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Expression and characterization of recombinant murine integrin β3 modified to express the human platelet antigen-1a epitope

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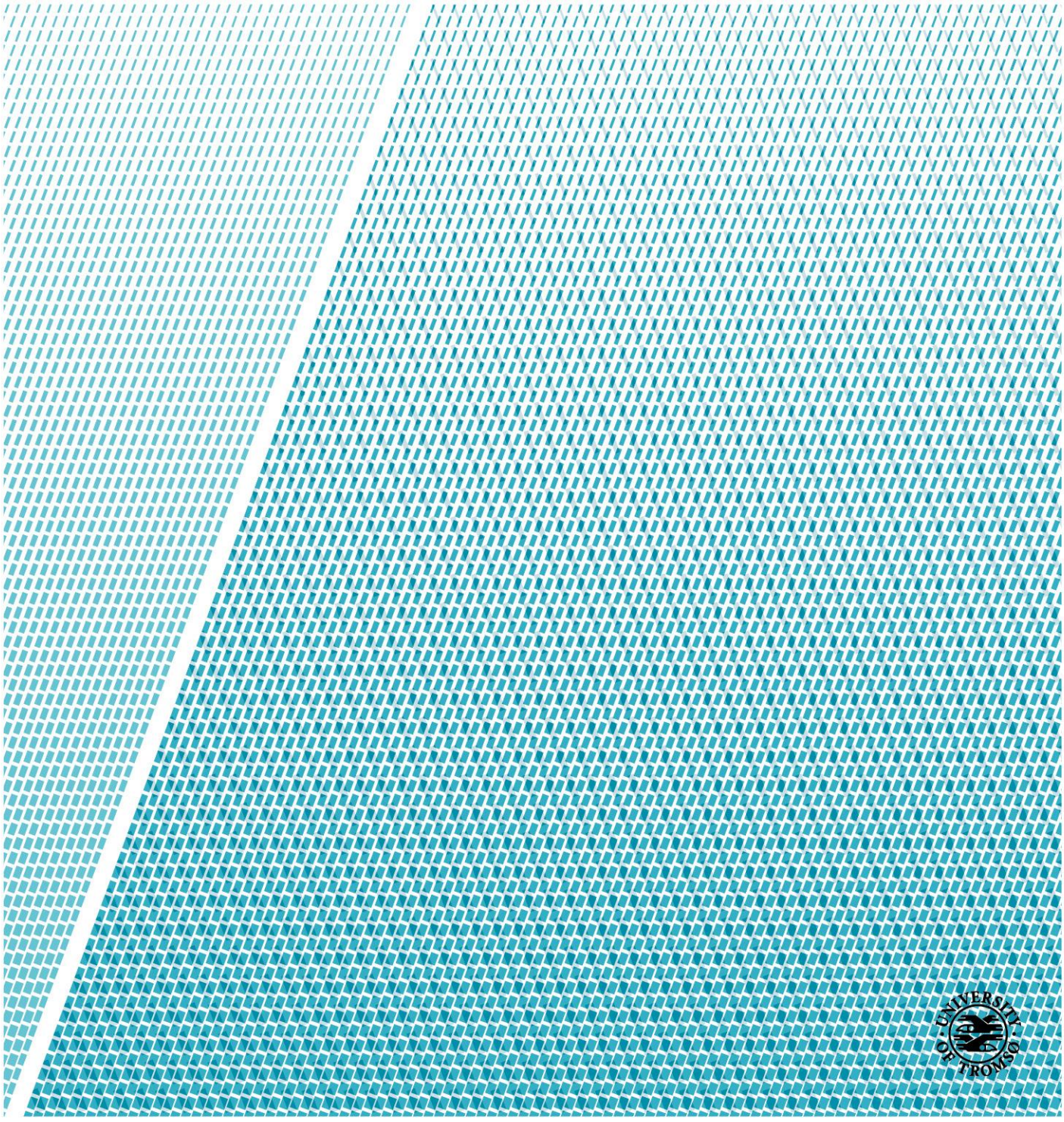

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Tromsø, May 2017

Trude Victoria Rasmussen

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Abbreviations

AcNPV	*Autographa californica* nuclear polyhedrosis virus
AP	Alkaline phosphatase
APC	Antigen-presenting cell
BAC	Bacterial artificial chromosome
Bp	Base pair
BSA	Bovine Serum Albumin
BV	Budded virus
CFSE	Carboxyfluorescein succinimidyl ester
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
GP	Glycoprotein
GV	Granuloviruses
HLA	Human leukocyte antigen
HPA	Human platelet antigen
HRP	Horseradish peroxidase
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITGB3	Integrin β3
Kb	Kilobase
KDa	Kilodalton
LB	Luria-Bertani
mAb	Monoclonal antibody
mITGB3	Murine ITGB3
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MWCO	Molecule weight cut off

NPV	Nucleopolyhedrovirus
NTC	No template control
OB	Occlusion body
ODV	Occlusion-derived virus
pAb	Polyclonal antibody
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBSA	Bovine serum albumin in PBS
PE	R-phycoerythrin
PMT	Photomultiplier Tube
PSI	Plexin-semaphorin-integrin
RPM	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal broth with Catabolite repression
TACE	TNFα converting enzyme
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
X-Gal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside
YFP	Yellow fluorescence protein

Abstract

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition where the mother produces alloantibodies against fetal platelets during pregnancy most commonly due to an incompatibility in the human platelet antigen (HPA)-1 system; a single amino acid polymorphism located on integrin β3 with two common variants, HPA-1a and HPA-1b. Antibody responses against HPA-1a can be formed in women who are HPA-1b homozygous. No effective treatment or prevention to the FNAIT condition are available today, and much is unknown about the immunization process.

Antibody production by HPA-1a-specific B cells is most likely dependent on helper T cells specific for the same antigen. To model these cellular and molecular interactions in mice, we aim to introduce the HPA-1 system in murine integrin β3 by altering four key amino acids, and to use recombinant protein to induce HPA-1a-specific B and T cell responses. Predictably, this will more closely mimic FNAIT associated immune responses compared to using human integrin β3 as an immunogen since the latter has many antigenic differences that can induce additional T cell responses.

The aim of the current study was to produce recombinant HPA-1a and HPA-1b variants of murine integrin β3 and to characterize these biochemically and functionally in comparison to human integrin β3. Thus, in this study, these recombinant murine integrin β3 proteins were designed and expressed using the baculovirus system. Concurrently, recombinant human integrin β3 was expressed using an already prepared virus stock. Produced proteins containing 6xHis were captured from the supernatants on beads coated with anti-His-tag antibody. The protein production was confirmed using SDS-PAGE and western blot, and the presence of the HPA-1a epitope was verified by flow cytometry using HPA-1a-specific antibodies.

HPA-1a-specific T cells isolated from an HPA-1bb mother with a child affected by FNAIT, were incubated with HLA-DRB3*01:01 positive monocytes co-cultured with antigen. HPA-1a platelets, integrin β3-derived peptide comprising the Leu33 residue and recombinant murine and human integrin β3 protein expressing the HPA-1a epitope stimulated T cell proliferation and tumor necrosis factor (TNF) production. Thus, the produced proteins can potentially be used to further study the interaction between HPA-1a specific B cells and T cells in a future murine model.

1 Introduction

1.1 Integrin structure and function

Integrins constitute a large family of transmembrane adhesion molecules, important for cell adhesion to the extracellular matrix but also adhesion between cells. The molecules are also significant in signal transduction and have the ability to transmit signals in both directions; inside-out and outside-in. Integrins have important roles in embryonic development, immunity, cancer and hemostasis. However, they are also in the focus of different autoimmune diseases and are receptors for many bacteria and viruses [1, 2]. Integrins are heterodimers consisting of two non-covalently associated glycoprotein subunits called α and β. These subunits are transmembrane with a short intracellular C-terminal and a large extracellular N-terminal. There are 18 α subunits and 8 β subunits known that combine to form at least 24 different integrins [1].

To control the activity of the integrins, the molecules have two states, active and inactive states (Figure 1.1), controlled by allosteric regulation [1]. For instance, a white blood cell has to change integrins from inactive to active state to attach to the endothelium at requested sites to be able to crawl out of the blood vessel, so regulation of the integrins have to be fast. In the absence of a signal, the integrin is in an inactive conformation. Here, the intracellular tails lie close together and the extracellular part is folded. When stimulated from ligand binding, either to the head region or to the tails, the intracellular parts separate allowing binding to intracellular cytoskeleton, and the extracellular domain unfolds extending the subunits. The headpiece is then open and ready for ligand binding. Due to the coupling of structural change to both ends of the integrin, the signal can be transmitted in both directions [2, 3].

1.1.1 Integrin ß3 chain can associate with αV and αIIb

The different integrins, made by combinations of α and β chains, all have distinct functions and properties, also depending on the cell type they exist on. Due to the different integrin variations, harmful changes can happen if there are defects in α or β chains.

Integrin β3, also named Glycoprotein (GP) IIIa, is encoded by the *ITGB3* gene. The integrin β3 chain only combines with α chains αIIb or αV [1]. αIIbβ3 is a receptor that binds to fibrinogen,

fibronectin, von Willebrand factor and vitronectin [4], while αvβ3 is a receptor that binds to vitronectin and osteopontin *in vitro* [5, 6]. Both integrins are present on platelets; the integrin αvβ3 is present in a low degree, only a few thousand per platelet [7], while the integrin αIIbβ3 is the major integrin on platelets, constituting approximately 80 000 integrins per platelet [8]. The two α chains have a 36.1 % identity in their protein sequences [9].

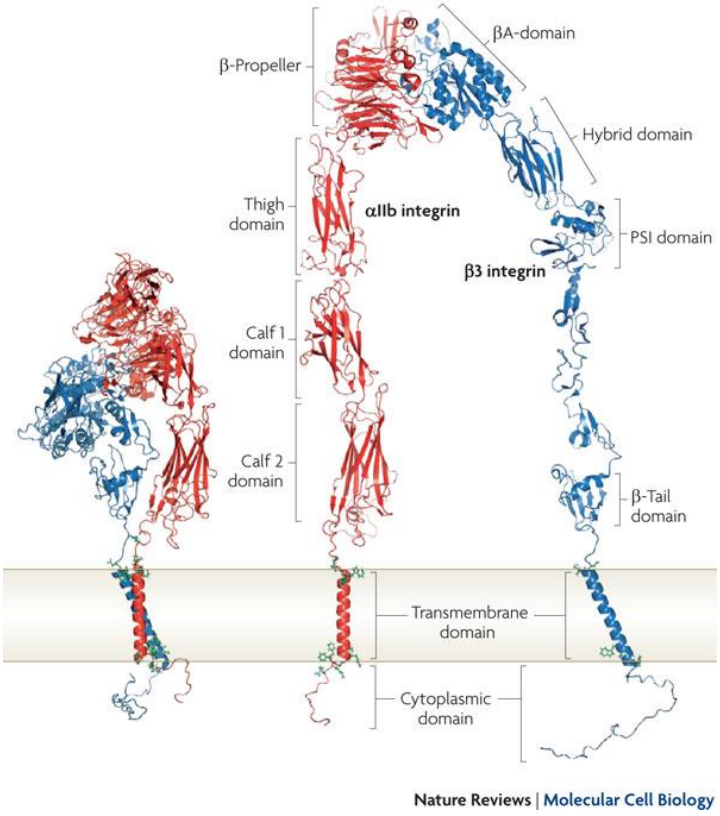

Figure 1.1: Domain structures of integrin αIIbβ3. Integrins are transmembrane adhesion molecules consisting of an α and β subunit. Most subunits have a short cytoplasmic domain and a large extracellular domain, where the two subunits assembles non-covalently. In their inactive state, the integrins form a bent conformation with their tails close together (left). When activated, the tails separate and the extracellular domain unfolds (right). In αIIbβ3, the β-propeller (αIIb subunit) and the βA-domain (β3 subunit) assemble to form the head region, which contains the ligand binding site. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology [10] (modified from EMBO j [11] (2009)), copyright (2010).

1.1.2 Human platelet antigens

Platelet glycoproteins exists in different polymorphic forms caused by single nucleotide polymorphisms (SNPs) in the genes expressing the proteins. These different polymorphic forms have structural differences resulting in the generation of antigens defined as human platelet antigens (HPA). Integrin β3 has a SNP generating an amino acid difference in position 33 giving rise to the HPA-1 Leu33Pro polymorphism [12]. When leucine is present, which is the major

antigen, it is defined as HPA-1a. On the other hand, a proline in this position defines HPA-1b. Only 2 % of the Caucasian population are homozygous (HPA-1bb) for the proline version [8].

1.1.3 Integrin ß3 is associated with FNAIT

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition caused by the production of alloantibodies in the mother during pregnancy, reactive with fetal platelets. The most common cause of this condition is an incompatibility in the HPA-1 system between the mother and fetus [12].

If an HPA-1bb woman is carrying an HPA-1a positive fetus, the mother produces alloantibodies against the fetal platelets. The alloantibodies can then travel over the placenta and into the fetal circulation where they opsonize the fetal platelets causing an immune response. The destruction of the fetal platelets by the maternal immune system, can result in thrombocytopenia (low platelet count) in the fetus. Consequently, if the platelet count becomes too low, the fetus is rendered with a high bleeding risk which in severe cases can cause intracranial hemorrhage and permanent brain damage or death [13-15].

1.1.4 FNAIT alloimmunization is associated with MHC class II allele HLA-DRB3*01:01

Several studies have shown that the alloimmunization in HPA-1bb women is strongly associated with human leukocyte antigen (HLA)-DRB3*01:01 [16-19]. A Norwegian prospective study showed that approximately 90 % of the immunized woman expressed this HLA molecule [15], compared to approximately 28 % in the normal population [16]. Integrin β3-derived peptides comprising the Leu33 residue have been shown to stably bind HLA-DRB3*01:01, but not peptide comprising Pro33 [20, 21].

Due to the binding of peptides with Leu33 to HLA-DRB3*01:01, but not peptides with Pro33, Leu33 was proven to be an important anchor residue for peptide binding to the MHC molecule. This was shown by Wu et al. who tested the binding of different truncated and mutated peptides to the MHC molecule. The peptides were aligned to determine the core motif [20]; the peptide anchor residues showed to have a 1-4-9 motif, corresponding to amino acid number 25, 28 and 33 in the mature integrin β3 protein (amino acids underlined in Figure 1.3).

The 1-4-9 peptide binding motif was confirmed in 2007 by Parry et al., when HLA-DRB3*01:01 bound by a modified HPA-1a peptide (Herein, "HPA-1a peptide" is defined as short, 20 amino acids or less, integrin β3 peptides spanning residues 25 to 33, with leu33) was crystallized [21]. In the peptide binding groove of HLA-DRB3*01:01, three pockets, P1, P4 and P9, accommodating the three anchor residues of HPA-1a peptide was shown. Of the HPA-1a peptide, the P1 pocket harbors Trp25 and the P4 pocket harbors Asp28. The P9 pocket is hydrophobic and very prominent, and Leu33 is entirely submerged into this pocket due to its hydrophobic side group. Proline contains a cyclic structure in its side-chain, which probably obstruct the binding in the P9 pocket.

1.1.5 T cell responses to FNAIT

T cells from HPA-1bb mothers immunized by paternally inherited HPA-1a present in the fetus, have been shown to proliferate when stimulated with HPA-1a peptide. On the other hand, they showed no proliferation when stimulated with HPA-1b peptide, showing the importance of HPA-1a in the generation of the T cell epitope [22, 23]. Later, clonal HPA-1a-specific CD4 positive T cells were generated from PMBC isolated from immunized women [19, 24]. In the study by Ahlen et al. [19], the clonal T cells proliferated in response to HPA-1a peptide stimulation through HLA-DRB3*01:01 positive antigen-presenting cells (APCs).

The association between FNAIT and HLA-DRB3*01:01 and the binding of HPA-1a (but not HPA-1b peptide) to the MHC molecule, indicates that HLA-DRB3*01:01-restricted, HPA-1a-specific T cells are significant in the antibody production in maternal immune responses in FNAIT. Thus, it could be possible to study and manipulate this T cell response leading to the production of anti-HPA-1a antibodies in controlled conditions through the peptide-MHC complex.

1.1.6 Murine integrin ß3

The murine integrin β3 shows sequence similarity to the human integrin β3 (Figure 1.2), but it does not does not comprise HPA-1a/b.

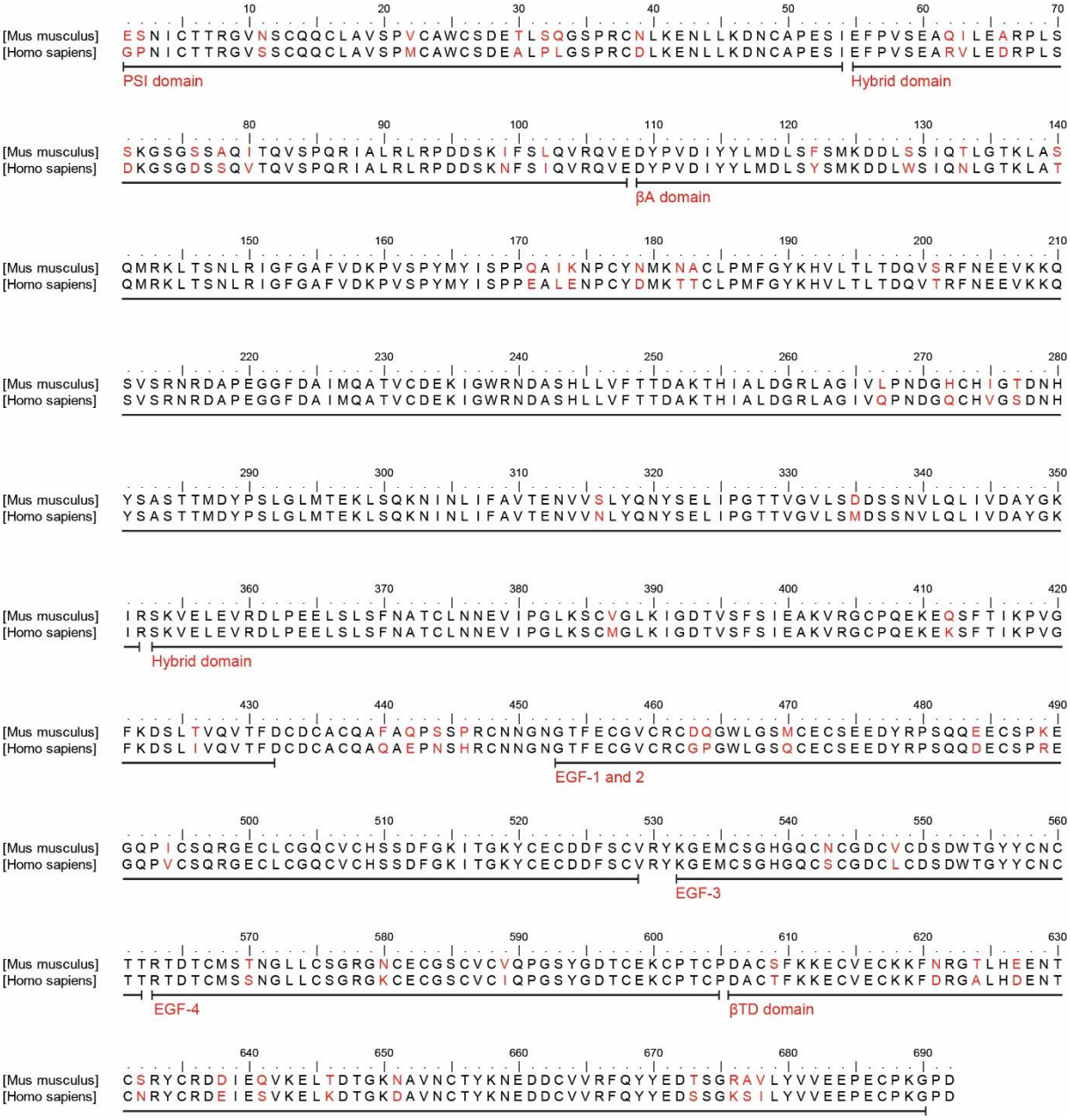

Figure 1.2: Comparison of extracellular domains in integrin β3 of human and murine peptide chains (reference sequence NP_000203.2 and NP_058060.2, respectively). The sequences start with the N-terminal of the mature integrin β3 protein, with amino acid variations between the two species in red. Protein domains are under lined [25].

When comparing the human and murine extracellular domain of the mature integrin β3 protein reference sequences (NP_000203.2 and NP_058060.2, respectively), 65 amino acid differences are present. The HPA-1a epitope is present in the plexin-semaphorin-integrin (PSI) domain, and it has recently been shown that four amino acid substitutions is sufficient to design the HPA-1a epitope in the murine protein [26].

These four amino acid substitutions designed to create the epitopes corresponding to HPA-1a "APLD" and HPA-1b "APPD" are shown in Figure 1.3: T30A, S32P, Q33L/P and N39D in the mature murine integrin β3.

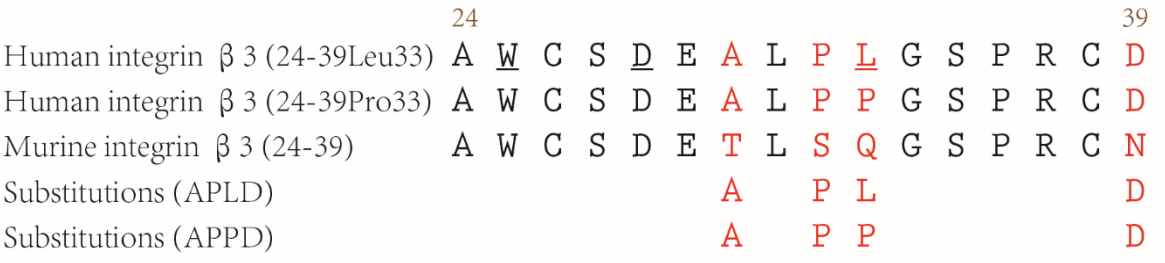

Figure 1.3: Amino acid substitutions in mature murine integrin β3 to resemble the human platelet antigen-1a (HPA-1a). Murine integrin β3 does not contain HPA-1a, and four amino acid substitutions were designed to generate the epitope **[26]**. Residues shown to anchor in HLA-DRB3*01:01 is underlined **[20]**.

