends support to the notion that these T cells are indeed activated by rabbit integrin β3.

Rabbit integrin β3 antigen in peptide, (protein) and platelet form could be successfully used to activate HPA-1a specific T-cells and imitate immunization of mothers pregnant with a FNAIT afflicted child. Rabbit integrin may therefore be a realistic candidate for future tolerization studies as a treatment for FNAIT.

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Appendix

I Calculations

I - Calculation of affinity column capacity:

- In the column ~5 mg of mAb 26.4 was used. Which was bound to the resin by the Fc region of the mAb and the mAb will thus have two binding regions.
- mAb's are approx. 150 kDa
- HPA-1a/rabbit integrin β3 weigh approx. 87 kDa but are usually detected at 110 kDa.

$$mAb\ mol = \frac{5*10^{-3}\ gram}{150.000\ kDa} = 3.3 * 10^{-8}$$

$$mass\ of\ HPA-1a\ or\ rabbit\ integrin\ \beta3\ expected\ to\ bind$$
$$= (3.3 * 10^{-8} * 2) * 110.000 = \sim 0{,}0073\ gram \rightarrow \sim \mathbf{7{,}3\ mg}$$

II - Calculation of protein we expect in our platelet sample:

- both human and rabbit platelets have ~80.000 molecules of integrin β3 on its surface/platelet (14, 48). There is about $300*10^9$ platelets/liter of blood in both human and rabbits → we use around 5 mL of blood per isolation cycle.

$$amount\ of\ integrin\ \beta3\ per\ 5\ mL\ of\ blood = 300 * \frac{10^9}{1000} * 5mL * 80.000$$
$$= 120.000 * 10^9\ plt \rightarrow to\ mol = 1{,}99\ \times\ 10^{-10}\ mol$$

$$Rabbit\ integrin\ \beta3\ mass\ per\ 5\ mL\ blood = 1{,}99\ \times\ 10^{-10}\ mol * 110.000 =$$
$$2{,}2 * 10^{-5}\ gram \rightarrow \sim \mathbf{22\ \mu g}\ rabbit\ integrin\ \beta3\ per\ 5\ mL\ blood.$$

II Additional information

I - Method for HPA-1a/rabbit integrin β3 isolation with a size-exclusion and affinity (mAb 26.4) column.

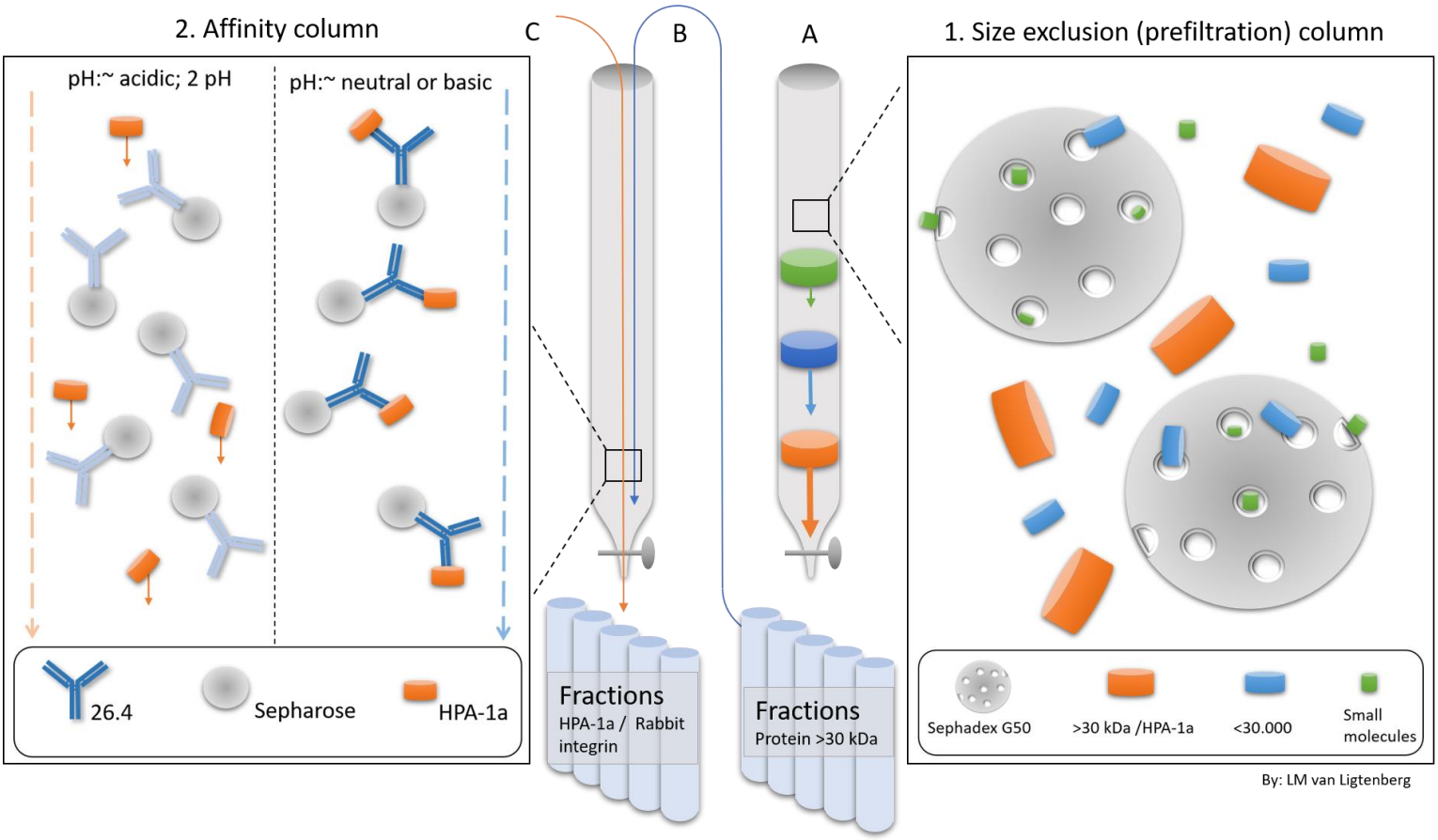

Figure Appendix 1 ***schematic of the HPA-1a/ rabbit integrin β isolation setup.***

Platelet lysate of rabbit or HPA-1a type is prefiltered in a column containing Size-exclusion resin called Sephadex G50. 1. This resin has small holes through which mainly the molecules under 30 kDa can travel (small molecules in green). Bigger molecules do not enter the holes of the resin and have therefor a quicker path through the resin (medium and big molecules in blue and orange). A The big molecules over 30 kDa will exit the column first. B The fractions are measure with nanodrop for protein concentration and the fractions containing the first peak in protein concentration are entered in to the affinity column. 2. The HPA-1a and rabbit integrin β3 will bind the 26.4 linked CNBr-activated resin while all other proteins will exit the column by washing. C After washing the column is eluted with a low pH buffer and target protein will loosen from the 26.4 and be captured in fractions.

II - Increased loading stability of Peptides in MHC II on B-LCL enables T-cell activation by L33 but not LolP1.

Previously the loading stability and thus the quantity of MHC II loaded peptide was assessed in B-LCL's in the presence or absence of Adamantane Ethanol. LolP1 and L33 peptides were loaded into B-LCL MHC II by a 4 hour coincubation with Adamantane Ethanol or DMSO in the controls. Some B-LCL were then used for a peptide loading quantification by flow cytometry (Section 4.2.1). With the remaining B-LCL's a CFSE proliferation assay was performed if a significant difference in HPA-1a specific T-cell activation could be detected.

In figure appendix 3 the HPA-1a specific T-cell clone D8T104 is represented in 4 flow cytometry histograms. D8T104 was incubated with B-LCLs pulsed with negative control peptide LolP1 with or without AdEtOH (left top and bottom Fig 4-3) . Both respective D8T104 population only showed the brightly CFSE-stained population with no populations with decreased CFSE fluorophore intensity indicating no proliferation. L33 pulsed B-LCL's can be seen on the right, where AdEtOH-less pulsing showed no populations decreased in CFSE intensity. However L33 pulsed B-LCL's in the presence of AdEtOH did show 1 to 2 D8T104 populations with decreased CFSE intensity, indicative for T-cell proliferation (Fig 4-3 lower right histogram). Indicating that AdEtOH increased loading stability in B-LCL enabled T-cell activation by L33 presentation but not lolP1, while non AdEtOH treated B-LCL L33 presentation was not able to activate HPA-1a specific T-cells.