1.2 Recombinant protein production

In biomedical science, the behavior and interaction of proteins are in focus due to their essential functions in living organisms. To be able to study proteins, an adequate amount is needed. In some cases, the protein can be isolated from its native source, but often it must be produced *in vitro* to gain practical and functional quantities. The production and purification of protein can be very comprehensive and time consuming. Often recombinant or mutant versions are produced to simplify the production and study.

Depending on the origin of the protein, a suitable protein expression system must be determined. Many systems are available such as bacteria, yeasts, insect cells and mammalian cell lines. Protein production in prokaryotic expression systems are often not ideal for eukaryotic proteins due to the post-translational modifications and protein folding present in eukaryotic organisms. Choosing an appropriate system for the desired protein is therefore important.

1.2.1 Baculoviruses

Baculoviridae are a group of rod-shaped viruses that infect invertebrates and contains circular, double-stranded, supercoiled DNA, which varies from 80-180 kilobases (kb) [27]. During infection, the virus is present as two phenotypes; occlusion-derived virus (ODV) and budded virus (BV). ODVs are important in the survival of the virus in the external environment (insect to insect), while the BV is important in spreading the virus inside the host (cell to cell) [27].

The life cycle of the virus is divided into three phases. In the early phase, also named the virus synthesis phase, the virus infects the cell and starts early viral gene expression. The hosts own gene expression is also turned off. In the next phase, named the late phase or the viral structural phase, viral DNA is replicated and BV is produced. The BV is released from the cell enveloped in the cell membrane, also containing glycoproteins that enable endocytosis by other insect cells. In the last phase, named the very late phase or the viral occlusion protein phase, the polyhedrin and p10 genes are expressed and the ODV are formed leading to cell lysis [28]. The ODV is occluded in a crystalline protein matrix, named the occlusion body (OB). Depending on the morphology of the ODV, baculoviruses are divided into nucleopolyhedrovirus (NPV) and granuloviruses (GV) [29].

1.2.2 Baculovirus expression vector

Autographa californica nuclear polyhedrosis virus (AcNPV) is the most commonly used isolate in recombinant gene expression [30]. When used as a baculoviral expression vector, some genes have been substituted to generate highly efficient protein expression. In wild-type AcNPV, the OBs are made by polyhedron, which is produced in the very late phase together with large amounts of p10 protein. These two proteins are important in horizontal insect cell infection, and are not needed in the late phase during the BV production. Thus, replacing the polyhedrin and p10 genes by genes of interest, the desired protein is produced in large amounts in the very late phase under the control of polyhedrin (polh) and p10 promoters [31].

1.2.3 The MultiBac system

The MultiBac system is a baculovirus expression system that is adapted to produce eukaryotic protein complexes with multiple subunits [32]. The baculovirus expression vector (bacmid) MultiBac is a derivate from the AcNPV genome and contains approximately 130 kb [33]. To produce a protein using the baculovirus expression system, the gene of interest is incorporated into a transfer vector and transfected into insect cells through a baculovirus expression vector. The MultiBac bacmid is present as a bacterial artificial chromosome (BAC) in *Escherichia coli* (DH10MultiBac cells) [32], simplifying the integration of recombinant protein genes.

In the MultiBac system, multiple transfer vectors, separated into acceptor and donor plasmids, have been produced [34]. Both types of vectors contain a LoxP site used for recombination by Cre-fusion, while only acceptor vectors contain a second recombination element, a Tn7L and

Tn7R recombination site for recombination into Tn7 sites. Cre-recombination is typically used for the creation of transfer vector dimers or trimers.

EMBacY is a modified version of the original MultiBac bacmid, created to simplify the detection of proteins produced in insect cells. Integrated in the bacmid backbone, is a gene expressing yellow fluorescence protein (YFP), where the fluorescence intensity increases in parallel with the amount of protein produced. Consequently, the protein production can be monitored using flow cytometry measuring YFP production. *E. coli* containing the EMBacY bacmid (DH10EMBacY cells), contains resistance genes for kanamycin and ampicillin [34].

1.2.4 Tn7 transposition

Integration of a transfer vector into the MultiBac bacmid through Tn7 transposition is provided in trans by transposase genes present on a helper plasmid also existing in the *E. coli* cells [35]. This helper plasmid also confers resistance to tetracycline [32]. The mini-attTn7 site in EMBacY is constructed inside a *lacZ* gene in the genome [32]. If the gene is undisturbed, the *lacZ* gene produces an enzyme named β-galactosidase, which hydrolyses lactose. 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-Gal) is a lactose analog, which when hydrolyzed generates a blue derivative. Isopropyl-β-D-thiogalactopyranoside (IPTG) is a transcription factor for the l*acZ* gene increasing expression. If X-Gal and IPTG is present on the LB agar plates, the colonies will turn blue instead of white [36]. Vectors containing a Tn7L and Tn7R recombination site can be integrated into MultiBac Tn7 sites. If integrated, the *lacZ* gene will not be expressed and the colonies will be white when cultured on plates with X-Gal and IPTG (Figure 1.4) [34, 36].

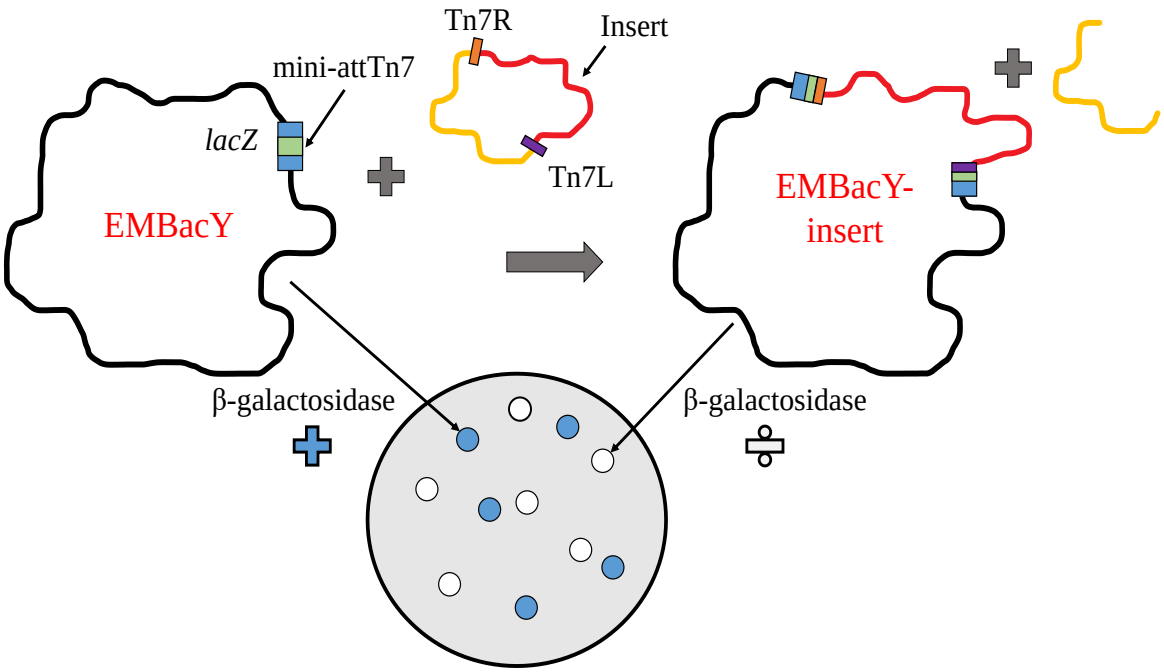

Figure 1.4: Blue and white screening of *E. coli* containing the EMBacY genome with successfully incorporated insert. Transfer vector is incorporated into the EMBacY genome by Tn7-recombinase mediated transposition. Tn7 is present inside the *lacZ* gene causing gene disruption when insertion of transfer vector. By culturing the transformed cells on LB agar plates with IPTG and X-Gal, the negative colonies will be blue due to expressed β-galactosidase enzyme making a blue derivative when hydrolyzing X-Gal. If insert is incorporated, the enzyme is not expressed resulting in white colonies.

This screening for cells with successfully integrated transfer vector in their bacmid is referred to as blue and white screening. The next step in protein production is to purify the EMBacY bacmid from the *E. coli* cells and use it to transfect insect cells.

1.2.5 Protein production using insect cells

As an expression system, insect cells, which are eukaryotic, are more expensive and time consuming than bacteria, but the problems with the post-translational modifications are mostly avoided. The most common method for insect cell protein expression is by the use of the baculovirus expression system. First, insect cells are cultured to a desired concentration, and then they are infected with baculovirus, which uses the host machinery to replicate itself, thus, co-producing the protein of interest incorporated in the baculovirus genome [30].

The most commonly used insect cell lines in protein production are Sf9, Sf21 and High Five. Sf9 and Sf21 are clonal isolates derived from the fall army worm *Spodoptera frugiperda* pupal ovarian tissue [37, 38], while High Five is a clonal isolate derived from the cabbage looper

Trichoplusia ni ovarian cells [39]. The three cell lines can grow in both adherent and suspension cultures, and can be adapted to serum-free medium.

When transfecting insect cells with isolated bacmid, the result is often a low-yield protein and virus production. Thus, it is normal to use the initial virus product to produce a high-titer virus stock, which is further used to make a high-yield protein production. All cell lines produce both virus and protein, but Sf9 and Sf21 cells are generally good at producing virus, while High Five cells are especially good at producing protein [40]. An overview of protein production using EMBacY bacmid and insect cells, which is the strategy in this study, is shown in Figure 1.5.

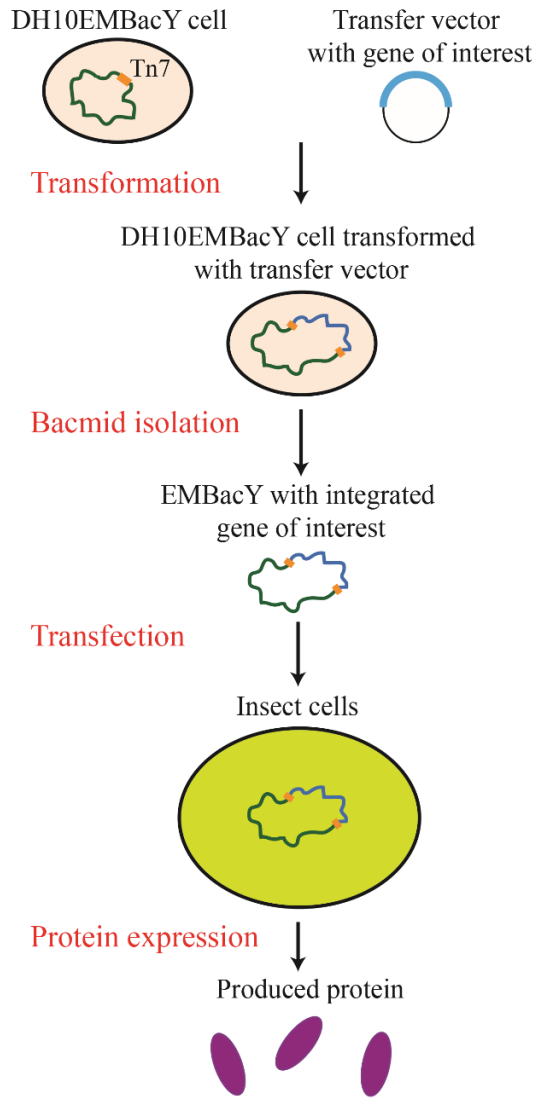

Figure 1.5: Overview of protein production using the baculovirus expression vector EMBacY in the transfection of insect cells. Transfer vector incorporated with gene of interest is transformed into DH10EMBacY cells (*E. coli*). EMBacY genome with integrated gene of interest is next isolated and introduced to into insect cells. Cells transfected with EMBacY co-express yellow fluorescent protein, which is incorporated into the bacmid backbone, together with the desired protein.

1.3 Rationale and aim of the study

Today there are no effective methods to prevent or to treat FNAIT, and details surrounding maternal immunization are still unknown. In order to examine the T cell dependent antibody production further, Immunology research group has acquired mice expressing transgenes for human CD4 and human MHC class II DR3-DQ2, including DRA and DRB3*01:01 (hCD4.DR3-DQ2.MHCII$^{\Delta/\Delta}$ Tg) [41]. Measurement of HPA-1a-specific immune responses in these mice upon immunization with human platelets has been hampered by over-shadowing immune responses against a myriad of other antigenic differences between mouse and human platelets. To recreate the immunization process in FNAIT, it is desired to immunize these transgenic mice with recombinant murine integrin β3, or platelets from mice expressing integrin β3, containing the human HPA-1a epitope designed by the substitution of only four amino acids [26]. Predictably, these four amino acid changes will recreate both B cell and T cell epitopes recognized in FNAIT. Importantly, by eliminating human antigens other than HPA-1a, there is little chance that other T and B cells are activated leading to unwanted immune responses. The latter principle is important for studies of the impact of antigen-specific manipulation of HPA-1a-specific T cells on anti-HPA-1a antibody production.

The aim of the current study was to produce recombinant murine integrin β3 comprising the HPA-1a and HPA-1b epitope and to characterize these biochemically and functionally in comparison to recombinant human integrin β3.

2 Methods

2.1 Preparation of transfer vectors

Recombinant human integrin β3 protein was recently produced in our laboratory and thus, the viruses needed to infect insect cells for protein production were ready to use. However, murine proteins had not been produced, and the expression design and virus production must be carried out prior to the protein production. Therefore, recombinant murine integrin β3 expression constructs were made as described in method 2.1-2.3.

2.1.1 Plasmid amplification and purification

The genes of interest were designed, and ordered using GeneArt from ThermoFisher Scientific (Waltham, Massachusetts). Gene constructs for recombinant murine integrin β3 with substitutions comprising both the HPA-1a epitope and the HPA-1b epitope were ordered. These recombinant integrin β3 (ITGB3) proteins are in this study defined as mITGB3-APLD-6xHis and mITGB3-APPD-6xHis, respectively. The gene constructs arrived in a pMK-T backbone (with kanamycin resistance). In this project, all the LB agar plates and LB broths were ordered from the Bacteriology and Medium production unit in the Microbiology and Infection control section at the University Hospital of North Norway. The different antibiotics concentrations used are described in Appendix I.

To amplify the pFL vector and the pMK-T vector (containing the gene constructs), the vectors were transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen by Life Technologies, Carlsbad, California) according to manufacturer's protocol [42]. Briefly, thawed competent cells were supplied 50-100 ng vector and heat shocked at 42 °C. Next, the cultures were incubated shaking overnight with SOC (Super Optimal broth with Catabolite repression) medium and plated on selective LB agar plates.

The next day, single colonies were cultured in 3 ml LB broth with antibiotics overnight at 37 °C (250 rpm). The desired plasmids were then purified from the culture using QIAprep 2.0 Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol [43]. Briefly, the cells were pelleted, resuspended and lysed under alkaline conditions. After pelleting cell

debris, the DNA was transferred to and absorbed by a unique silica membrane. Next, the membrane was washed, the DNA eluted in dH_2O and quantified by NanoDrop.

2.1.2 Cloning of genes by restriction enzyme digestion

Restriction enzymes and a ligation enzyme (New England Biolabs, Ipswich, Massachusetts) were used to transfer the gene constructs from the pMK-T vector into the pFL vector. Master mix for restriction digestion of pMK-T containing the gene constructs (Table 2.1) and master mix for restriction digestion of pFL (Table 2.2) were made.

Table 2.1: Master mix for restriction enzyme digestion of pMK-T with ITGB3 inserts. 1000 ng vector were supplied master mix to a total reaction of 30 µl. Reagents from New England Biolabs, Ipswich, Massachusetts.

Reagent	Stock concentration	Volume 1x reaction (µl)	Final concentration 1x reaction (30 µl)
NEBuffer 2	10x	3	1x
Bovine Serum Albumin	10x	3	1x
XhoI	20 U/µl	1	20 U/reaction
NheI	10 U/µl	1.5	15 U/reaction
MscI	3 U/µl	1	3 U/reaction

Table 2.2: Master mix for restriction enzyme digestion of pFL. 3000 ng vector were supplied master mix to a total reaction of 30 µl. Reagents from New England Biolabs, Ipswich, Massachusetts.

Reagent	Stock concentration	Volume 1x reaction (µl)	Final concentration 1x reaction (30 µl)
NEBuffer 2	10x	3	1x
Bovine Serum Albumin	10x	3	1x
XhoI	20 U/µl	1	20 U/reaction
NheI	10 U/µl	1.5	15 U/reaction

Each of the reactions; cutting of pMK-T-mITGB3-APLD-6xHis, pMK-T-mITGB3-APPD-6xHis and pFL, were done in triplets. 1000 ng pMK-T-mITGB3 and 3000 ng of pFL were supplied to the proper wells. Master mix without restriction enzymes were included as control showing uncut plasmids. Restriction digestion was performed using a PCR thermal cycler by the following program: 4 °C for 2 minutes, 37 °C for 12 hours, 80 °C for 20 minutes.

Next, to separate the cut mITGB3-inserts from the pMK-T backbone and the pFL backbone from the cut part, the digested products were supplied with loading buffer and run on a 0.9 % 1xTAE agarose gel. A 1 kb DNA Extension Ladder (Invitrogen by Life Technologies) was included. The gel was run at 70 V for 90 minutes and post stained with 3x GelRed (Biotium, Fremont, California) for 10 minutes.

Using a low-intensity UV-light, the correct bands were cut from the gel and purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Chicago, Illinois) according to manufacturer's protocol [44]. Briefly, Capture buffer was supplied to the gel slices and the mix warmed at 60 °C until the agarose completely dissolved. The solution was supplied to a column containing a silica membrane for DNA absorption. After a wash, the sample was eluted and quantified by NanoDrop.

2.1.3 Ligation of inserts into pFL backbone

Ligation reactions were made by mixing ligation master mix (Table 2.3) with ~75 ng pFL backbone and 150 ng insert to a total reaction volume of 10 µl.

Table 2.3: Master mix for ligation of pFL and insert. 75 ng pFL backbone and 150 ng insert were supplied master mix to a total reaction of 10 µl. Reagents from New England Biolabs, Ipswich, Massachusetts.

Reagent	**Stock concentration**	**Volume 1x reaction (µl)**	**Final concentration 1x reaction (10 µl)**
T4 DNA Ligase Buffer	10x	1	1x
T4 DNA ligase	400 U/µl	1	400 U/reaction

The molar ratio between backbone and insert were 1:5. Ligation was performed using a PCR thermal cycler by the following program: 16 °C for 35 minutes and 65 °C for 20 minutes.

The ligated vectors were transformed as before into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen by Life Technologies) according to manufacturer's protocol [42] as described earlier.

60 µl transformation mix was spread on LB/Amp agar plates and incubated overnight at 37 °C. Each colony was then streaked onto a new LB/Amp agar plate and inoculated in LB/Amp broth. The agar plates and the broth were incubated overnight at 37 °C, the broth shaking at 250 rpm.

2.1.4 PCR amplification of inserts

The colonies were verified by amplifying the inserts using PCR. Primers specific for the pFL vector flanking the insert site (Appendix II) were ordered from Eurogentec (Seraing, Belgium).

24 µl master mix for PCR amplification of pFL-inserts (reagents from QIAGEN) was mixed with 1 µl culture from the different pFL-insert candidates (Table 2.4).

Table 2.4: Master mix for PCR amplification of pFL-inserts. 1 µl culture containing pFL-insert candidates were supplied master mix to a total reaction of 25 µl. Reagents from QIAGEN (Hilden, Germany) and primers from Eurogentec (Seraing, Belgium).

Reagent	Stock concentration	Volume 1x reaction (µl)	Final concentration 1x reaction (25 µl)
dNTP	10 mM of each dNTP	0.25	100 µM of each dNTP
SeqFwd	5 µM	3.5	0.7 µM
SeqRev	5 µM	3.5	0.7 µM
HotStarTaq	5 U/µl	0.25	1.25 U/reaction
PCR Buffer	10x	2.5	1x

A no template control (NTC) using water instead of culture and purified pFL without insert were included as controls. PCR amplification was performed by the following cycles: 95 °C for 15 minutes, then 35 cycles of 94 °C for 20 seconds, 62 °C for 1 minute, 72 °C for 30 seconds, and finally 72 °C for 10 minutes.

PCR products were qualitatively analyzed on a 0.9 % 1xTAE agarose gel. The gel was run at 70 V for 90 minutes, post stained using 3x GelRed (Biotium) for 10 minutes and photographed using GBox EF Gel Doc Fluorescent Imaging System (Syngene, Bangalore India).

2.1.5 Sequencing of gene constructs

The cultures verified with an insert by PCR, were purified from the inoculation culture using QIAprep 2.0 Spin Miniprep Kit (QIAGEN) following manufacturer's protocol [43] as described earlier. Next, insert amplification was executed as earlier, but the products were not run on a gel. Instead, the products were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to manufacturer's protocol [44] as described earlier; the only exception being that the mix was not warmed.

Sequencing of the PCR segments were done using Sanger sequencing. Primers covering the insert with a few hundred base pairs (bp) apart (Figure 2.1) were ordered from Eurogentec.

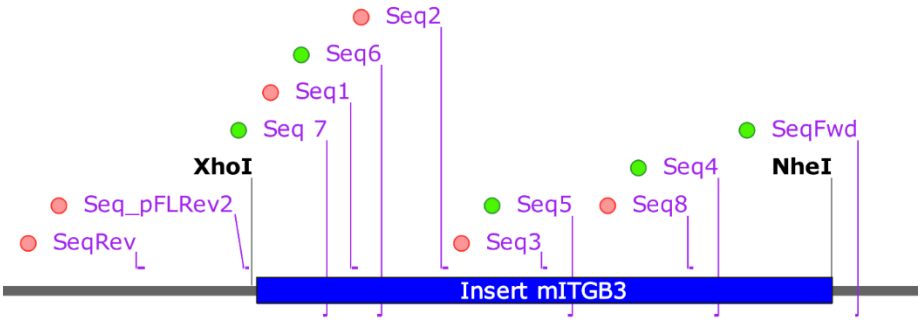

Figure 2.1: The binding location of primers in the recombinant protein sequence. The inserts were sequenced using primers binding inside and close to the insert. Red dots indicate a primer with a reverse direction and green dots indicate a primer with a forward direction (opposite of gene direction).

Master mix for sequencing using BigDye 3.1 (Applied Biosystems, Foster City, California) was mixed with 1 µl purified template (Table 2.5).

Table 2.5: Master mix for sequencing of PCR templates. Template (1 µl purified PCR product) were supplied master mix generating a total reaction of 20 µl. Reagents from Applied Biosystems (Foster City, California) and primers were ordered from Eurogentec (Seraing, Belgium).

Reagent	**Stock concentration**	**Volume 1x reaction (µl)**	**Final concentration 1x reaction (20 µl)**
BigDye Terminator v1.1, v3.1 Sequencing Buffer	5x	4	1x
BigDye Terminator v3.1 Cycle Sequencing Mix	-	1	-
Primer	1 µM	2	0.1 µM

All the primers were supplied template in separate reactions. The sequencing reaction were run using PCR with the following cycles: 96 °C for 1 minute, then 10 cycles of 94 °C for 10 seconds, 58 °C for 20 seconds, 72 °C for 30 seconds, 20 cycles of 94 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 30 seconds, and finally 72 °C for 10 minutes.

The product was purified and analyzed by the DNA sequencing core facility at the University Hospital of North Norway.

2.2 Integration of transfer vector into the EMBacY bacmid

2.2.1 Production of competent DH10EMBacY cells

Next, DH10EMBacY cells (gift from EMBL Grenoble), was made competent prior to transformation. Thawed DH10EMBacY cells were spread on LB/Kan/Tet/IPTG/X-gal agar plates and incubated at 37 °C overnight.

10 ml LB/Kan/Tet broth was inoculated with a single blue colony and incubated at 37 °C overnight shaking at 250 rpm to generate a starter culture. A medium control was included. The next day, 2 ml from this culture was transferred to 25 ml medium in a T75 flask. The flasks were incubated at 37 °C shaking at 250 rpm until OD_{600} reached 0.5-0.6. Concurrently, 80 mM $MgCl_2$/20 mM $CaCl_2$ and 0.1 M $CaCl_2$ were prepared, sterile filtered using a 0.2 µm PES filter (Polyethersulfone Membrane; VWR, Radnor, Pennsylvania) and cooled on ice. From this point, efforts were made to keep the cells ice-cold, using pre-chilled pipette tips and centrifugation steps performed in the cold room. The culture was divided between two 50 ml tubes and cooled on ice for 5 minutes. Next, the cells were pelleted by centrifugation at 1000 x g for 5 minutes at 4 °C and the supernatant was discarded. The cells were carefully resuspended in 10 ml ice cold $MgCl_2$/$CaCl_2$ (80 mM/20 mM), pelleted and the supernatant discarded. Next, the cells were carefully resuspended in 5 ml ice cold $CaCl_2$ (0.1 M), pelleted and supernatant discarded. Finally, the cells were carefully resuspended in 200 µl cold $CaCl_2$ (0.1 M) and aliquoted into 5 tubes and put on ice.

2.2.2 Transformation of DH10EMBacY cells

100 ng transfer vector with insert was added to the competent cells, and the tubes were flicked gently to mix the culture (no DNA as a negative control). The tubes were incubated on ice for 25 minutes, heat shocked for 45 seconds in a 42 °C water bath, and cooled on ice for 5 minutes. Then 500 µl SOC medium was added, and cells were incubated at 37 °C overnight shaking at 250 rpm.

The next day, the suspensions were spread on warm LB/Kan/Tet/Gent/IPTG/X-gal agar plates. Gentamycin is very toxic to the cells, and thus 200 µl culture was spread to allow the cells to start growing on the plates. The EMBacY control was spread on a LB/Kan/Tet/IPTG/X-gal agar plate as a positive control and on a LB/ Kan/Tet/Gent/IPTG/X-gal agar plate as a negative

control. The agar plates were incubated overnight at 37 °C. From the incubated plate, white colonies (based on blue and white screening) were picked and transferred to new LB agar plates using streak-plate technic. The plates were incubated overnight at 37 °C. When selecting colonies based on blue and white screening, X-gal and IPTG (Appendix I) were supplied on top of warm LB agar plates 20 minutes before use.

2.2.3 Isolation of bacmid from DH10EMBacY cells

In order to make baculovirus, insect cells are transfected with purified bacmid. The protocol used for isolation of bacmid is adapted from a published protocol from Fitzgerald et al. [34]. Single white colonies from both the EMBacY-mITGB3-APLD-6xHis and the EMBacY-mITGB3-APPD-6xHis agar plates were transferred to 5 ml LB/Kan/Tet/Gent broth. EMBacY alone (blue colonies) were used as a positive control, inoculated in 5 ml LB/Kan/Tet broth. These tubes were incubated shaking overnight at 37 °C and 250 rpm.

The next day, the cultures were pelleted at 4000 x g for 5 minutes. The supernatant was discarded and the pellet resuspended in the remaining liquid and transferred to 1.5 ml tubes. The tubes were centrifuged at 4300 x g for 5 minutes, and the supernatant discarded. The pellet was then resuspended in 280 µl P1 buffer (without LyseBlue) from the QIAprep 2.0 Spin Miniprep Kit (QIAGEN). Next, 280 µl P2 buffer was supplied and the tubes turned carefully 6-10 times to mix the solution. Within 5 minutes, 280 µl N3 buffer was added and the tubes turned to homogenize the solution. The tubes were centrifuged at 11300 x g for 10 minutes. Because cell debris from the lysis reaction can obstruct the transfection reaction, a second centrifugation step was included. Next, the supernatant was transferred to a new tube and supplied 650 µl isopropanol. The tubes were then transferred to -20 °C at least two hours to increase the yield from the isopropanol precipitation of DNA.

The tubes were then centrifuged at 13000 x g for 10 minutes at 4 °C. A small pellet was then visible, and the supernatant was removed by careful vacuum suction. To wash the cells, 200 µl of 70 % ethanol was supplied drop-by-drop without disturbing the pellet. The tubes were centrifuged as before (only 5 minutes), and the supernatant removed by suction. 50 µl of 70 % ethanol was supplied and the tubes centrifuged again. Next, the tubes were taken to the sterile hood and the supernatant removed by pipetting. The pellet was air-dried for approximately 10 minutes and then carefully resuspended in 30 µl sterile dH_2O. The solution was incubated 30-

45 minutes to allow the DNA to be completely resuspended. The bacmid concentration was quantified by NanoDrop. Purified bacmid was stored at -20 °C or used immediately.

2.3 Virus production

2.3.1 Insect cell culturing

The insect cell lines most commonly used in this project were Sf21, Sf9 and High Five. Sf21 and Sf9 are cultured in Sf-900 II SFM, and High Five in Express Five SFM (Gibco by Life Technologies, Carlsbad, California) supplied with L-Glutamine (Sigma-Aldrich, St. Louis, Missouri). The cells were cultured both as adherent and spin cultures. The non-CO_2, non-humidified incubator held 27 °C, and the spin was set to 120 rpm. The medium used for the insect cells is not supplied with antibiotics, so aseptic techniques were used when working with insect cell cultures.

2.3.2 Adherent and suspension cultures

When thawing insect cells, it is good to start with an adherent culture to allow the cells to settle. Stored, frozen insect cells were thawed using a 37 °C water bath as quickly as possible until only a small piece of ice was remaining. The content of the vial was transferred to a T-flask supplied with medium. The size of the T-flask, T25, T75 or T175, were chosen based on cell number. The total volume used in these T-flasks were respectively 4-7 ml, 15-20 ml and 25-30 ml. The cells were subcultured when the confluency reached 90 % by loosening the cells from the flask surface and transferring a subset to a new flask.

When the adherent culture was in better condition and the viability was higher (>95 %), a suspension culture was started. Sf21 and Sf9 cells grow quite slower than High Five cells. In our hands, the cells grow best when the culture is approximately 1.1×10^6 cells/ml for Sf21 and Sf9 and 0.8×10^6 cells/ml for High Five when the cells are passaged. When the cells were transferred to a suspension culture, the cell number, cell viability and general cell condition were calculated and checked every day. If the cell number had reached 2.0×10^6 cells/ml or more, the cells were subcultured. Concurrently, the adherent culture was still grown and used as a live cell stock.

2.3.3 Transfection of insect cells

Cells in good condition and high cell viability were seeded into 6-well tissue culture plates (Corning, Corning, New York). 15-60 minutes before transfection 1.3×10^6 cells/well were seeded in 3 ml medium. 2 wells were seeded per bacmid in addition to medium and cell controls. 20 µl bacmid DNA was supplied to 200 µl medium and mixed gently. Next, 10 µl Plus Reagent (Invitrogen by Life Technologies) was supplied to the DNA mix and the tube incubated for 15-30 minutes. 16 µl Cellfectin II Reagent (Invitrogen by Life Technologies) was added to 200 µl medium and incubated for 15 minutes. A total transfections mix was made by adding the Cellfectin mix to the DNA mix. The solution was incubated 10-15 minutes before dropwise being supplied to the seeded cells. Each transfection mix was divided between two wells. The plate was sealed with parafilm and incubated in 27 °C in a dark box. After approximately 60 hours, the 6 ml supernatant (initial virus, V_0) from each bacmid were centrifuged at 3000 x g for 5 minutes to remove excess cells. The supernatant was moved to a new tube and stored at 4 °C. 3 ml fresh medium was supplied to the cells in the 6 well plates, to further monitor the infection and protein production.

2.3.4 Amplification of virus

2 ml V_0 was supplied to fresh insect cells in a total volume of 22 ml. The cells had a starting concentration of 1.1×10^6 cells/ml, and were incubated at 27 °C shaking at 120 rpm. The cells were counted, viability calculated and the cells split to below 1.1×10^6 cells/ml every day until cell proliferation arrested. After 48 additional hours, the supernatant was harvested by spinning down the cells at 400 x g for 5 minutes and collecting the supernatant. The cells were resuspended in fresh medium to monitor protein production. The supernatant was centrifuged at 3000 x g for 5 minutes to remove remaining cells and culture debris. This supernatant is first-generation virus, V_1. From this virus stock, a second-generation virus, V_2, could be produced the same way as V_1 to increase the titer of the viral stock. Protein production in the cells can be monitored either by flow cytometer or by doing a small-scale protein purification of the supernatant after 2-3 days.

2.3.5 Measurement of YFP production by flow cytometry

Because YFP is expressed together with the desired protein from the EMBacY genome, flow cytometer is a very easy method to monitoring protein production. Flow cytometry is a method that can measure and analyze single particles, often cells, by flowing them one by one in a fluid stream through a laser light beam. Particles that are 0.2-150 µm in size is suitable for analysis by flow cytometry [45]. All the data in this study was collected using the FACSDiva software (BD Biosciences, San Jose, California) and further analyzed using FlowJo 7.6 (FlowJo LLC, Ashland, Oregon).

In this experiment, a FACSCanto (BD Biosciences) was used for YFP detection. Because the cells express their own fluorophore intracellularly, the samples do not need any preparations. Photomultiplier Tube (PMT) voltages were routinely set using BD CS&T Beads. Using forward and side scatter parameters, the cells were gated. Next, the gated cells were analyzed for their YFP (emission maximum at 527 nm) intensity using the FL1 detector (530/30 nm filter) on the blue (488 nm) laser.

2.4 Protein production

2.4.1 Small-scale production of recombinant human integrin ß3

The recombinant human integrin β3 proteins were made from a virus stock using High Five cells, known to produce more protein than Sf21 and Sf9 [46]. For small-scale protein production, 40 ml High Five cells with a concentration of 1×10^6 cells/ml were cultured in a 1-L spinner flask. 0.5 % virus stock (Often V_1 or V_2) was added to the suspensions (in this case 0.2 ml) based on an earlier titration of virus concentration. Cells not supplied with virus (cell control) and cells transfected with empty EMBacY bacmid were included as controls. After approximately 24 hours, the virus-inoculated High Five cultures were supplied with one additional volume of medium. The cell control was split below 1×10^6 cells/ml. 1 ml from each flask, including the cell control, were collected and YFP expression was measured by flow cytometry. After approximately 48 hours, YFP from each suspension were measured and cell control split as before. Next, the suspensions were harvested after approximately 70-80 hours evaluated based on the YFP intensity. It is desired that it is as high as possible, indicating a high protein production, but it should be harvested before 30-50 % of the cells dies. When ready, the suspensions were centrifuged using 50 ml tubes at 400 x g for 5 minutes (soft spin). Next, the

supernatant was transferred to new tubes and centrifuged at 3000 x g for 10 minutes. Using 0.2 µm PES filter, the supernatant was sterilized and stored at 4 °C for up to a week.

2.4.2 ELISA

After the collection of human integrin β3 supernatants, the protein production was verified using an enzyme-linked immunosorbent assay (ELISA). An ELISA is a method that utilizes antigen-antibody interaction to identify a substance, such as a protein, an antibody or a hormone. Multiple ELISA formats are possible. The most common enzymes used for detection are horseradish peroxidase (HRP) and alkaline phosphatase (AP), and a large number of substrates are available [47].

In this project, two different ELISAs were conducted for recombinant human integrin β3. First a sandwich ELISA, where the antigen was captured by an immobilized antibody, followed by a direct ELISA, where the antigen was bound directly to the ELISA plate without the use of a capture antibody. All antibodies used in this study are presented in Appendix III.

In the sandwich ELISA, a monoclonal antibody (mAb) AP3, known to bind human integrin β3 independent of HPA-1 allotype, was used as the capture antibody. The antibody was diluted to 10 µg/ml in a carbonate-bicarbonate buffer, and 100 µl supplied to a Nunc MaxiSorp flat-bottom 96-well plate (Invitrogen by ThermoFisher).

In the direct ELISA, 100 µl supernatant was supplied to each well without precoating with a capture antibody. Phosphate buffered saline (PBS), medium and the supernatants from cells only and cells transfected with EMBacY were included as negative controls (undiluted). Both ELISA plates were incubated over night at 4 °C.

The following day, the wells of both plates were washed 4 times with PBS 0.05 % Tween 20 (Sigma-Aldrich) 250 µl/well, and 2 % PBSA (2 % bovine serum albumin in PBS) was supplied 100 µl/well to block for unspecific binding. The plates were incubated 2 hours at room temperature. For the sandwich ELISA, the liquid was discarded, the samples and controls prepared in 0.2% PBSA and 100 µl mix supplied to each well. Recombinant human integrin β3 supernatants with both HPA-1 allotypes (defined as hITGB3-L33-6xHis and hITGB3-P33-6xHis), were diluted 1:50, 1:100 and 1:200. All the samples and controls were plated in parallel, and the plate incubated 2 hours at room temperature.

From here, both ELISAs were handled the same way. Each plate was washed and 100 µl mouse anti-His-tag mAb (Aviva Systems Biology, San Diego, California) diluted 1:5000 was supplied to all the wells. After 1 hour incubation, the plate was washed, and 100 µl goat anti-mouse IgG2b polyclonal antibody (pAb) conjugated to HRP (Invitrogen by Life Technologies) diluted 1:20 000 was supplied. The plate was incubated 45 minutes at room temperature and washed as before, but this time followed by one wash with PBS. TMB substrate (3,3′,5,5′-Tetramethylbenzidine; Sigma-Aldrich) was next supplied 100 µl/well and the plate incubated until the color turned blue (approximately 10 minutes). The reaction was stopped using 1 M H_2SO_4 100 µl/well and the absorbance read at 450 nm using a Multiskan Ascent 354 Microplate Reader (Thermo Labsystems, Helsinki, Finland).

2.5 Protein purification by capture on beads

Beads coated with anti-His-tag antibody were used to capture His-tagged protein from the protein production supernatants. Recombinant human integrin β3 proteins were purified from the supernatants made in the small-scale protein production using virus stocks, while recombinant murine integrin β3 proteins were purified from the transfection reaction supernatants (6-well plates).

2.5.1 Purification of His-tagged proteins using beads bound by anti-His-tag antibody

Dynabeads M-280 Streptavidin (Invitrogen by Life Technologies) are magnetic beads covalently coupled to streptavidin. 250 µl (for the human proteins) and 140 µl (for the murine proteins) 10 mg/ml beads were transferred to a 1.5 ml tube.

The stock solution was removed after placing the tube on the magnet (for at least 1 minute). Using the magnet, the beads were washed three times in 1 ml PBS by vortex. Next, the beads were resuspended in PBS; 180 µl (for the human proteins) and 140 µl (for the murine proteins). Next, the beads were supplied biotinylated rabbit anti-His pAb (GenScript, China) 4 µg/mg beads, followed by an incubation at room temperature for 3 hours by head-over-head mixing.

During the incubation, the protein supernatants were concentrated to 0.5-1 ml using Amicon Ultra centrifugal filter with a molecule weight cut off (MWCO) of 50 000 Da (Millipore, Billerica, Massachusetts). The beads were next washed three times as before, resuspended in

PBS and divided between 5 new tubes. Concentrated supernatants were supplied to the correct tube and incubated spinning overnight at 4 °C. The following day, the beads were washed three times and resuspended in PBS. The samples were stored at 4 °C.

2.6 Protein analysis

2.6.1 SDS-PAGE

Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) is a method widely used to analyze protein mixtures and is especially useful to study protein production. Proteins are separated according to size, and by the use of a ladder containing proteins with known size, the relative molecular mass of the proteins could be determined. The samples could be loaded to the gel in reducing or non-reducing conditions. In reducing conditions, the sample is supplied with a reducing agent (which reduces disulfide bridges in the protein) and SDS (which binds strongly to the protein causing denaturation and gives the protein a net negative charge). In non-reducing conditions, only SDS is supplied and the protein preserves some of its native structure [48].

To verify protein purification, the beads were supplied to a SDS-PAGE gel under reducing conditions. All reagents for SDS-PAGE are supplied by Invitrogen by Life Technologies. Using the magnet, bead samples were adapted to 10 µl. Each 10 µl sample was added 3.8 µl NuPAGE 4x LDS Sample Buffer and 1.5 µl NuPAGE 10x Sample Reducing Agent. Next, the samples were incubated at 70 °C for 10 minutes on a heating block. 1x SDS Running Buffer was prepared from NuPAGE 20x MES SDS Running Buffer. 500 µl NuPAGE Antioxidant was supplied in the inner chamber of the running tank. Subsequently, 7 µl of each ladder, Novex Sharp Pre-stained and Unstained Protein Standard, and 12 µl of each sample were loaded to the gel, NuPAGE Novex 4-12% Bis-Tris Gel (1.5 mm, 15-well gel). To avoid too many residual beads on the gel, the tubes were placed on the magnet when loading the sample. The gel was run at 35 minutes at 200 V.

The gel was stained using SimplyBlue SafeStain. First, the gel was supplied 100 ml deionized water and heated in the microwave until the solution almost boiled. The gel was shaked at an orbital shaker for 1 minute, the water discarded and the washing process repeated 2 more times. Next, the gel was supplied 20 ml SimplyBlue SafeStain, heated in the microwave until the solution almost boiled and shaked for 20 minutes. The solution was replaced with 100 ml

deionized water and the gel shaked for 10 minutes before 20 ml 20 % NaCl was supplied. The gel was incubated shaking overnight.

2.6.2 Western blot

Western blot is a method that detects proteins in a sample using protein-specific antibodies. After the protein separation on the SDS-PAGE, the proteins are blotted onto a nitrocellulose paper by electroblotting. Here, the membrane and gel is compressed in a cassette between two parallel electrodes. The current causes the proteins to transfer from the gel and onto the membrane. The most common detection method is by the use of an enzyme-linked antibody specific for the desired protein followed by incubation in the substrate solution [48].

A SDS-PAGE gel with reduced conditions was run as described in section 2.6.1, with the exception that instead of the unstained marker, a MagicMark XP Western Protein Standard (Invitrogen by Life Technologies) was used (only 1 µl was loaded of each ladder).

After running the gel, the proteins were blotted onto a membrane, Invitrolon PVDF Filter Paper Sandwich 0.45 um Pore Size (Invitrogen by Life Technologies). Before electroblotting, the membrane was prepared by first wetting it in 100 % methanol, then deionized water and last 5 minutes in 1x Transfer Buffer made by NuPAGE 20x Transfer Buffer (Invitrogen by Life Technologies). The wells and sides of the gel were removed, and the membrane put on top of the gel. Filter paper were supplied on both sides making a sandwich. The sandwich was assembled in the blot apparatus with 1x Transfer Buffer in the inner chamber and water in the outer chamber. The gel was electroblotted for 1 hour at 25 V.

Using a shaker, the membrane was washed three times for 10-15 minutes in PBS 0.05% Tween 20. Next, the membrane was blocked for 1 hour shaking using a blocking reagent. The blocking reagent was made by adding 0.2 % goat IgG (Thermo Scientific, Waltham, Massachusetts) to SuperBlock (PBS) Blocking Buffer (Thermo Scientific) supplied with 0.05 % Tween 20. After blocking, the membrane was washed three times. Mouse anti-His-tag mAb (Aviva) was diluted 1:1000 in Blocking Buffer with 0.05 % Tween 20 to 1 µg/ml. The membrane was incubated in the primary antibody at 4 °C shaking overnight.

The following day, the membrane was washed three times. Goat anti-mouse IgG pAb conjugated with HRP (Sigma-Aldrich) was diluted 1:10 000 in Blocking Buffer with 0.05 % Tween 20. The membrane was incubated in the secondary antibody 1 hour shaking followed

by washing of the membrane three times. Substrate solution was made using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and 2 ml was supplied to the membrane. The membrane was incubated 5 minutes in the dark, before photographing using ImageQuant LAS 4000 (GE Healthcare Life Sciences) at the Bioimaging Core facility, UiT.

2.6.3 Detection of proteins by flow cytometry

To obtain information about the recombinant protein conformation, the beads were analyzed by flow cytometry using various specific antibodies. Four different monoclonal antibodies were used in this experiment; 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 HPA-1a-specific at low concentrations [49]. 20 µl beads were incubated with 6 ng/µl antibody in a total reaction of 50 µl. Negative controls were made by incubating beads with PBS. The samples were incubated in dark for 15 minutes followed by a wash with 4 ml 0.2 % PBSA.

The gated cells were analyzed for their Alexa Fluor 488 and FITC (emission maximum at 519 nm) intensity using the FL1 detector (530/30 nm filter) on the blue (488 nm) laser.

2.6.4 T cell TNF secretion and proliferation assay

After the presence of integrin β3 on the beads were confirmed, their ability to stimulate HPA-1a-specific T cells were examined. T cell tumor necrosis factor (TNF) expression and proliferation due to stimulation were studied by incubating T cells with antigen pulsed APC. The cells were stained with CFSE (Carboxyfluorescein succinimidyl ester), which is an intracellular protein binding dye that is retained in the daughter cells after proliferation. The intensity is reduced by half in daughter cells compared to the mother cell, and decreased intensity indicates cell proliferation. T cells that are stimulated through their T cell receptor may also secrete TNF. This cytokine is initially produced as a membrane bound protein, which is subsequently cleaved by a TNFα converting enzyme (TACE) making TNF soluble [50]. Using Tapi-0 which is a metalloprotease inhibitor, TNF is retained on the cell membrane. Including an anti-TNF antibody makes it possible to measure TNF expression on T cells [51].