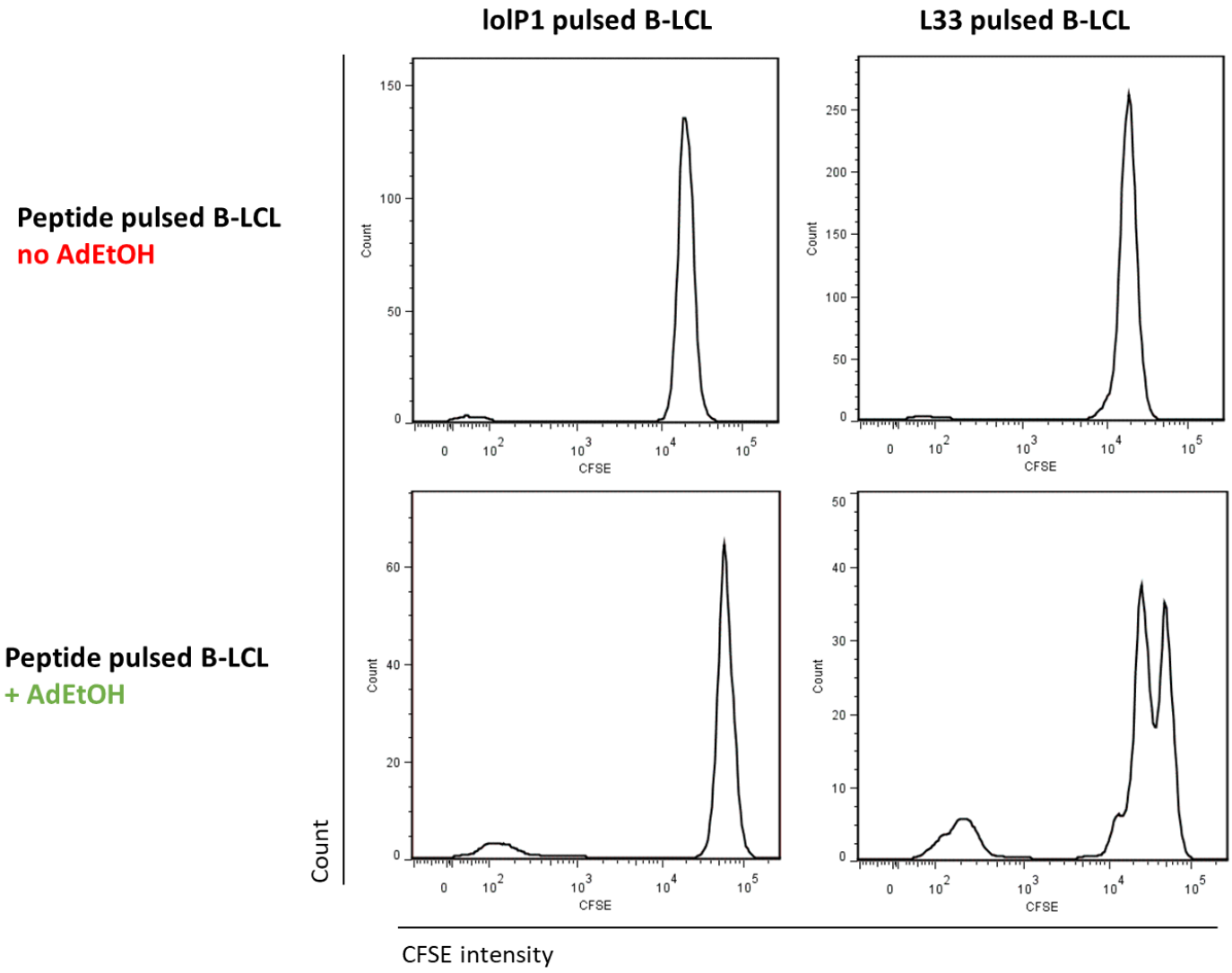

Figure Appendix 3 ***Proliferation assay with D8T104 T-cell clone incubated with B-LCL's pulsed with control peptide lolP1 or L33 with AdEtOH present or absent.***