In this experiment, HLA-DRB3*01:01 positive monocytes from an HPA-1bb donor were co-cultured with beads, platelets or peptides, prior to incubation with HPA-1a-specific T cell clone D8T108. Peptides used in this study are presented in Appendix IV. Both assays were set up

almost identically. PBMC (Peripheral blood mononuclear cell) HD#31 (HLA-DRB3*01:01-positive donor) were thawed in T cell medium (TCM) consisting 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 % Penicillin-Streptomycin (Sigma-Aldrich), and incubated at 37 °C overnight with 7.5 % CO_2.

The following day, the cells were washed and resuspended in 1.2 ml FBS, 800 µl red blood cells (isolated from an unrelated donor by two rounds of Lymphoprep gradient separation), and 100 µl RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, British Columbia). The mix was incubated 20 minutes at room temperature. The cell suspension was mixed with 2 ml PBS, layered on top of 3 ml Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 800 x g for 15 minutes without brakes. Monocytes were collected at the interface and washed twice with 0.2 % PBSA. The cells were resuspended in TCM.

1.2×10^5 monocytes were co-cultured with antigen; 20 µg beads (hCtr-EMBacY (empty EMBacY), hITGB3-L33-6xHis, hITGB3-P33-6xHis, mCtr-EMBacY, mITGB3-APLD-6xHis or mITGB3-APPD-6xHis), 12×10^6 HPA-1a positive or HPA-1bb homozygous platelets, or 2 µM Lol P1, L33 or P33 peptide. Lol P1 is used as a control peptide due to its binding to HLA-DRB3*01:01 [52]. The co-cultures were set up as 200 µl in 96-well flat bottom plates and were incubated overnight at 37 °C with 7.5 % CO_2.

The following day, D8T108 T cells [53] were dyed with CellTrace CFSE (Invitrogen, by Life Technologies). 3×10^6 cells were washed two times with PBS and resuspended 1:1 with 0.2 % PBSA to 0.1 % PBSA. The cells were supplied ~80 ng CFSE in PBS, and incubated at 37 °C for 12 minutes. To stop the dyeing, the cells were cooled on ice and washed three times in TCM. The cells were resuspended in TCM and 1×10^5 cells/well were supplied to two 96-well round-bottom plates, one TNF assay and one proliferation assay. The co-cultures were washed in TCM and divided between the two plates (approximately 6×10^4 cells/well).

The T cells in the TNF assay were stimulated in the presence of 1 µM Tapi-0 (Enzo Life Sciences, Farmingdale, New York) and 1 µl PE (R-phycoerythrin) Mouse Anti-human TNF (BD Bioscience) for 6 hours at 37 °C with 7.5 % CO_2. After the incubation, the cells were washed in 0.2 % PBSA and analyzed by flow cytometry. PMT voltages were routinely set using BD CS&T Beads, and compensation was calculated using single stained samples. For the TNF secretion assay, the PMT values were slightly adjusted, and compensation was optimized

manually, due to very bright CFSE staining of the T cells. The gated cells were analyzed for their CFSE (emission maximum at 517 nm) intensity using the FL1 detector (530/30 nm filter) and PE (emission maximum at 578 nm) intensity using the FL2 detector (585/42 nm filter) on the blue (488 nm) laser.

After 24 hours, the T cells in the proliferation assay were supplied 30 U/ml IL-2 (PeproTech, London, United Kingdom). The proliferation assay was incubated 4 days in total at 37 °C with 7.5 % CO_2. After the incubation, the cells were washed in 0.2 % PBSA and analyzed by flow cytometry.

The last part of the study (T cell TNF secretion and proliferation assay) uses human sample material. These studies are approved by the Regional Committee for Medical Research Ethics (REC North), University of Tromsø - The Arctic University of Norway (P REK Nord number 57/2008). Blood samples were drawn from donors after written informed consent was obtained in accordance with the Declaration of Helsinki.

3 Results

3.1 Gene constructs for recombinant murine ITGB3 were ordered and cloned into a transfer vector

To be able to produce recombinant murine integrin β3 (modified by four specific amino acid substitutions to resemble the human HPA-1a epitope), a gene construct was designed and assembled to an expression construct. As a control, a construct containing the human HPA-1b epitope was included.

3.1.1 Designed recombinant murine ITGB3 gene constructs

Murine integrin β3 does not comprise HPA-1a/b. Consequently, the murine molecular variant had to undergo modifications by substitution of 4 amino acids to resemble the HPA-1a or HPA-1b epitope (Figure 1.3). The recombinant murine integrin β3 proteins are in this study named mITGB3-APLD-6xHis (comprising the HPA-1a epitope) and mITGB3-APPD-6xHis (comprising the HPA-1b epitope).

Using the NCBI's Protein database, the protein sequence of the native signal peptide and the extracellular domain in the murine integrin β3 precursor was obtained (NCBI Reference Sequence: NP_058060.2). In front of the native sequence, a kozak sequence was inserted to improve protein expression [54]. Following the extracellular region of the protein, six histidine residues were inserted to generate a 6xHis-tag [55] , prior to a stop codon.

Due to a plan to integrate the recombinant protein sequence into a transfer vector in the following step, XhoI and NheI restriction sites were included in front and in the end of the sequence, respectively. For improved expression in insect cells the sequences were codon optimized for *Spodoptera frugiperda*. The designed gene constructs had a total gene length of 2193 bp (731 amino acids). An overview of the gene construct is shown in Figure 3.1. The synthetic constructs were ordered using GeneArt from ThermoFisher Scientific, in pMK-T vector (with kanamycin resistance).

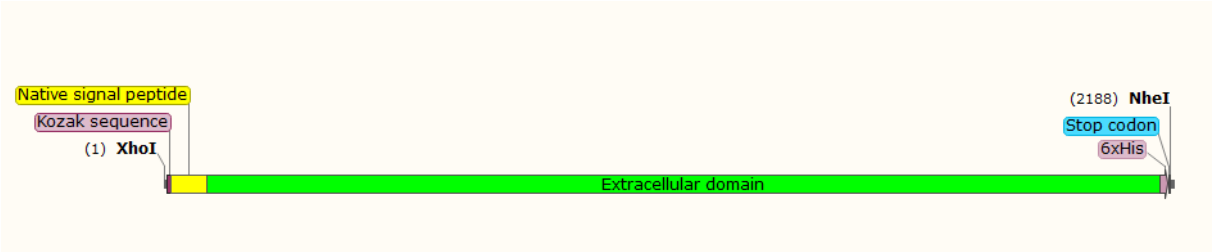

Figure 3.1: Overview of recombinant murine integrin β3 gene construct. The gene construct contains the native signal peptide (amino acids 1-25) and the extracellular domain (amino acids 26-717) of murine integrin β3 succeeded by a 6xHis-tag and a stop codon. A kozak sequence is present in front of the gene to increase expression. Restriction sites for XhoI and NheI are enveloping the construct for later cloning purposes. The total gene length was 2193 bp.

3.1.2 Gene constructs were efficiently cut by restriction enzymes

The first step in the cloning process was to cut the plasmids, both pFL and pMK-T (containing the gene construct), using restriction enzymes. Upon cutting the pMK-T with the two restriction enzymes, XhoI and NheI, it generates two bands of approximately the same length. In qualitative analysis of the restriction product on agarose gel, the two bands will migrate the same distance and be inseparable. Therefore, an additional restriction enzyme was included, MscI, which cuts the backbone of pMK-T into three bands (Figure 3.2).

After restriction enzyme digestion of the plasmids, the products were run on an agarose gel to separate the fragments. Subsequently, the desired DNA fragments were isolated from the gel. After isolation, the resulting concentrations were unsatisfactory to use in following DNA ligation. Since it is most essential to remove the small DNA sequence that is cut from the backbone of pFL due to the likely re-ligation, only the restriction enzyme digestion and purification of pFL was repeated. Inserts were used unpurified - directly from the restriction digestion reaction.

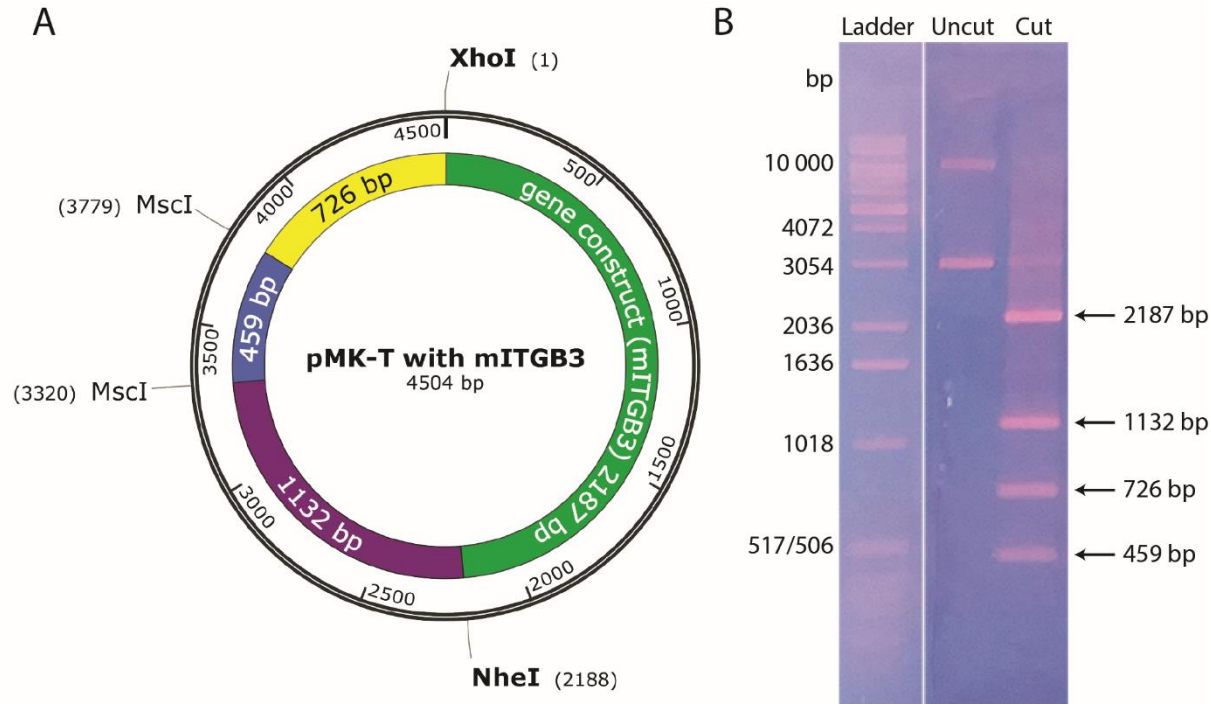

Figure 3.2: Restriction enzyme digestion of pMK-T vector containing the gene construct. The gene construct for recombinant murine integrin β3 was cut from the pMK-T vector using restriction enzymes XhoI, NheI and MscI. (A) Overview of vector made by SnapGene, showing restriction sites and calculated fragment sizes after digestion. (B) The digested products were run on a 0.9 % 1xTAE agarose gel together with undigested vector and a 1 kb DNA Extension Ladder. The gene construct (2187 bp), together with the backbone fragments, were seen on the gel at approximately the expected fragment lengths.

3.1.3 Verification of insert in pFL in transformed *E.coli*

E.coli transformed with ligation product, was analyzed with PCR to verify that the acquired plasmid contained the insert. The products were qualitatively analyzed on a 0.9 % 1xTAE agarose gel. Empty pFL vector generates a 548 bp band, while pFL vector with insert generates a 2722 bp band (insert 2193 bp and backbone 529 bp). As controls, NTC and empty pFL vector were included. *E.coli* colonies with successfully inserted gene of interest were found for both constructs (Figure 3.3); 4 out of 5 mITGB3-APLD-6xHis candidates and all 3 mITGB3-APPD-6xHis candidates.

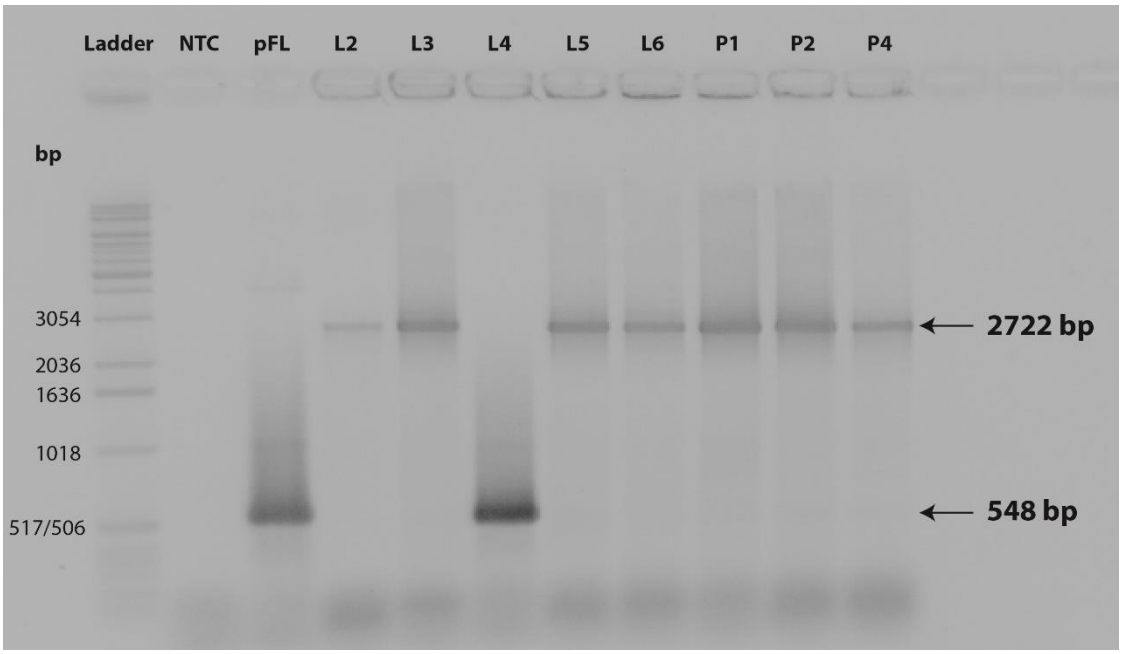

Figure 3.3: PCR amplification of gene insertion site in the pFL vector. pFL-mITGB3-APLD-6xHis (L) and pFL-mITGB3-APPD-6xHis (P) candidates were analyzed by PCR amplification of the gene insertion site. The product was run on a 0.9 % 1xTAE agarose gel. Empty pFL was included as a control. Vectors L2, L3, L5, L6, P1, P2 and P4 showed fragments lengths of approximately the expected length, 2722 bp, while vector L4 had a band length similar to the empty pFL, which is expected to be 548 bp. A 1 kb DNA Extension Ladder was used.

One of each HPA-1 allotype were selected; mITGB3-APLD-6xHis candidate L3 and mITGB3-APPD-6xHis candidate P1. These two candidates were sequenced and the sequences are shown in Appendix V. Both contained the correct HPA-1 version.

3.2 Protein production of recombinant murine and human ITGB3

3.2.1 Transfection of insect cells

After verifying the successful cloning of the gene construct into the transfer vector (pFL) the vectors were integrated into the bacmid EMBacY. The bacmid was isolated using isopropanol and used to transfect insect cells to make an initial virus stock (which further could be used to make more virus and protein).

When transfecting, empty EMBacY was always included as a positive control, named hCtr-EMBacY in the human protein production and mCtr-EMBacY in the murine protein production. From this point on the isolated bacmids are named the same as the gene constructs even though they are incorporated into EMBacY.

Many transfection attempts were conducted using different cell lines (Sf21, Sf9 and High Five), different DNA concentrations, different incubation times and temperatures and different transfection reagents. The transfection efficiency was measured using flow cytometry on transfected cells, 24 hours after V_0 supernatant collection. None of the attempts to produce initial virus generated more than 10 % YFP positive cells, and the following virus amplification attempts were also negative. However, when using a frozen bacmid (produced prior to this project) in one of the transfection attempts, it resulted in more than 50 % YFP positive cells (data not shown). The following virus amplification was also successful (50 % or more YFP positive cells). Results from transfection of Sf9 is representatively shown in Figure 3.4.

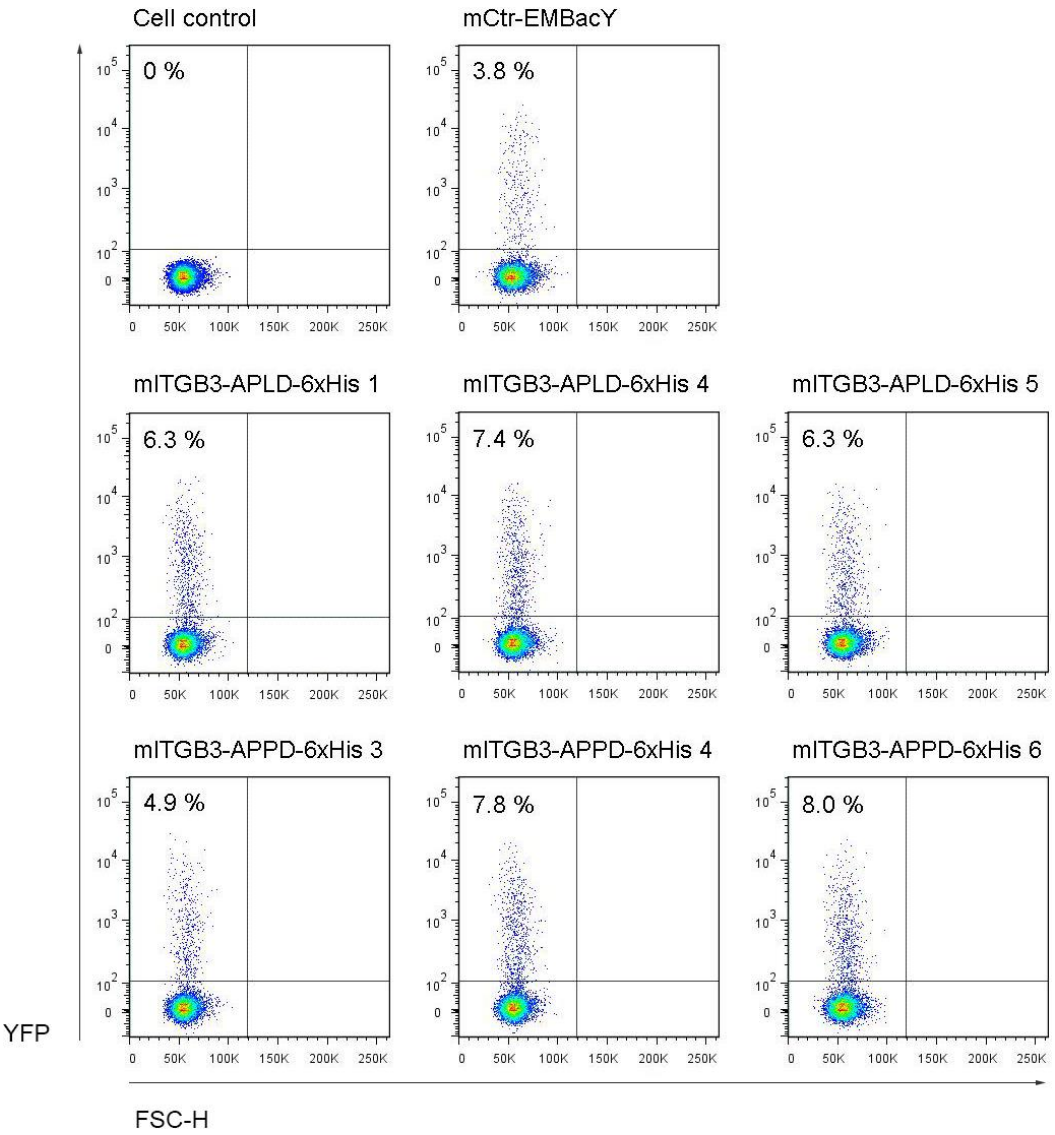

Figure 3.4: YFP intensity in Sf9 cells transfected with bacmid expressing recombinant murine integrin β3. Isolated mCtr-EMBacY, mITGB3-APLD-6xHis isolate 1, 4 and 5 and mITGB3-APPD-6xHis isolate 3, 4 and 6 bacmid were used to transfect Sf9 cells in 6-wells plates using Plus reagent and Cellfectin II. 24 hours after collection of V_0, the YFP intensity in the cells was measured by flow cytometry. The cell control showed no increase in YFP intensity, while cells infected with bacmid showed 3.8-8 % YFP positive cells. Mean fluorescence intensity (MFI) is presented.

Despite the unsuccessful virus amplifications, supernatants from the transfection were collected 72 hours after harvesting initial virus for further analyses of the produced recombinant proteins.

3.2.2 Recombinant human ITGB3 were produced using a virus stock

The recombinant human integrin β3 proteins were produced by infecting High Five with a high titer virus stock recently produced in our laboratory, with same methods. The cell cultures were monitored by flow cytometry at 26, 50 and 75 hours, with analysis of both cell size and YFP intensity. Suspension cultures transfected with empty EMBacY (hCtr-EMBacY), hITGB3-L33-6xHis or hITGB3-P33-6xHis viruses clearly showed swelling in the populations already at 26 hours, compared to control (Figure 3.5). By 75 hours almost the entire population showed increased cell size, but also slightly increased presence of debris. The supernatants were harvested at 75 hours.

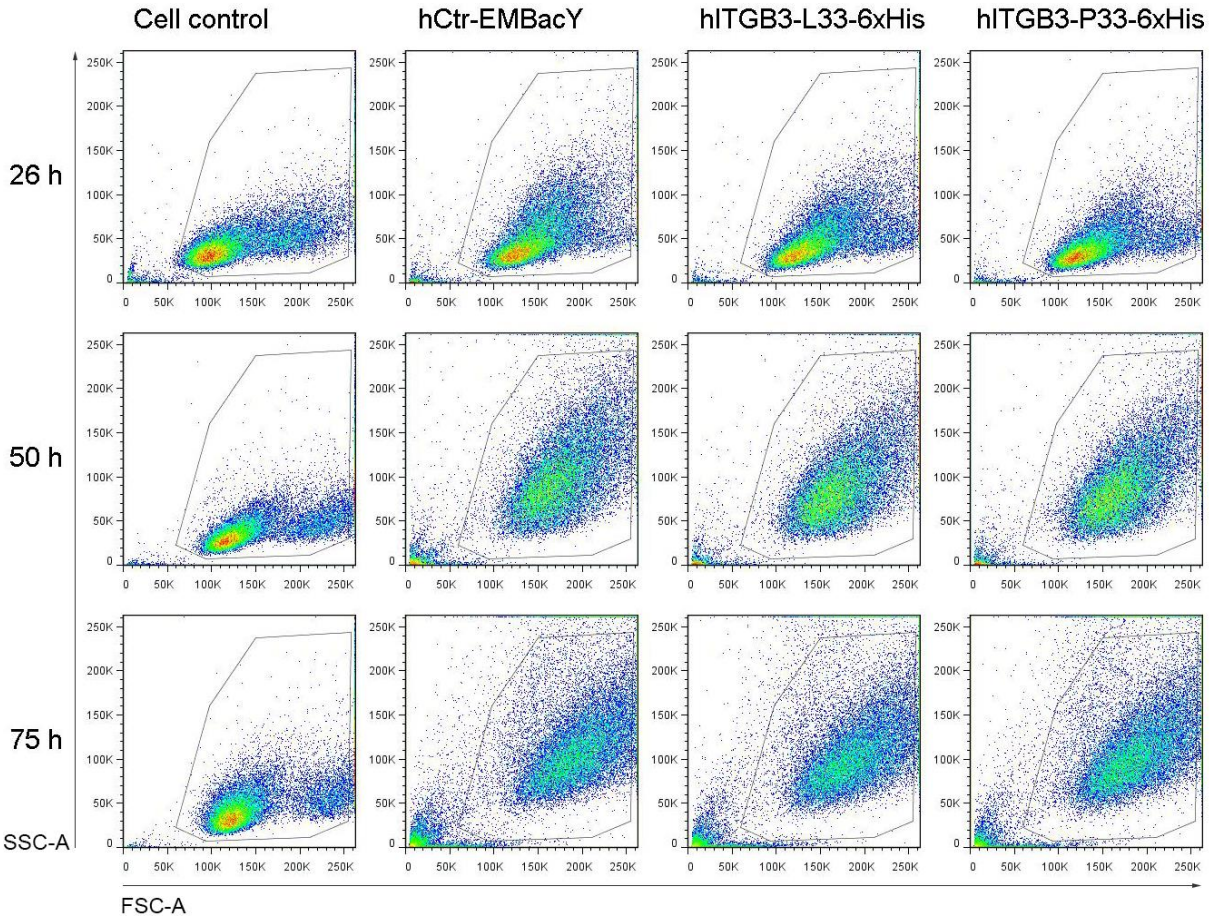

Figure 3.5 High Five cells measured by flow cytometry at 26, 50 and 75 hours after virus infection showed increased cell size. High Five cells in suspension cultures were infected with hCtr-EMBacY, hITGB3-L33-6xHis or hITGB3-P33-6xHis bacmid and incubated at 27 °C shaking at 120 rpm for 75 hours. The cell control showed a continuous cell size in the population, while the virus infected cells showed enhanced cell size at increasing time points.

The suspension cultures inoculated with virus, (hCtr-EMBacY, hITGB3-L33-6xHis or hITGB3-P33-6xHis) clearly showed a successive increase in YFP intensity as the time passed (26, 50 and 75 hours), compared to control (Figure 3.6). At time of harvest, 75 hours, all three populations had a mean fluorescence intensity above 33000.

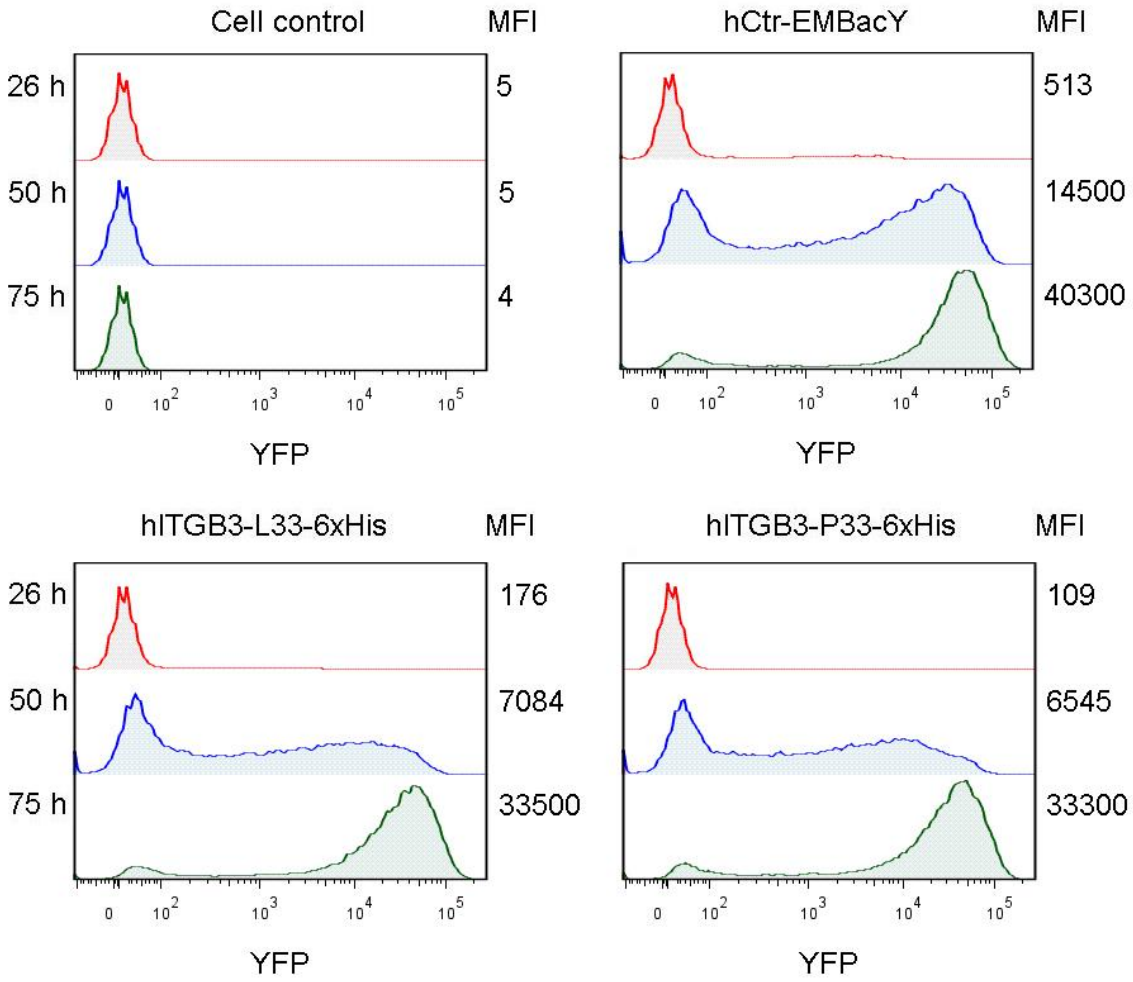

Figure 3.6: Virus infected High Five cells had increased YFP intensity at 50 and 75 hours. High Five cells in suspension cultures were infected with hCtr-EMBacY, hITGB3-L33-6xHis or hITGB3-P33-6xHis virus and incubated at 27 °C shaking at 120 rpm for 75 hours. The cultures were measured at 26, 50 and 75 hours after initial virus infection. At 75 hours, the virus infected cells showed increased YFP intensity in nearly the whole cell population, while the cell control showed negative YFP intensity. The virus infected cells were harvested at 75 hours. Mean fluorescence intensity (MFI) is presented.

3.3 Detection of recombinant human His-tagged protein by ELISA

The recombinant human integrin β3 protein supernatants were analyzed in a sandwich ELISA, using anti-human integrin β3 antibody for capture and anti-His antibody for detection.

Purified recombinant human integrin β3 (100 µg/ml), produced prior to this project, was used to make a standard curve. Six samples with different protein concentrations were made from the stock solution by making a twofold serial dilution starting at 1:500. A standard curve was made by plotting the mean OD_{450nm} value against the known protein concentrations (Figure 3.7).

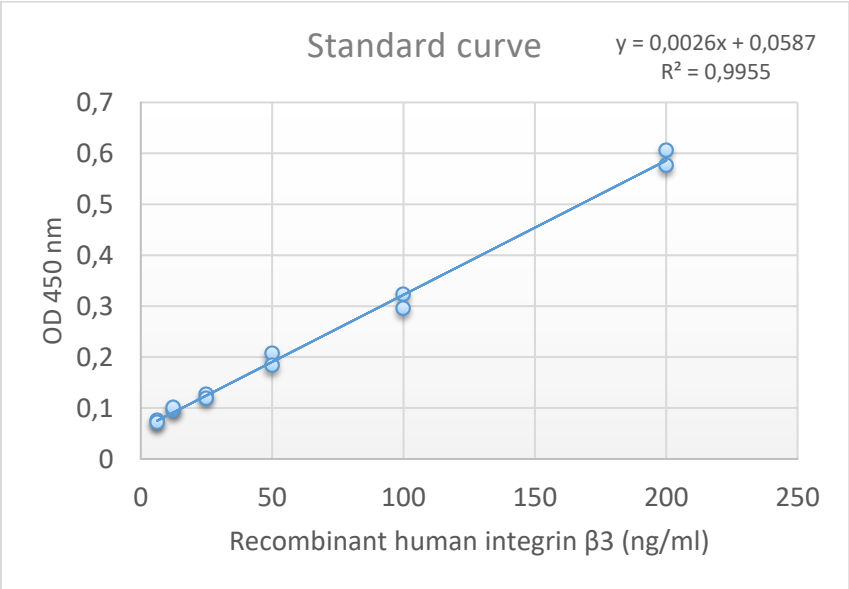

Figure 3.7: ELISA standard curve made by purified recombinant human integrin β3. ELISA coated with AP3 were supplied recombinant human integrin β3 with known concentrations (in parallel) to generate a standard curve. The mean OD_{450nm} values (Y-axis) were plotted against known protein concentrations (X-axis).

The ELISA verified that the supernatants showed presence of His-tagged integrin β3 protein (Figure 3.8). The dilutions displayed approximately half the OD_{450nm} value, as expected by the twofold dilution. The negative controls; 0.2 % PBSA, medium, cell control and EMBacY, did not indicate His-tagged protein.

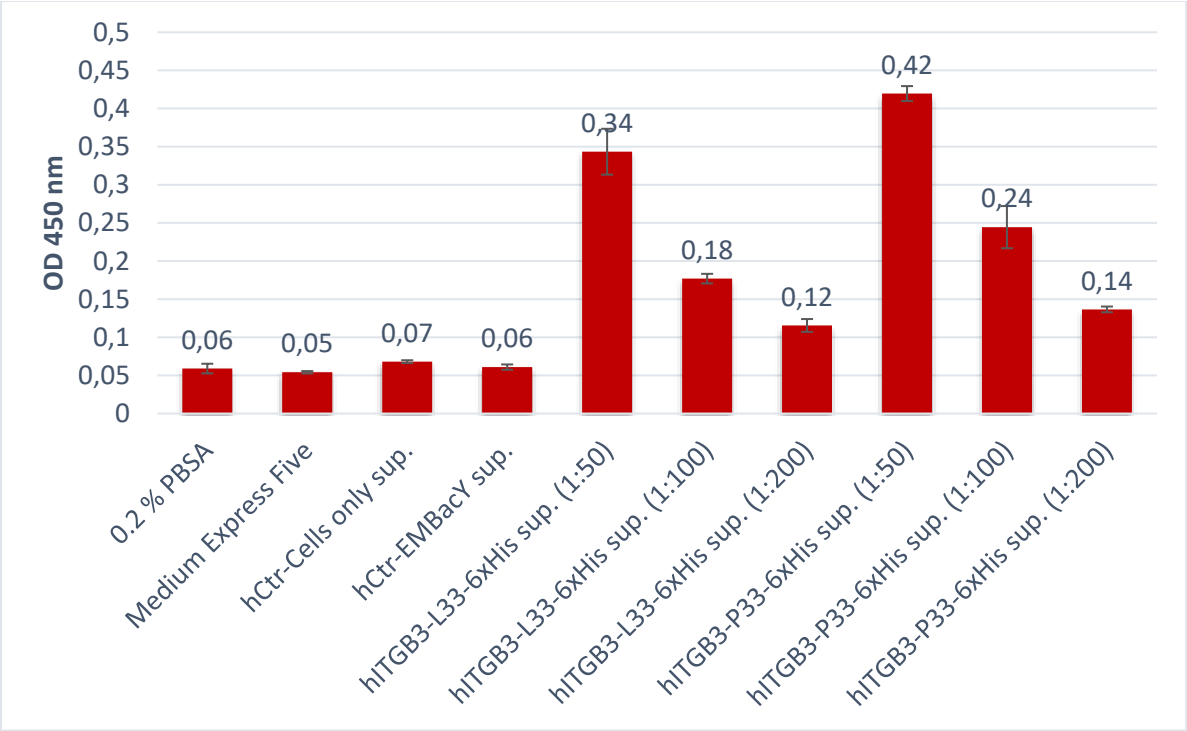

Figure 3.8: Sandwich ELISA of insect cell-produced recombinant human integrin β3 supernatants showed the presence of His-tagged protein in both hITGB3-L33-6xHis and hITGB3-P33-6xHis supernatants. The produced supernatants were analyzed by ELISA using anti-human integrin β3 (AP3) mAb as capture antibody, and detection by an anti-His-tag antibody. Each sample was run in parallel and the diagram is based on the mean OD_{450nm} value. hITGB3-L33-6xHis and hITGB3-P33-6xHis supernatants were diluted 1:50, 1:100 and 1:200 in 0.2 % PBSA. His-tagged proteins were detected in both protein supernatants, but not in the negative controls; 0.2 % PBSA, medium, cells only supernatant and EMBacY supernatant.

In addition to the sandwich ELISA, the recombinant human integrin β3 supernatants were also tested in an ELISA without any anti-human integrin β3 capture of integrin β3. Supernatants were then incubated directly in the plates, and detected by anti-His-tag antibody.

The recombinant human integrin β3 supernatants show presence of His-tagged protein, but not the negative controls; 0.2 % PBSA, medium, cell control and EMBacY (Figure 3.9).

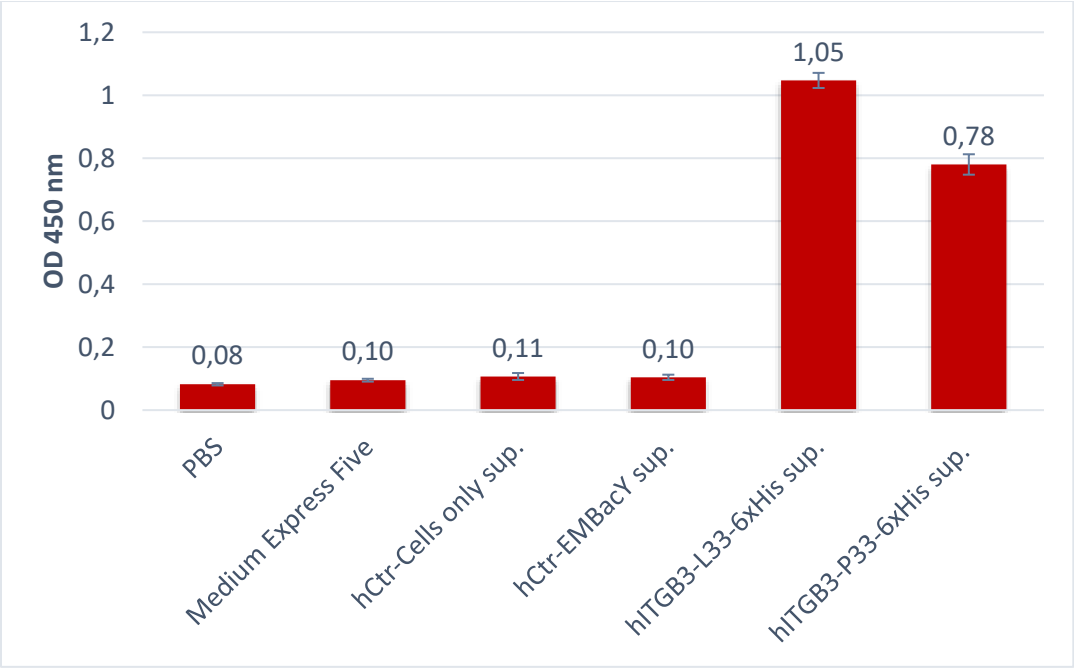

Figure 3.9: Direct ELISA of insect cell-produced recombinant human integrin β3 supernatants showed the presence of His-tagged protein. The produced supernatants were analyzed by ELISA by coating MaxiSorp plates directly with produced supernatants, and detection by an anti-His-tag antibody. Each sample was run in parallel and the diagram is based on the mean OD_{450nm} value. His-tagged proteins were detected in the recombinant human integrin β3 supernatants, but not in the negative controls; PBS, medium, cells only supernatant and EMBacY supernatant.

3.4 Recombinant proteins were detected by SDS-PAGE and western blot

The recombinant human integrin β3 supernatants (small-scale protein production) and the recombinant murine integrin β3 supernatants (low-yield protein production from transfection attempts to produce virus stock) were purified by incubating concentrated supernatants with M-280 beads coated with anti-His-tag antibody, in a capture regimen.

The theoretical molecular weight of the mature recombinant human and murine integrin β3 proteins were calculated using a compute pI/Mw tool (ExPASy, SIB Bioinformatics Resource Portal, retrieved from: http://web.expasy.org/compute_pi/). By supplying the sequence of the extracellular domain and 6xHis-tag of the recombinant human and murine integrin β3 (NP_000203.2 and NP_058060.2, respectively), the calculation showed that the theoretical molecular weight of the human protein was 77.3 kDa and the murine protein was 77.1 kDa.

3.4.1 SDS-PAGE

Beads incubated with recombinant human integrin β3 supernatant L33-6xHis and P33-6xHis resulted in a band corresponding to ~80 kDa under reducing conditions (Figure 3.10), although having an estimated theoretical molecular weight of approximately 77 kDa. In the well with beads only, a band was seen at approximately 13 kDa, likely representing streptavidin subunits. In all the samples with beads coated with anti-His-tag antibody, three additional bands were seen. The smallest band (25 kDa) is most likely the light chains of the antibody, the middle band (55 kDa) is most likely the heavy chains of the antibody, and the larger band (70 kDa) is unknown.

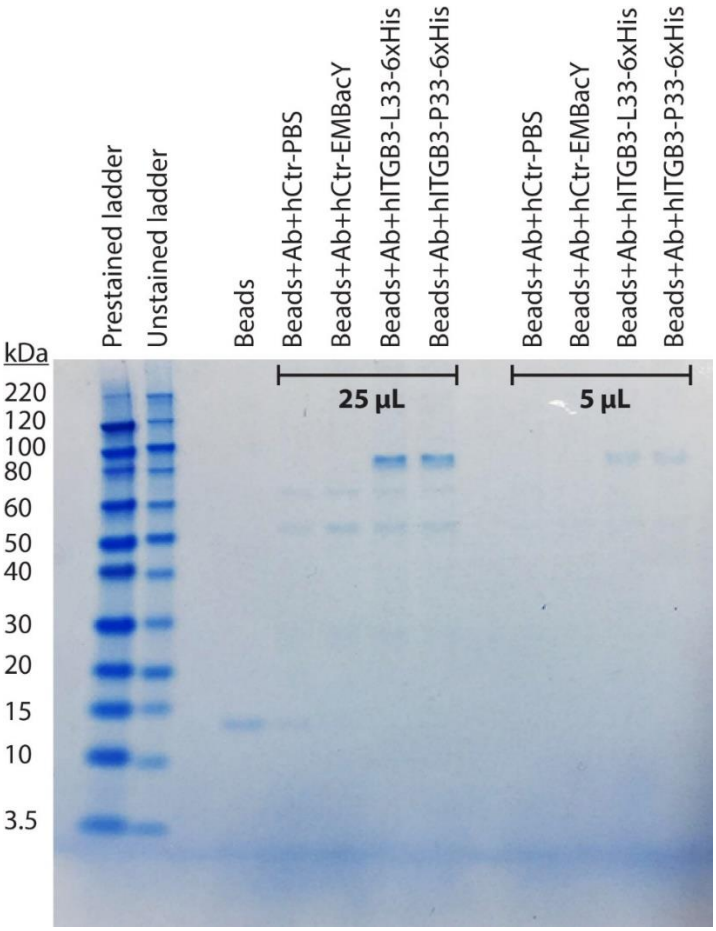

Figure 3.10: Beads coated with anti-His-tag antibody captured an 80 kDa protein in hITGB3 supernatants. The insect cell-produced recombinant human integrin β3 captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by SDS-PAGE under reducing conditions (NuPAGE Novex 4-12% Bis-Tris Gel). The beads were supplied in two volumes, 25 and 5 µl. hITGB3-L33-6xHis and hITGB3-P33-6xHis showed a protein band at approximately 80 kDa, which is not present in the samples with beads alone, beads with Ab and PBS or in beads with Ab and EMBacY supernatant. Novex Sharp Pre-stained and Unstained Protein Standards were used.

The SDS-PAGE with recombinant murine integrin β3 (Figure 3.11) showed comparable bands as the recombinant human integrin β3 samples (Figure 3.10). The samples with beads incubated with recombinant murine integrin β3 supernatant APLD-6xHis and APPD-6xHis also showed a band of approximately 80 kDa. Also here, the streptavidin and antibody chains are visible in some of the samples.