The T-cells activation was assessed by a CFSE proliferation assay to determine the positive or negative effect of increased peptide loading through added AdEtOH. The top left shows the D8T104 population incubated with B-LCL pulsed with LolP1 in absence of AdEtOH. The lower left shows the D8T104 population incubated with B-LCL pulsed with LolP1 in presence of AdEtOH. The top right histogram shows the D8T104 population incubated with B-LCL pulsed with L33 in absence of AdEtOH. The lower right shows the D8T104 population incubated with B-LCL pulsed with L33 in presence of AdEtOH, with the parent as well as 2 daughter populations. CFSE intensity is presented on the X-axis and cell count on the Y-axis.

Appendix Table 1 Media, buffers, materials and reagents used during this study.

Phosphate Buffered Saline (PBS)	**Medicago (Uppsala, Sweden)**	**Washing**
PBS + 0.2% Bovine Serum Albumin (PBSA 0.2%)	In house (UNN)	Washing, flow cytometry
Iscove's Modified Dulbecco Medium (IMDM)	Lonza (Basel, Switzerland)	Cell culture
HPA-1a$^-$ plasma	Blood donor blood bank UNN Tromsø	Cell culture
Fetal Bovine Serum (FBS)	Thermo Fisher (Waltham, MA)	Cell culture
Penicillin-Streptomycin	Sigma (St. Louis, MO)	Cell culture
IL-2	PreproTech (London, UK)	Cell culture
IL-15	PreproTech (London, UK)	Cell culture
CFSE, CellTrace	Thermo Fisher	Staining for flow cytometry
Dimethyl Sulfoxide (DMSO)	Wak-Chemie Medical GmbH (Steinbach, Germany)	Cryo storage
Lymphoprep ™	Axis-Shield (Dundee, Great Britain)	Density gradient for fractioning of whole blood or buffy coat
Anti-CD3 Ab	Thermo Fisher	Cell culture
PHA (R30852801)	Thermo Fisher	Cell culture
Adamantane ethanol		B-cell pulsing
UltraComp eBeads	Thermo Fisher	Flow cytometry
Dynabeads antibody coupling kit	Novex life science	Beads coupling
Dynabeads M-270 Epoxy	Novex life science	Beads antigen pulsing
RosetteSep monocyte enrichment cocktail	Stemcell technologies	Monocytes enrichment
EDTA		HPA-1a antibody assay
Sodium Azide		Antibody/peptide storage
TRITION X-100		Cell lysis
TAPI-0		TNF-α
Sephadex G50 superfine	GE healthcare	Protein isolation
MilliQ water	In house	Protein isolation
Ethanol		Protein isolation
CNBr- activated Sepharose 4B	GE healthcare	Protein isolation
HCl		Protein isolation
$NaHCO_3$		Protein isolation

NaCl		Protein isolation
Tris-HCl		Protein isolation
Acetate buffer		Protein isolation
Protease inhibitor cOmplete	Roche	Protein isolation
Tris		Protein isolation
trisodium citrate dehydrate		Protein isolation
NuPAGE 4x LDS Sample Buffer	Novex	SDS-PAGE
NuPAGE 10x Sample Reducing Agent	Novex	SDS-PAGE
20x NuPAGE Running buffer	Novex	SDS-PAGE
NuPAGE® Novex® 4-12% Bis-Tris Gels	Novex	SDS-PAGE
SimplyBlue SafeStain		SDS-PAGE
HRP		WB
MagicMark XP western protein standard	Invitrogen	WB
Invitrolon PVDF Filter Paper Sandwich 0.45 um Pore Size	Invitrogen	WB
NuPAGE 20x Transfer Buffer	Invitrogen	WB
SuperBlock (PBS) Blocking Buffer	Thermo Scientific	WB
blocking reagent	Thermo Scientific	WB
PBS 0.05% Tween 20	In house	WB
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Scientific	WB