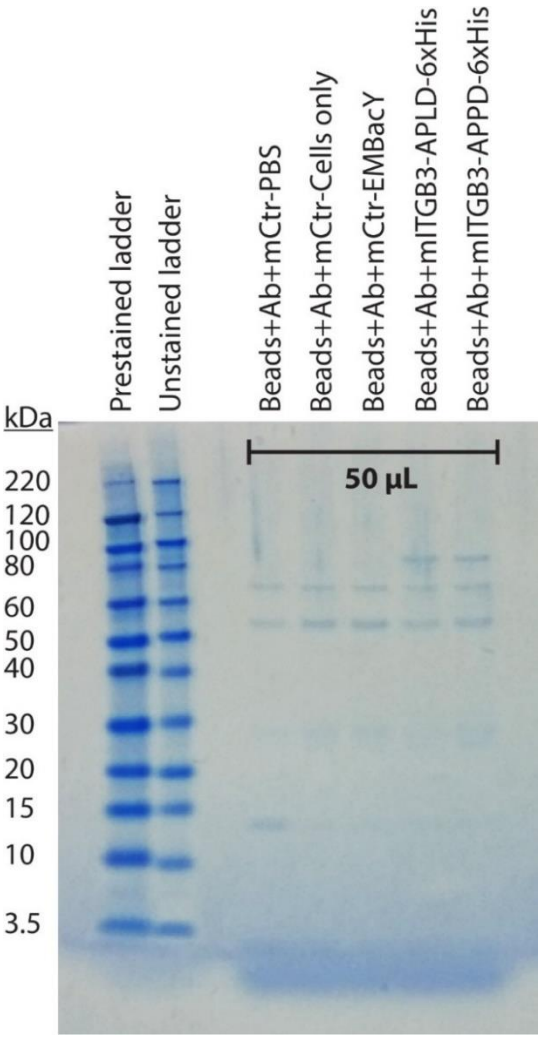

Figure 3.11: Beads coated with anti-His-tag antibody captured an 80 kDa protein in mITGB3 supernatants. The insect cell-produced recombinant murine integrin β3 captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by SDS-PAGE under reducing conditions (NuPAGE Novex 4-12% Bis-Tris Gel). mITGB3-APLD-6xHis and mITGB3-APPD-6xHis showed a protein band at approximately 80 kDa, which is not present in the samples with beads with Ab and PBS, beads with Ab and cells only supernatant and beads with Ab and EMBacY supernatant. Novex Sharp Pre-stained and Unstained Protein Standards were used.

3.4.2 Western blot

The beads coated with anti-His-tag antibody and protein supernatants were also analyzed by western blot. The samples were run on a SDS-PAGE in different sample amounts, which was next blotted onto a membrane and detected by anti-His-tag antibody.

A band of approximately 80 kDa was seen in the recombinant human integrin β3 L33-6xHis and P33-6xHis samples (Beads+Ab+hITGB3-L33-6xHis and Beads+Ab+hITGB3-P33-6xHis, respectively), but not in the negative controls (Figure 3.12).

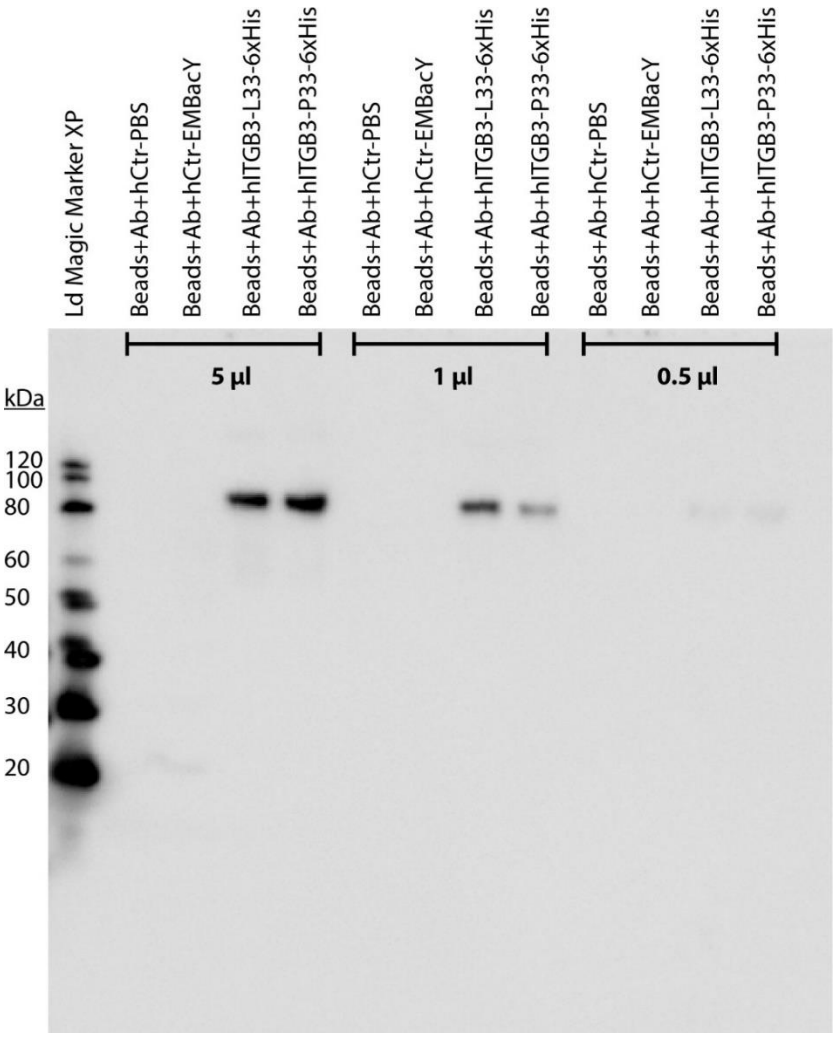

Figure 3.12: Western blot of beads captured with recombinant hITGB3 showed the presence of a His-tagged protein at approximately 80 kDa. The insect cell-produced supernatants captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by western blot under reducing conditions (NuPAGE Novex 4-12% Bis-Tris Gel). hITGB3-L33-6xHis and hITGB3-P33-6xHis showed a protein band at approximately 80 kDa detected by anti-His-tag antibody, which is not present in the samples with beads with Ab and PBS and beads with Ab and EMBacY supernatant. Novex Sharp Pre-stained Protein Standard (not included in the figure) and MagicMark XP Western Protein Standard were used.

Two bands of approximately 80 and 60 kDa were seen in the recombinant murine integrin β3 APLD-6xHis and APPD-6xHis samples (Beads+Ab+mITGB3-APLD-6xHis and Beads+Ab+ mITGB3-APPD-6xHis, respectively), but not in the negative controls (Figure 3.13).

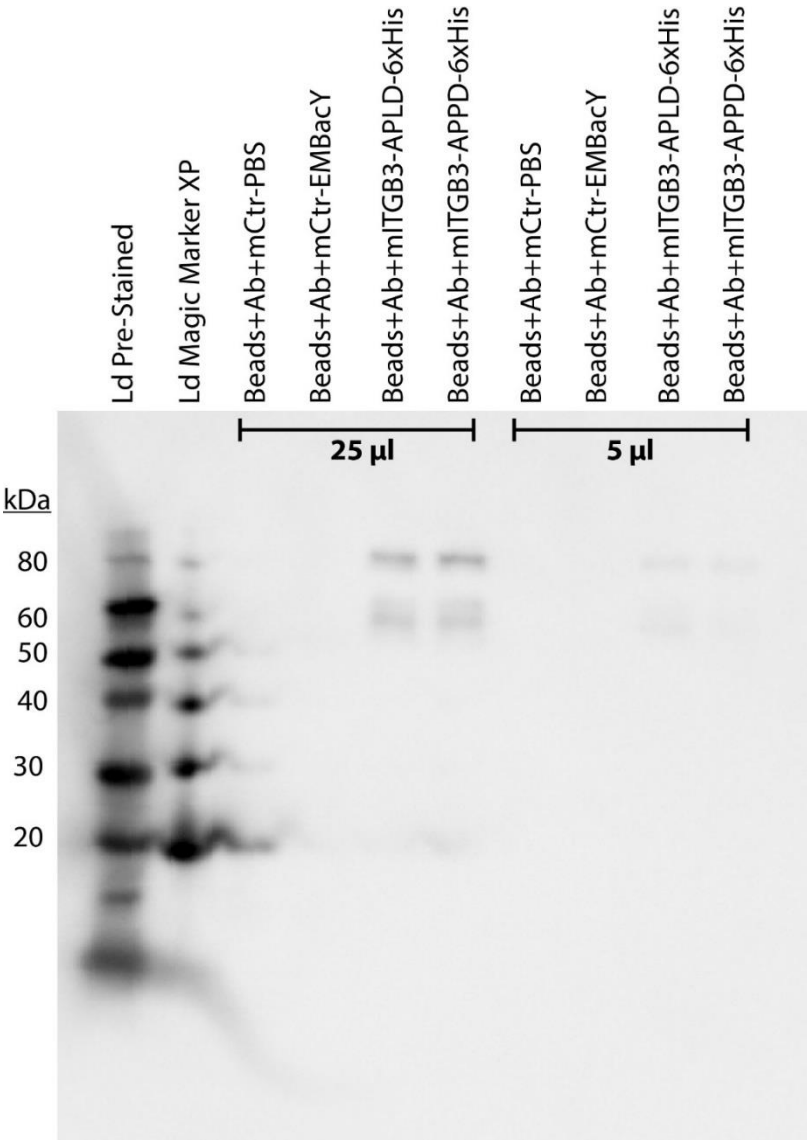

Figure 3.13: Western blot of beads captured with recombinant mITGB3 showed the presence of two bands when using anti-His-tag antibody detection. The insect cell-produced supernatants captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by western blot under reducing conditions (NuPAGE Novex 4-12% Bis-Tris Gel). mITGB3-APLD-6xHis and mITGB3-APPD-6xHis showed protein bands at approximately 60 kDa and 80 kDa detected by anti-His-tag antibody, which is not present in the samples with beads with Ab and PBS and beads with Ab and EMBacY supernatant. Novex Sharp Pre-stained Protein Standard and MagicMark XP Western Protein Standard were used.

3.4.3 Flow cytometry

Verification of intact HPA-1a epitope on recombinant integrin β3 proteins

To see if the HPA-1a epitope was intact in the recombinant proteins, the beads with captured proteins were analyzed by flow cytometry. Analysis of recombinant human integrin β3 showed that the anti-human integrin β3 antibodies (AP3 and Y251) bound to both recombinant hITGB3 versions, while the anti-HPA-1a antibodies (26.4 and Sz21) bound hITGB3-L33-6xHis and not hITGB3-P33-6xHis (Figure 3.14).

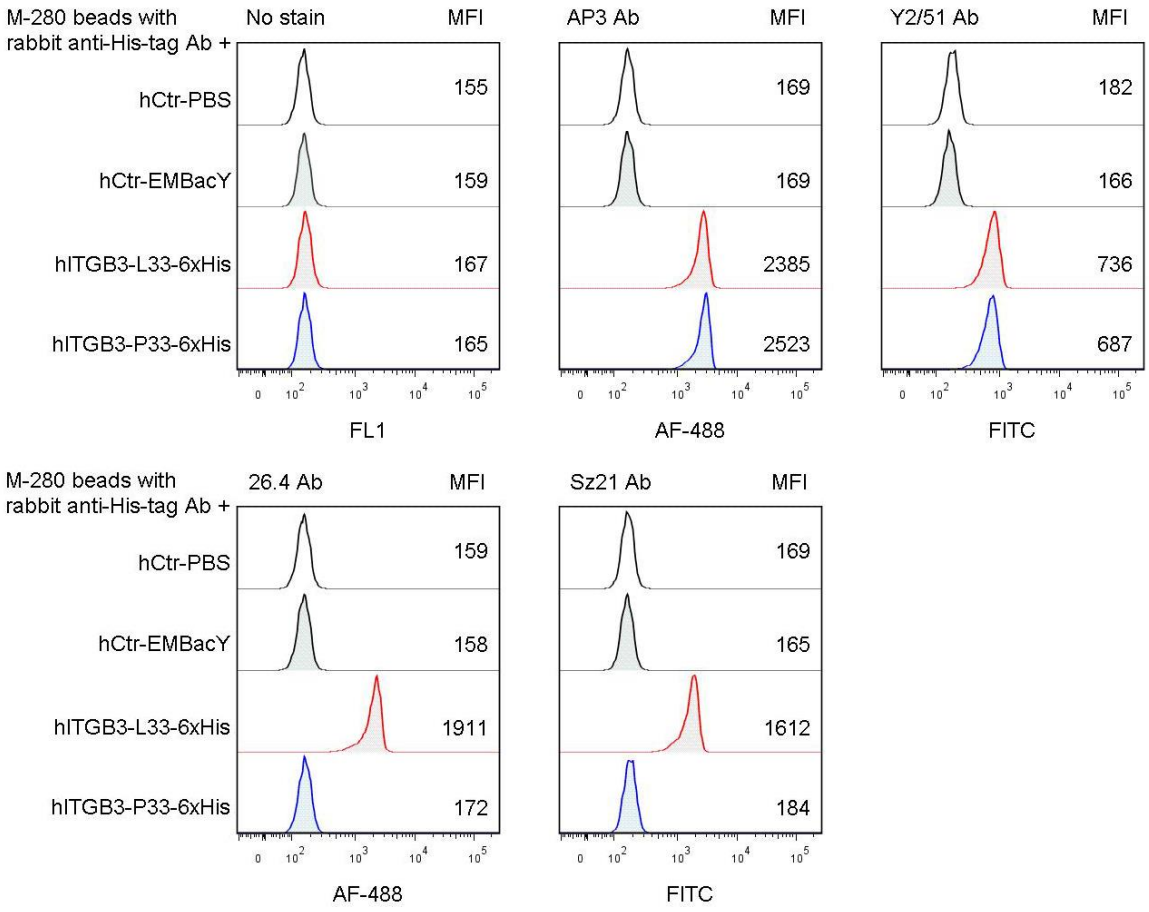

Figure 3.14: Binding of anti-human integrin β3 and anti-HPA-1a antibodies to recombinant hITGB3 captured on beads. The insect cell-produced supernatants captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by flow cytometry using antibodies conjugated with dye. The anti-human integrin β3 antibodies, AP3 and Y251, bound both recombinant hITGB3 versions, while the anti-HPA-1a antibodies, 26.4 and Sz21, only bound hITGB3-L33-6xHis. Mean fluorescence intensity (MFI) is presented.

Analysis of recombinant murine integrin β3 showed no binding to neither anti-human integrin β3 antibodies (AP3 and Y251) nor the anti-HPA-1a antibody, 26.4. However, reactivity was seen with Sz21 antibody, which showed binding to mITGB3-APLD-6xHis only (Figure 3.15).

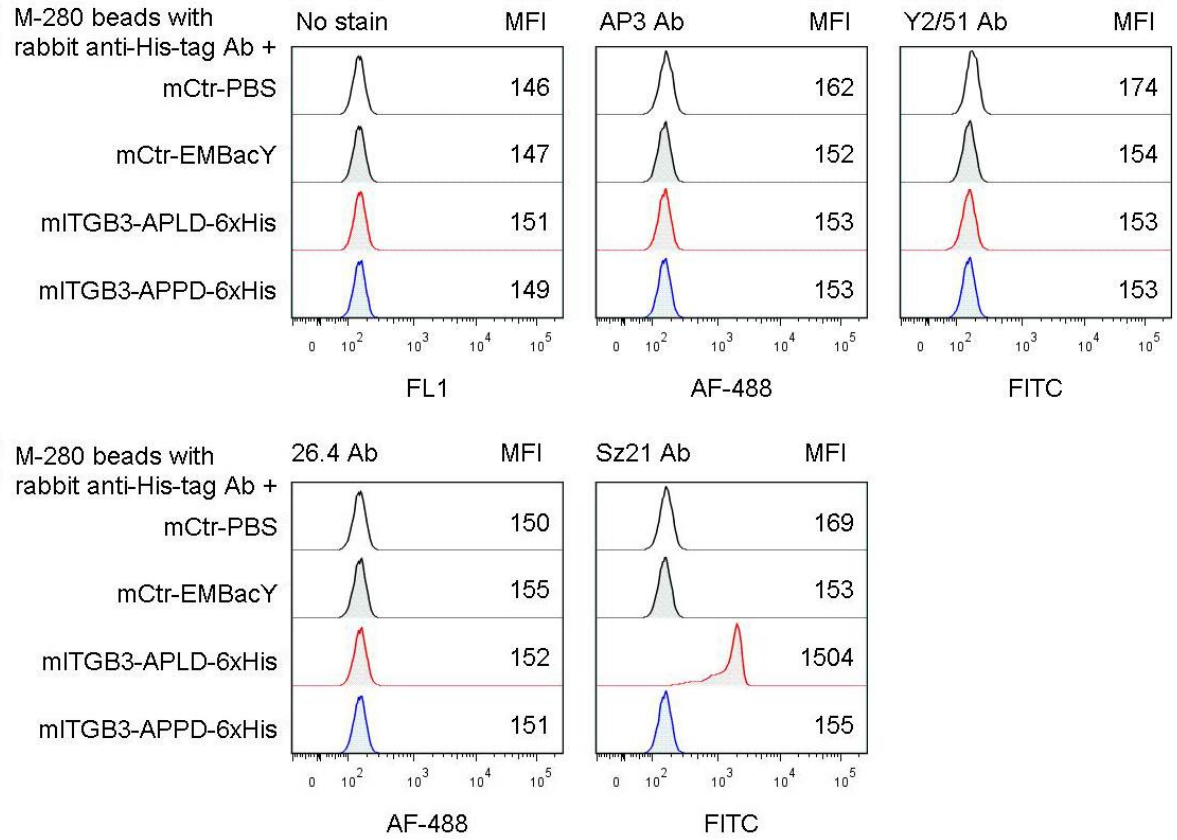

Figure 3.15: Binding of anti-human integrin β3 and anti-HPA-1a antibodies to recombinant mITGB3 captured by beads. The insect cell-produced supernatants captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody and analyzed by flow cytometry using antibodies conjugated with dye. The anti-HPA-1a antibody Sz21 bound mITGB3-APLD-6xHis, but not mITGB3-APPD-6xHis or the controls beads with PBS and EMBacY. AP3, Y2/51 and 26.4 did not bind any of the beads. Mean fluorescence intensity (MFI) is presented.

Measurement of antigen present on a new batch of recombinant murine integrin β3 beads

A new batch of recombinant murine integrin β3 had to be prepared to perform any further experiments. The beads were run on SDS-PAGE for confirmation, and a protein of 80 kDa was present (data not shown). The concentration of recombinant integrin β3 captured by beads were analyzed by Sz21 (HPA-1a-specific) antibody detection. M-280 streptavidin beads coated with anti-His-tag antibody with captured recombinant human and murine integrin β3 were analyzed to compare protein concentrations on the new batch of beads with murine integrin β3. Noteworthy, Sz21 (FITC) generated a lower FITC intensity on mITGB3-APLD-6xHis (439 MFI) compared to hITGB3-L33-6xHis (1532 MFI) (Figure 3.16).

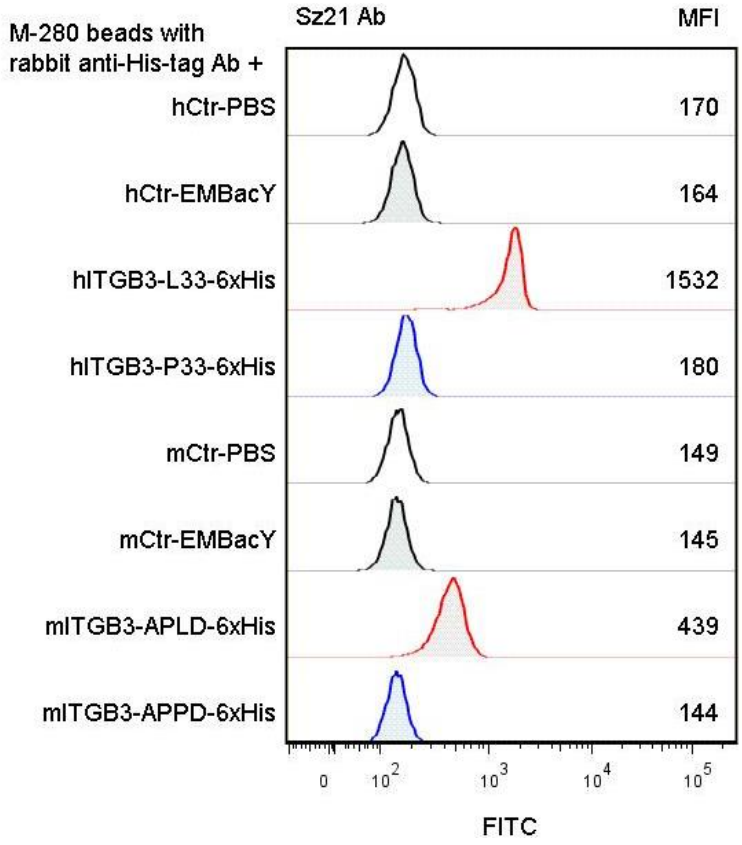

Figure 3.16: Binding of Sz21 to beads with recombinant human and murine integrin β3 showed a lower FITC intensity on mITGB3-APLD-6xHis beads compared to hITGB3-L33-6xHis beads. The insect cell-produced supernatants captured by M-280 streptavidin beads coated with biotinylated anti-His-tag antibody were analyzed by flow cytometry using Sz21 antibody conjugated with FITC. The FITC intensity was less in the beads with captured recombinant mITGB3-APLD-6xHis than in the beads with captured recombinant hITGB3-L33-6xHis.

3.5 T cell stimulation by recombinant proteins

3.5.1 Isolation of monocytes from PBMC

To see if the recombinant proteins could activate HPA-1a-specific T cells, the T cells were stimulated with APC co-cultured with antigen. In this project, HLA-DRB3*01:01 positive monocytes, purified from PMBC from an HPA-1bb donor, were used as APC. Successful enrichment of monocytes was demonstrated (Figure 3.17) by monocytes constituting 87.1 % cells of live cells after enrichment, compared to 13.6 % in the original PBMC samples.

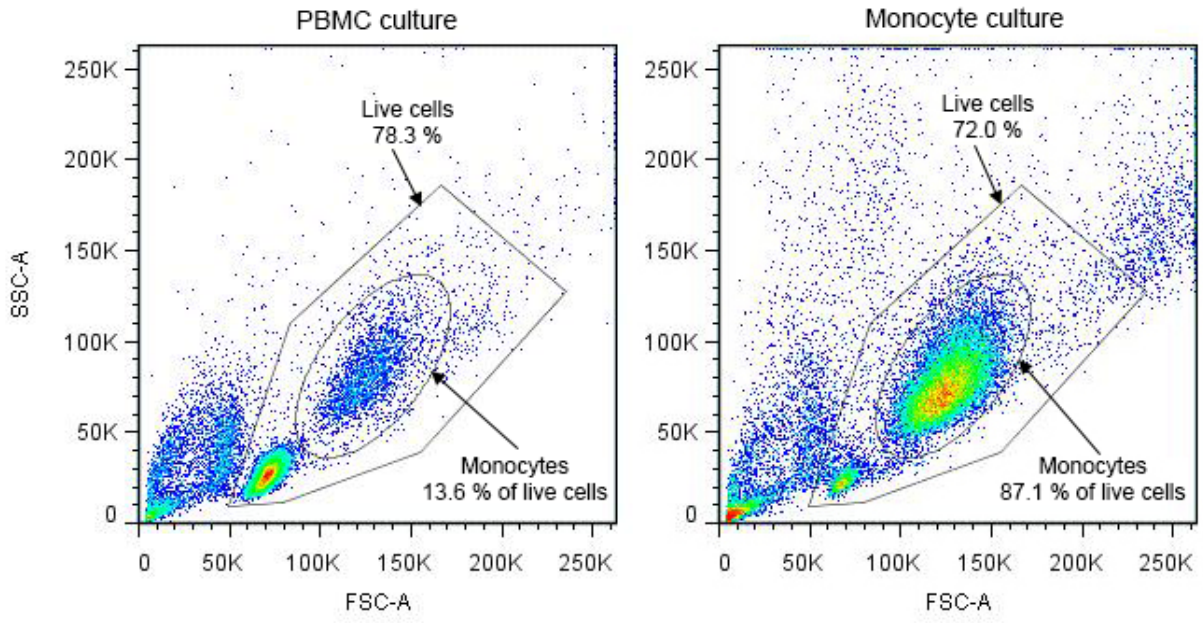

Figure 3.17: Monocytes were enriched from PBMC donor HD#31. The cells were measured by flow cytometry before and after the isolation showing increased percent of monocytes after the enrichment.

3.5.2 HPA-1a-specific T cells produced TNF and proliferated when stimulated with recombinant ITGB3 comprising the HPA-1a epitope

CFSE-stained HPA-1a-specific T cell clone D8T108 was stimulated with APC cocultured with antigen.

TNF secretion assay

HPA-1a-specific T cells incubated with HLA-DRB3*01:01 positive monocytes co-cultured with HPA-1aa platelets, L33 peptide, beads with hITGB3-L33-6xHis and mITGB3-APLD-6xHis, showed TNF secretion after 6 hours (Figure 3.18). In comparison, incubation with monocytes co-cultured with the controls HPA-1bb platelets, P33 or Lol P1 peptide, beads with hITGB3-P33-6xHis, mITGB3-APPD-6xHis, hCTr-EMBacY or mCtr-EMBacY showed no TNF production.

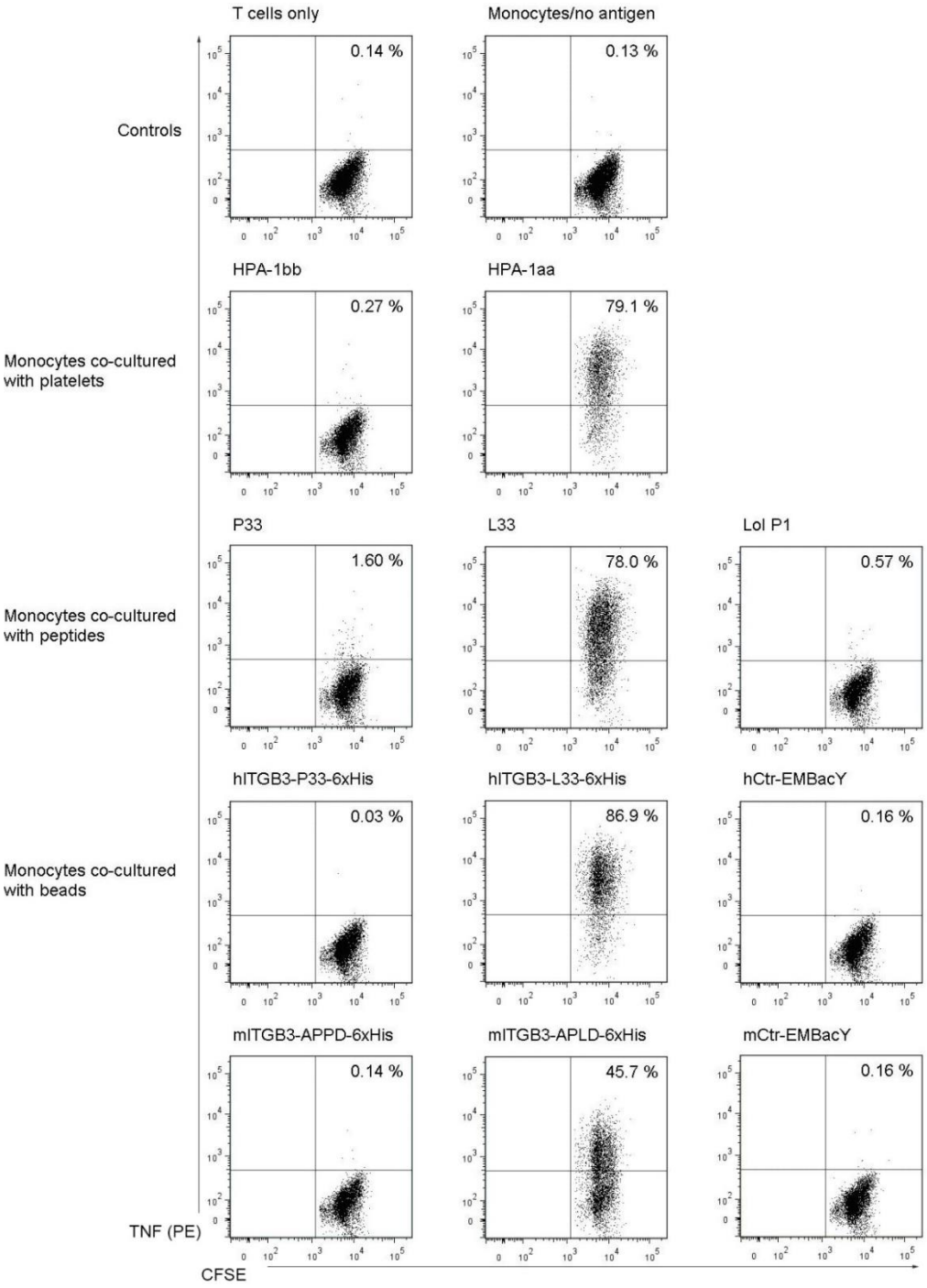

Figure 3.18: HPA-1a-specific T cells showed TNF production when incubated with HLA-DRB3*01:01 positive monocytes co-cultured with HPA-1aa platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis. HLA-DRB3*01:01 positive monocytes isolated from an HPA-1bb donor (HD#31) were pulsed overnight with platelets, peptides or beads with captured recombinant integrin β3 protein. This was followed by an incubation for 6 hours with CFSE labelled HPA-1a-specific T cell clone D8T108 in the presence of Tapi-0 and anti-TNF antibody (PE conjugated) to stimulate TNF production. T cells showed TNF production when stimulated with monocytes pulsed with HPA-1aa platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis, but not with monocytes pulsed with HPA-1bb platelets, P33 or Lol P1 peptide or beads coated with hITGB3-P33-6xHis, mITGB3-APPD-6xHis or control EMBacY.

Proliferation assay

CFSE labelled HPA-1a-specific T cells incubated with HLA-DRB3*01:01 positive monocytes co-cultured with HPA-1aa platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis showed decreased CFSE intensity after 4 days, indicating proliferation (Figure 3.19). mITGB3-APLD-6xHis did not yield as high response as the other positive results, and this was also seen in the TNF assay (Figure 3.18). HPA-1a-specific T cells incubated with monocytes co-cultured with the controls HPA-1bb platelets, P33 or Lol P1 peptide or beads with hITGB3-P33-6xHis, mITGB3-APPD-6xHis, hCTr-EMBacY or mCtr-EMBacY did not show extensive decrease in CFSE levels.

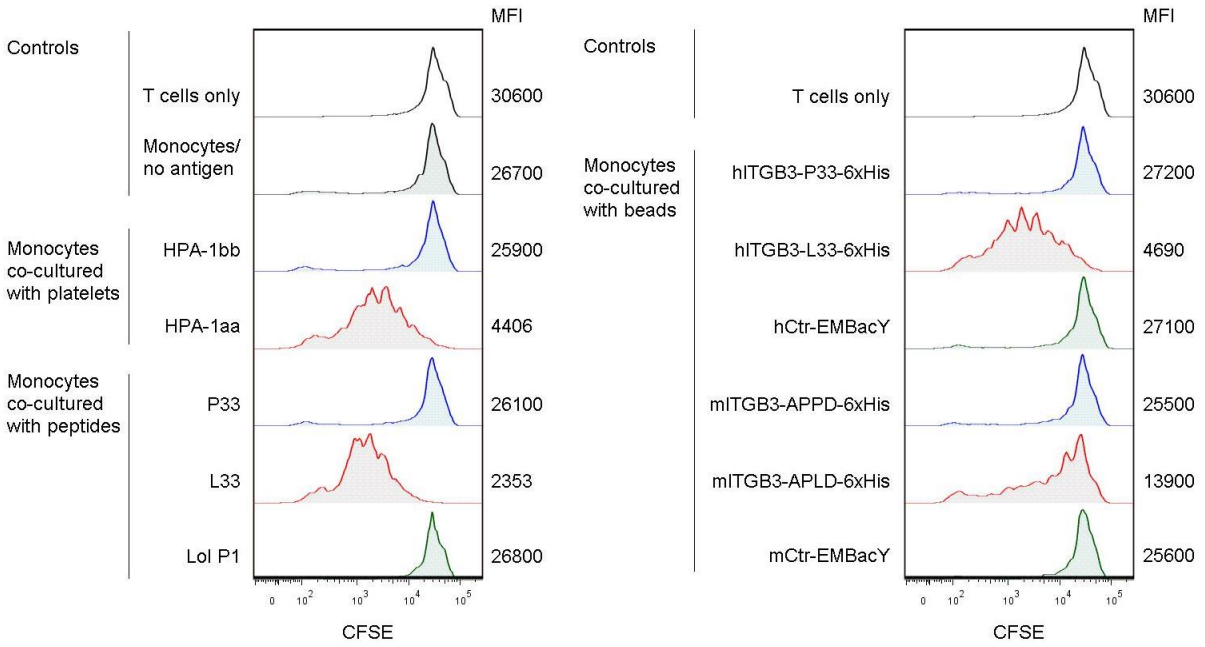

Figure 3.19 HPA-1a-specific T cells proliferated when incubated with HLA-DRB3*01:01 positive monocytes co-cultured with HPA-1aa platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis. HLA-DRB3*01:01 positive monocytes from an HPA-1bb donor (HD#31) were pulsed overnight with platelets, peptides or beads with captured recombinant integrin β3 protein. This was followed by an incubation for 4 days with CFSE labelled HPA-1a-specific T cell clone D8T108. Proliferation was assessed by flow cytometry as CFSE dye dilution. T cells proliferated when stimulated with monocytes pulsed with HPA-1aa platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis, but not when incubated with monocytes pulsed with HPA-1bb platelets, P33 or Lol P1 peptide or beads coated with hITGB3-P33-6xHis, mITGB3-APPD-6xHis or control EMBacY. Mean fluorescence intensity (MFI) is presented.

4 Discussion

The aim of this thesis was to produce recombinant murine integrin β3 proteins by incorporating a designed gene construct into the baculovirus expression vector, EMBacY. Next, the proteins were expressed by transfecting insect cells with the isolated and purified bacmid. Subsequently, already produced virus incorporated with the recombinant human integrin β3 gene were used to infect insect cells, expressing the recombinant human integrin β3 proteins. After the protein production, the recombinant proteins were captured on beads coated with anti-His-tag antibody and analyzed by SDS-PAGE and western blot. The presence of HPA-1a epitope was verified for the recombinant human and murine integrin β3 using antibodies specific for this antigen. HPA-1a-specific T cells were also incubated with HLA-DRB3*01:01 positive monocytes pulsed with the produced proteins to see if they could stimulate T cell proliferation and TNF production.

4.1 Construct design

Due to the desire of producing a soluble protein, only the native signal peptide and the extracellular domain of integrin β3 were included in the gene construct. This was based on a study by Peterson et al. [56], where truncated mutant versions of integrin β3 were produced, all containing the native signal peptide. The signal peptide in the N-terminal of the protein is important in the translocation of the proteins in the secretory pathway. Integrin β3 is normally membrane bound, but due to expression of the extracellular domain only, the protein is secreted from the cell. The selection of an efficient signal peptide is therefore important for efficient protein secretion. In protein production, different signal peptides could be favorable [57], but in this current project, the native signal peptide was utilized. To generate recombinant murine integrin β3, four amino acids were substituted creating either the human HPA-1a or HPA-1b epitope, as these four modifications have been showed to make the HPA-1a epitope in mice by our collaborators [26].

4.2 Protein production

In this project, the recombinant integrin β3 proteins, both human and murine versions, were produced using the baculovirus system. Many other protein production systems are available such as bacteria, yeast and other eukaryotic cell lines. Bacteria is probably the most cheap and simple method used to produce proteins, but since bacteria are prokaryotic, they do not possess

the cell machinery to correctly fold or perform post-translational modifications required in a eukaryotic protein. Insect cells can generate a high protein yield, correct folding of proteins and post-translational modifications. Thus, baculovirus and insect cells were used in the production of recombinant integrin β3 in this project, even though the system is relatively expensive and time-consuming.

Today, the baculovirus system is highly used and a wide range of proteins have been made [58]. The MultiBac system used in this study, is a system that uses a baculovirus expression vector adapted to produce eukaryotic protein complexes with multiple subunits [32]. This system is based on the use of standard operating procedures - from the insertion of the gene into the bacmid to the protein production [59]. The bacmid used in this project, EMBacY, was a gift from EMBL Grenoble. This is a new baculovirus genome that expresses YFP co-dominantly with the protein, which was created to simplify the monitoring of the protein production [32]. In this project, the use of this new bacmid was quite essential. Simply by measuring YFP intensity in the cells by flow cytometry, virus and protein expression was monitored at critical time points.

In this project, the transfer vector containing the gene of interest was integrated into the EMBacY genome present in *E. coli* cells by Tn7-recombinase mediated transposition. In the alternative method, which also was the original method when using baculovirus, the transfer vector and the bacmid were combined by homologous recombination. Here, both vectors are transfected separately into the insect cells, and then combined by homologous recombination. This recombination occurs at a low percentage, and multiple plaque assays must be conducted to separate the parental virus from the recombined virus [60]. An advantage using Tn7 recombination is that it is conducted prior to insect cell transfection. The correctly inserted genes are selected by blue and white screening, and a bacmid stock containing the desired gene can be generated. When using homologous recombination, the complex must be made during every experiment [32].

4.2.1 Insect cell transfection with isolated bacmid gave low transfection ratio

In this current project, the high-titer virus producing recombinant human integrin β3 was already created. However, the recombinant murine integrin β3 had not been created in our laboratory before, and an adapted version of the protocol for protein complex expression using MultiBac published from Fitzgerald et al. was used to produce the protein [34].

E. coli with EMBacY were made competent chemically. When the transfer vectors were supplied to these competent cells, the blue and white screening showed that only a few colonies were successfully integrated with a transfer vector. However, the few white colonies were enough to make a stock of EMBacY-mITGB3-APLD-6xHis and EMBacY-mITGB3-APPD-6xHis.

Following isopropanol bacmid isolation, insect cells were transfected with bacmid using transfection reagents. Usually, the whole process of protein production in insect cells using baculovirus takes 2-4 weeks, including the integration of transfer vector into bacmid, but this was not the case in the current project. The transfection efficiency of each transfection attempt was measured 24 hours after collecting initial virus, V_0. In the first transfection attempt, the level of YFP positive cells did not exceed 1 % and the following virus amplification attempt did not generate any YFP positive cells. Numerous attempts of transfection were conducted over several months, but the transfection ratio was still too low to generate a virus stock.

The low transfection ratio could result from problems in the original EMBacY *E. coli* cells, the bacmid isolation process, the transfection process or the insect cells. Several variables in the production were changed to achieve a positive outcome. In the earlier transfection attempts, the Sf21 cell line was used. However, the cells only generated transfection ratios of approximately 0-1 % YFP positive cells and had a high cell death. The cell concentration was increased to compensate for the dying cells, but it did not increase transfection efficiency. Both thawing of fresh cells and ordering of new cells from supplier were done before other cell lines as Sf9 and High Five were tried, still without sufficient ratios.

In the original transfection method, high amounts of DNA were used. Both increase in transfection reagent amounts and decrease of DNA were attempted without successful transfection. Also, a different transfection reagent was tried, but it did not solve the problem. To see if the problem with transfection arose in the insect cells, the different cultures were transfected with frozen bacmid made prior to this project. The transfection generated more than 50 % YFP positive cells, indicating that the insect cells were not the problem. Also, this showed that the problem did not arise from the transfection reagents or transfection process.

Following, the bacmid isolation process became the focus. The DNA isolation is dependent on precipitation with isopropanol, so increased freezing time (overnight) was tested. This still generated low transfection efficiency. Next, a short incubation of DNA and isopropanol at room

temperature was tested to avoid co-precipitation of salts from the lysis reaction, but the results were the same.

EMBacY without insert was included as a positive control in all the transfection attempts. Transfected insect cells should make viruses from the EMBacY bacmid (without insert) easier than when a insert is present due to less DNA to replicate and translate. Still, the insect cells transfected with only EMBacY were just as poor as the ones with recombinant integrin β3 inserted. A theory could therefore be that the quality of the original EMBacY stock was poor. In the last attempt with the original protocol, transfection ratio of approximately 5 % YFP positive cells were generated. This was not enough virus for a virus amplification, but the supernatants were collected and analyzed further. The problems with the generation of virus have not been encountered earlier at the Immunology research group and troubleshooting will be performed, but there was not time for more testing in this master thesis.

The recombinant human integrin β3, both L33-6xHis and P33-6xHis molecular versions, were made using virus stocks made prior to this project. Here, we clearly see that the cells in culture have swollen, indicating virus infected cells (Figure 3.5). Almost the whole population is YFP positive, and the intensity is satisfactory (Figure 3.6). These observations further suggest the problem with transfection may be due to issues with the current EMBacY construct.

4.3 Analysis of recombinant ITGB3

4.3.1 Human ITGB3 were detected by ELISA

The recombinant human integrin β3 were examined in two different ELISAs. The first one was done using AP3 (which binds both molecular versions) as capture antibody, and detecting with anti-His-tag antibody. Here, His-tagged proteins are clearly present in the supernatants containing hITGB3-L33-6xHis and hITGB3-P33-6xHis (Figure 3.8).

An antibody that binds both molecular versions of the recombinant murine integrin β3 are not available at our laboratory at the moment. Due to the lack of a specific antibody other than the anti-His-tag, the murine proteins could not be bound by a separate capture antibody for ELISA. As a solution to this problem, an ELISA plate was coated directly with recombinant human integrin β3 supernatants, followed by detection by anti-His-tag antibody. His-tagged protein was clearly present in the supernatants (Figure 3.9), and this protein detection method could be applicable for the recombinant murine proteins when the protein production issues are solved.

4.3.2 SDS-PAGE showed a protein of approximately 80 kDa

Further, these recombinant human integrin β3 supernatants, together with low-yield recombinant murine integrin β3 supernatants from the transfection attempt, were concentrated and captured by M-280 streptavidin beads coated with biotin conjugated anti-His-tag antibody. Next, the beads were run on SDS-PAGE to see if a protein band in the correct size was present.

Both gels (Figure 3.10 and 3.11) showed protein bands of approximately 80 kDa, in the integrin β3 samples. The theoretical molecular weight of the proteins is approximately 77 kDa, and it is a little smaller than the predicted size on the gel. Due to the fact that proteins may migrate differently from their predicted molecular weight on the gel, the protein seen at approximately 80 kDa is probably the recombinant integrin β3 proteins.

A band of approximately 13 kDa was seen in the wells containing only beads, likely representing streptavidin subunits. This band is not seen clearly in the other samples, but this is possible due to high concentration of beads in this sample. In the samples containing beads coated with anti-His-tag antibody, three bands are present. A weak band is seen at 25 kDa and a stronger band at 55 kDa, likely representing the light and heavy chains of the antibody, respectively. A band is also seen at 70 kDa, and because all three bands are seen in the sample with beads, antibody and PBS, but not in the sample with beads alone, something corresponding to the beads with antibody is making the additional band. In the washing process of the beads, PBS was utilized and not PBSA to avoid the albumin (66.5 kDa) protein on the gel. A possible explanation to the additional band could be that the antibody solution contained albumin prior to the biotinylation process by the producer, leading to biotinylated albumin in the product and thus binding to the streptavidin beads. The bands potentially corresponding to the integrin β3 proteins, show that the protein purification by capture on beads generated pure samples.

4.3.3 His-tagged proteins were detected by western blot

To confirm that the bands seen on the gels were His-tagged protein, the beads were also analyzed by western blot detected with anti-His-tag antibody. The blot with the human samples clearly show bands corresponding to integrin β3 (Figure 3.12). The western blot of the recombinant murine integrin β3 did not generate a satisfactory blot as the recombinant human integrin β3 blot (Figure 3.13). Here, the blot generated double bands, which might be due to a poorly executed western blot. Another possibility could be that there are additional His-tagged

proteins in the supernatant, but this is unlikely because the additional band is not present in the western blot of recombinant human integrin β3. The band seen at 80 kDa is most likely the desired protein. Summing up, the western blot showed His-tagged proteins in the samples containing human and recombinant murine integrin β3, but not in the samples containing only beads, beads and antibody or beads, antibody and EMBacY supernatant. Further, other antibodies should be tested in western blot to generate integrin β3-specific detection.

4.3.4 The HPA-1a epitope was present in human and murine ITGB3

To test if the HPA-1a epitope was intact in the produced proteins, recombinant human and murine integrin β3 captured on beads were analyzed with different antibodies. When reacting beads with AP3 mAb and Y2/51 mAb, only the human ITGB3-L33-6xHis and ITGB3-P33-6xHis were positive, but not the murine versions. This was expected, because these antibodies are anti-human ITGB3. Sz21 mAb bound to both the human and the murine ITGB3 comprising the HPA-1a epitope, showing that the epitope was present in both proteins. On the other hand, 26.4 mAb bound the hITGB3-L33-6xHis protein, but not the mITGB3-APLD-6xHis protein.

The lack of binding of 26.4 to mITGB3-APLD-6xHis was not as expected. The Sz21 antibody is showing that the epitope is present, so other factors must influence the binding of 26.4. A study done by Valentin et al. [61] on anti-HPA-1a antibody responses show that antibodies could be classified into two groups; antibodies binding near the epitope without the demand of other domains (Type I antibodies) and antibodies that reacts with the epitope only when interacting with cysteine rich domains around residues 433-692 (Type II antibodies). It is possible that 26.4 ends up in the second category, not binding this murine integrin β3 even though the four amino acid substitutions are resembling the HPA-1a epitope.

4.3.5 Beads coated with recombinant ITGB3 could be used in T cell stimulation

HPA-1a-specific T cells were in this project incubated with HLA-DRB3*01:01 positive monocytes co-cultured with platelets, peptides or beads captured with the produced proteins. TNF production and proliferation in the T cells were measured. Incubation with APC pulsed with HPA-1a platelets, L33 peptide or beads with hITGB3-L33-6xHis or mITGB3-APLD-6xHis generated T cell TNF production and proliferation (Figure 3.14 and 3.15, respectively). The response to mITGB3-APLD-6xHis was not as great as in the stimulation by hITGB3-L33-6xHis. Based on the HPA-1a detection by flow cytometry using Sz21 (Figure 3.16), the most

likely reason for the low stimulation is caused by lower protein concentration on the beads with the murine protein compared to beads with the human protein. The low stimulation, both in the TNF and the proliferation assay, indicates that not all T cells were presented with proteins. In the assays, 100 platelets were supplied per monocyte, but only 10 beads were supplied per monocyte due to low material amount. However, the beads with recombinant human ITGB3-L33-6xHis generated an equivalent response to the platelets, indicating that 10 beads per monocyte was sufficient, but that the beads with murine protein had a sub-optimal protein concentration.

4.4 Future prospects

4.4.1 New purification methods are needed for the murine ITGB3

The recombinant human integrin β3 has been made previously by the Immunology research group, and due to this, a standard purification method has been developed. This involves the capture of protein from the supernatant by columns containing AP3 mAb. Because the murine versions of the protein have not previously been produced, a purification method has not been established. The purification model for the human protein could not be applied because AP3 mAb only binds human integrin β3, as shown in this project. However, anti-murine integrin β3 antibodies are available on the market, but due to the four amino acid substitutions made in the gene to comprise the HPA-1a epitope and the fact that only the extracellular protein domain is included, the binding of these antibodies could not be guaranteed. Development of a purification methods will become a priority when the problems with the virus production and then consequently the protein production is solved.

In this project, the proteins were purified from the supernatants by capture on beads, which showed to be a good method for analyzing the presence of proteins on SDS-PAGE, western blot and flow cytometry. But due to the binding of biotin and streptavidin between the beads and the anti-His-tag antibody, the beads must be warmed at above 70 degrees to release the protein from the beads [62]. This treatment will denature the proteins, leading to the loss of protein structure, so this purification method is not ideal when doing a large-scale protein production.

4.4.2 Protein production with both α and β chain is desired

In an immune response, antigen-presenting cells internalize antigen, process it and present the antigen to T cells. The T cells use their T cell receptor to recognize the epitope on the peptide bound to MHC class II on the APC. On the other hand, B cells recognize epitopes on the surface of the antigen through their membrane-bound immunoglobulin. Therefore, the epitopes that activate T and B cells can be very different. When integrin β3 is expressed in the human body, the protein is combined with an α-chain, either αIIb or αV protein, generating a functional integrin heterodimer [1]. The HPA-1a antigen is present on the β-chain, and the HPA-1a variant only differs from the HPA-1b variant with one amino acid, respectively a leucine or a proline. The T cell epitope is presented to the T cell by the MHC molecule, independent of the integrin β3 composition; only integrin β3, integrin αVβ3 or integrin αIIbβ3.

In this thesis, we only produce recombinant integrin β3 without any α-chain. In an immune reaction generated against the HPA-1a integrin β3 variant, B cell responses generated against the whole αVβ3 or αIIbβ3 complex could be missed due to the lack of an α-chain since some anti-HPA-1a antibodies have been shown to depend on association with an α-chain for efficient binding [63]. Also, if using the produced recombinant integrin β3 to detect antibodies generated in an immune response against this antigen, the antibodies specific for an integrin complex are not detected in such an assay. To solve this potential problem, recombinant integrin β3 in dimer with both αV and αIIb could be produced. The production and spontaneous dimerization of both α-chain and β-chain have been shown in a study by Peterson et al. [64], where a recombinant soluble form of αIIbβ3 is produced, and the dimer assumes an active, ligand-binding conformation. Therefore, by including the α-chains, more HPA-1a-specific antibodies could be detected if the recombinant protein is used in an antibody detection assay, or produced if used in immunization. Also, a suitable tag could be included in the α-chains, generating a method to purify the proteins from the supernatants [55]. Due to the use of the MultiBac system in the integrin β3 production, only minor additions in the protocol is necessary if integrin β3 protein with α-chains are to be produced [32]. The only addition is the creation of multigene transfer vectors prior to the insertion of vector into the bacmid [34].

4.4.3 Murine model mimicking FNAIT immunization

At the Immunology research group, a transgene mouse strain is currently available. This strain, hCD4.DR3-DQ2.MHCII$^{\Delta/\Delta}$ Tg [41], express human MHC class II DR3-DQ2 and human CD4, and a knockout on murine MHC. The mice express the MHC DRA/DRB3*01:01 which as mentioned is strongly associated with immunization with HPA-1a [16-19].

Human platelets contain a lot of different epitopes that could be presented by human MHC [8]. Therefore, using human HPA-1a positive platelets for immunization of these transgenic mice would most certain generate a high number of antibodies with different specificity caused by immune responses against different epitopes on the platelets. An alternative is therefore to immunize these mice with murine platelets from mice expressing a recreated HPA-1a epitope [26]. The only difference between the integrin β3 on the immunizing platelets and wild-type murine integrin β3 would be amino acid substitutions recreating the HPA-1a epitope. This will hopefully lead to less unwanted immune responses. Using these modified platelets would allow measurement of T cell activation, but also anti-HPA-1a antibody production. Immunizing these mice with murine integrin β3-APLD platelets can potentially generate immune responses in a similar manner as in the immunization of an HPA-1bb mother to an HPA-1a positive fetus. Alternatively, these mice can also be immunized with the recombinant murine integrin β3-APLD-6xHis protein produced in the current project. This protein antigen will likely also induce both HPA-1a-specific B cell and T cell responses in these human MHC transgenic mice, with minimum responses against other antigenic differences since the rest of the protein is murine integrin (self). Importantly, in this model, production of HPA-1a-specific antibodies will be dependent on HPA-1a-specific T cells only; not other T cell responses, which is likely the case when immunizing with human platelets carrying many potential T cell epitopes. The advantage of this model system is that manipulation of HPA-1a-specific T cell responses can directly affect production of anti-HPA-1a antibodies. Also, mice expressing HPA-1b epitope is yet to be generated, and the use of recombinant murine integrin β3-APPD-6xHis protein could be a good negative control when immunizing the transgenic mice. Meanwhile, the murine wild type will be a valid control.

To see if a T cell population specific for HPA-1a have been expanded (indicating an immune response), murine T cells are isolated from the spleen after immunization of the mouse. T cells are stimulated *in vitro* with HPA-1a negative APC incubated with synthetic peptides comprising the L33 residue. This peptide includes the three anchor residues shown to bind HLA-

DRB3*01:01 [21], and some surrounding residues. HPA-1a-specific T cells growing in proliferation study could be isolated and analyzed further [19]. Today, peptides are used to select HPA-1a-specific T cells, but a possibility is to use the recombinant murine integrin β3-APLD-6xHis produced in this study as a replacement. Here, the same anchor residues and HPA-1a epitope are present, so the ingestion and presentation of protein and epitope by HLA-DRB3*01:01 positive APC is likely, as shown in the TNF and proliferation study. Due to the differences in epitope recognition between T cells and B cells, recombinant integrin β3 protein could also be used to isolate B cells and generate *in vitro* antibody production by HPA-1a-specific B cells. Another application of the recombinant murine integrin β3 protein could be to measure anti-HPA-1a antibody levels produced in immunized mice. By binding the protein to beads as done in this project, antibodies from immunized mice could be measured by flow cytometry with secondary anti-mouse antibody conjugated with dye as detection.

The applications of the recombinant murine protein are major, and the desire to solve the protein production challenges are of high priority.

5 Conclusion

In this master thesis, the first aim was to design recombinant murine integrin β3 comprising the HPA-1a or HPA-1b epitope by changing four amino acids in the PSI domain. Also, a His-tag was included by inserting 6 histidines at the C-terminal. Next, the proteins were expressed using the baculovirus system, but a low insect cell transfection ratio lead to an unsuccessful virus stock generation. However, the transfection supernatants were qualitatively analyzed together with recombinant human integrin β3 proteins produced using already made virus stocks.

Analyzing the recombinant human integrin β3 supernatants by ELISA showed the presence of His-tagged proteins. The recombinant human and murine proteins were purified from the supernatants using beads coated with anti-His-tag antibodies. The presence of recombinant proteins on the beads was confirmed by SDS-PAGE and western blot, and the expression of the HPA-1a epitope was confirmed by flow cytometry using anti-HPA-1a antibodies.

Also, HPA-1a-specific T cells were incubated with HLA-DRB3*01:01 positive monocytes co-cultured with beads bound by recombinant proteins expressing HPA-1a. The T cells showed proliferation and TNF production indicating T cell activation. Due to the correct ingestion and presentation by the APC, these recombinant proteins could be further used in the study of HPA-1a-specific T cell and B cell interaction. Recombinant murine integrin β3 expressing HPA-1a epitope could possibly be used in immunization of mice expressing human HLA-DRB3*01:01 and CD4, mimicking immunization of mothers with a FNAIT affected child.

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Appendix

Appendix I. Overview of antibiotics used in the study

Table A 1: Antibiotics and reagents present in LB broth and LB agar plates used in the study. LB medium with different antibiotics were ordered from the Bacteriology and Medium production unit in the Microbiology and Infection control section at the University Hospital of North Norway. IPTG and X-gal were supplied to the plates directly before use.

Antibiotic	Concentration	Abbreviation	Supplier
Ampicillin	100 µg/ml	Amp	-
Kanamycin	50 µg/ml	Kan	-
Tetracyclin	10 µg/ml	Tet	-
Gentamycin	5 µg/ml	Gent	-
Isopropyl-β-D-thiogalactopyranoside	40 µl/plate of 100 mM stock solution	IPTG	TaKaRa (Tokyo, Japan)
5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside	17 µl/plate of 100 mg/ml stock solution	X-gal	Invitrogen by Life Technologies (Carlsbad, California)

Appendix II. Overview of primers used in the study

Table A 2: Nucleotide sequence of primers used to sequence recombinant mITGB3 gene construct inserted into pFL (Section 2.1.5). Primers were ordered from Eurogentec (Seraing, Belgium).

Primer	Sequence 5'-3'
SeqFwd	CGCGCTATGACGGCAATA
SeqRev	TATGATCCTCTAGTACTTCTCGACAAGCT
Seq_pFLRev2	AATCACTCGACGAAGACTTGATCA
Seq 1	ACTCCAAGATCTTCAGCCTGCA
Seq 2	AGTCTGTCTCCCGTAACCGTGA
Seq 3	ACTCCTCCAACGTGCTGCA
Seq 4	TAGTAACCGGTCCAGTCGGAGT
Seq 5	TAGCGTTGAAGGACAGGGACA
Seq 6	GGTCTGGATAGAGGACAGGTCGT
Seq 7	TCCAGGATCTGAGCCTCGG
Seq 8	AGTACTGCGAGTGCGACGACT

Appendix III. Overview of antibodies used in the study

Table A 3: Monoclonal (mAb) and polyclonal (pAb) antibodies used in the different analyses executed in this study.

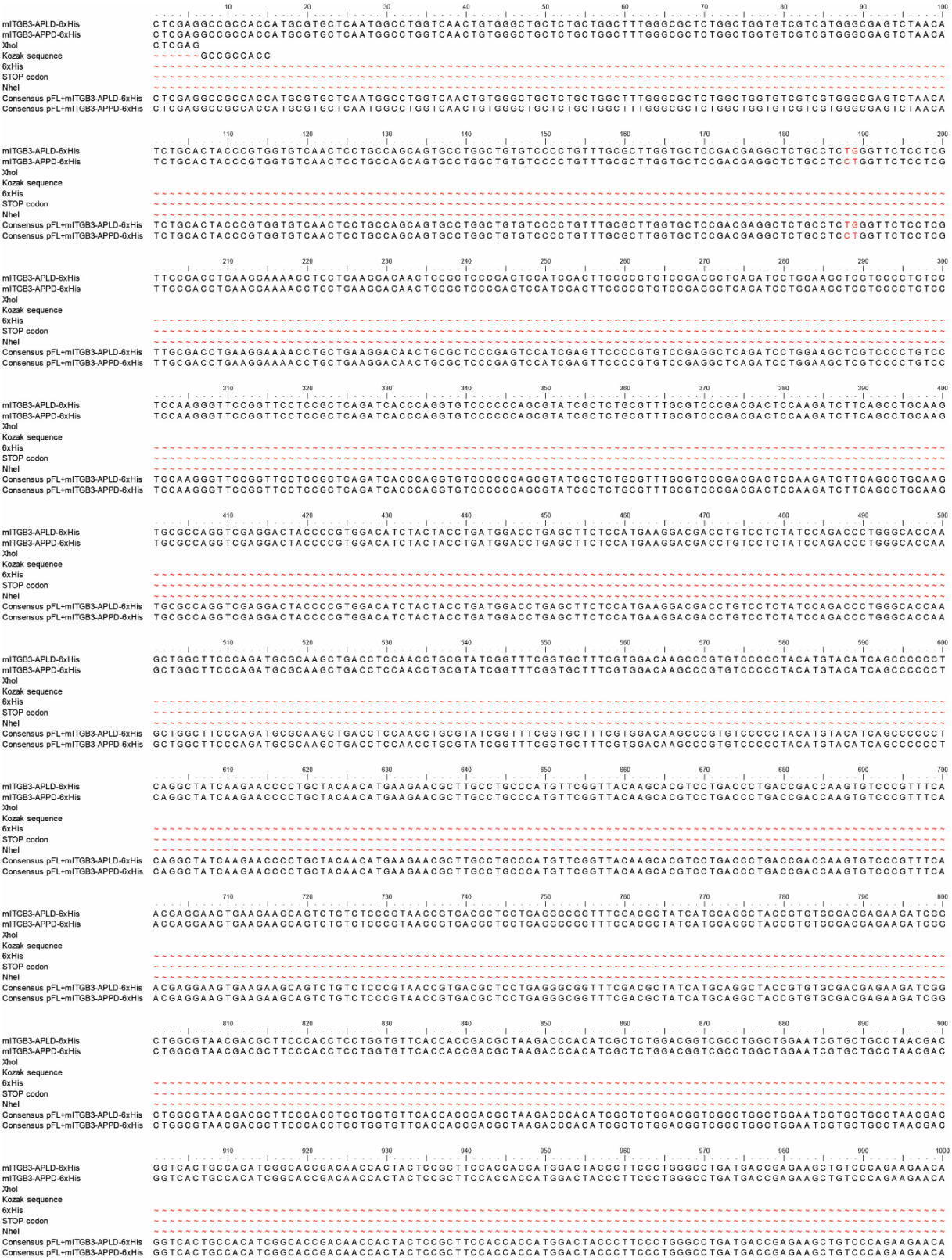
Antibody	Target	Conjugate	Host	Supplier	Usage
AP3, mAb	Human ITGB3	Alexa Fluor 488 and unconjugated	Mouse hybridoma	Hybridoma from ATCC (Manassas, Virginia)	ELISA (capture Ab) and flow cytometry
Anti-His, mAb	His-Tag	-	Mouse	Aviva Systems Biology (San Diego, California)	ELISA and Western blot (primary detection)
Anti-mouse IgG2b, pAb	Mouse IgG2b	HRP	Goat	Invitrogen by Thermo Fisher Scientific (Waltham, Massachusetts)	ELISA (secondary detection)
Anti-His, pAb	His-Tag	Biotin	Rabbit	GenScript (China)	Protein purification on beads
Goat IgG, Whole molecule control	Unspecific	-	Goat	Invitrogen by Life Technologies (Carlsbad, California)	Western blot (block)
Anti-mouse IgG, pAb	Mouse IgG	HRP	Goat	Sigma-Aldrich (St. Louis, Missouri)	Western blot (secondary detection)
Y2/51, mAb	Human ITGB3	FITC	Mouse	Dako (Santa Clara, California)	Flow cytometry
26.4, mAb	Human HPA-1a	Alexa Fluor 488 (Conjugated by Immunology research group)	Human hybridoma	Developed by Immunology research group	Flow cytometry
Sz21, mAb	Human HPA-1a in low concentrations	FITC	Mouse	Beckman Coulter (Brea, California)	Flow cytometry
Anti-human TNF, mAb	Human TNF	PE	Mouse	BD Pharmingen by BD Bioscience (San Diego, California)	TNF assay

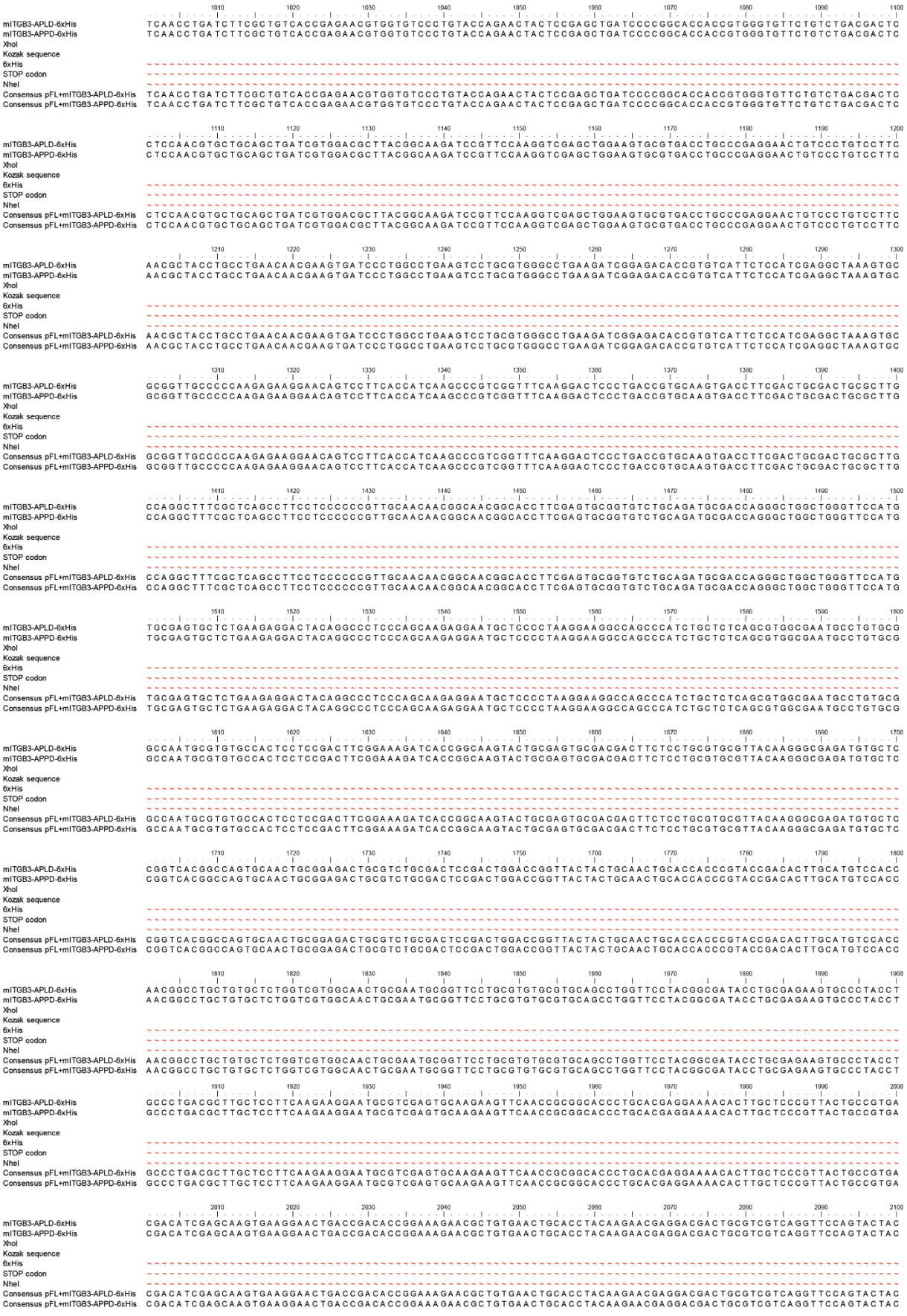
Appendix IV: Overview of synthetic peptides used in the study

Table A 4: Amino acid sequence of synthetic peptides used in stimulation of HPA-1a-specific T cells (Section 2.6.4). Peptides were supplied by Eurogentec, (Seraing, Belgium).

Peptide	Antigen	Amino acids	Peptide length
L33 (integrin β3 19-38)	HPA-1a	VSPMCAWCSDEALPLGSPRC	20-mer
P33 (integrin β3 19-38)	HPA-1b	VSPMCAWCSDEALPPGSPRC	20-mer
Lol P1 (191-210)	Ryegrass pollen	ESWGAVWRIDTPDKLTGPFT	20-mer

Appendix V: Alignment of consensus sequences from sequencing of pFL with insert




mITGB3-APLD-6xHis
mITGB3-APPD-6xHis
XhoI
Kozak sequence
6xHis
STOP codon
NheI
Consensus pFL+mITGB3-APLD-6xHis
Consensus pFL+mITGB3-APPD-6xHis

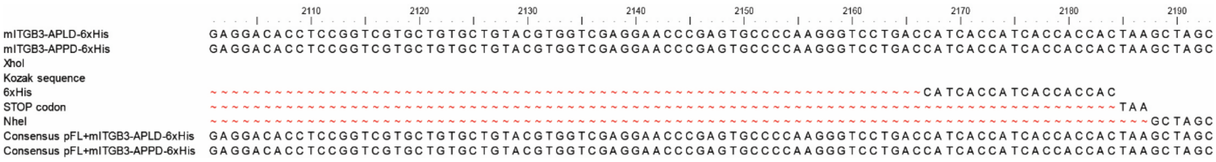


Figure A 1: Consensus sequence alignment of sequenced mITGB3-APLD-6xHis and mITGB3-APPD-6xHis inserted into pFL vector (Section 3.1.3).