Medizinische Hochschule Hannover

(Hannover Medical School)

Institute for Transfusion Medicine

THE MOLECULAR BASIS OF MICROPOLYMORPHISM AT RESIDUE 156 AND ITS FUNCTIONAL ROLE IN PEPTIDE SELECTION BY HLA-B*35 ALLOTYPES

Doctoral thesis

In partial fulfilment of the requirements for the degree of

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Submitted by

Ms. Trishna Manandhar

from Kathmandu, Nepal

Hannover, 2014

Medizinische Hochschule Hannover

Institut für Transfusionsmedizin

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INAUGURAL DISSERTATION

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Ms. Trishna Manandhar

aus Kathmandu, Nepal

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Dedicated to my mother

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Dissertation (The molecular basis of micropolymorphism at residue 156 and its functional role in peptide selection by HLA-B*35 allotypes) selbstständig verfasst habe.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Ich habe die Dissertation an folgenden Institutionen angefertigt: Institut für Transfusionsmedizin, Medizinische Hochschule Hannover.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
µg	Microgram
µl	Microlitre
µM	Micromolar
AA	Amino acid
ABC	ATP-binding cassette
ADP	Adenosine di-phosphate
Ala (A)	Alanine
APCs	Antigen presenting cells
ATP	Adenosine tri-phosphate
BCR	B cell receptor
CDR	Complementarity determining regions
CID	Collision-induced dissociation
CNX	Calnexin
Conc.	Concentration
CRT	Calreticulin
DAS-ELISA	Double antibody sandwich ELISA
DC	Dendritic cells
ddH2O	Double-distilled water
ddNTP	2', 3'-Dideoxynucleotide triphosphates
DMSO	Dimethyl sulfoxide
DNA	Deoxyibonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EBV	Epstain barr virus
ELISA	Enzyme linked immune sorbent assay
ER	Endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
et al.	*et alii* (Latin) and other

g	grams
GvHD	Graft versus host disease
hc	Heavy chain
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
HLA-B*35/156	HLA-B*35 alleles that differ at a single residue at position 156
HPLC	High performance liquid chromatography
HPLC-H_2O	HPLC grade water
HRP	Horse-radish peroxidase
HSCT	Hematopoietic stem cell transplantation
HSP	Heat shock protein
HSPA	Heat shock protein 1A
IFN-γ	Interferon- γ
IL	Interleukin
IMGT	International immunogenetics
kb	Kilo basepair
kDa	Kilo Dalton
LC-ESI-MS/MS	Liquid chromatography-Electrospray ionization-Tandem MS
LCL	Lymphoblastoid cell line
Leu (L)	Leucine
LMP	Large multifunctional protease / Low molecular mass polypeptide
LTA	Lymphotoxin alpha
LTA	Lymphotoxins A
LTB	Lymphotoxin beta
LTB	Lymphotoxins B
Lys (K)	Lysine
M	Molar
mab	Monoclonal antibodies
MALDI-TOF	Matrix assisted laser desorption- Time of flight
MFI	Median florescence intensity
MWCO	Molecular weight cut off

Met (M)	Methionine
mg	Miligram
MHC	Major histocompatibility complex
MICA	Major histocompatibility complex class I chain genes A
MICB	Major histocompatibility complex class I chain genes B
ml	Mililitre
mM	Milimolar
MS	Mass spectrometry
NBD	Nucleotide binding domain
nMFI	normalized Median florescence intensity
NK	Natural killer
ON	Overnight
ON	Overnight
pab	Polyclonal antobodies
PAGE	Polyacrylamide gel electrophoresis
PBR	Peptide binding region
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PDB	Protein data bank
PDI	Protein disulphide isomerase
PDIA3	Protein disulphide isomerase A3
Phe (F)	Phenylalanine
pHLA	Peptide HLA complex
Pi	Inorganic phosphate
PLC	Peptide loading complex
pmol	Picomol
Pro (P)	Proline
PSMB8	Proteasome β 8
PSMB9	Proteasome β 9
RCSB	Research collaboratory for structural bioinformatics
RNA	Ribonucleic Acid

SDS	Sodium dodecyl sulfate
TAP	Transporter associated with antigen processing
TAPBP	TAP binding protein (tapasin)
TBS	Tris-buffered saline
TCR	T cell receptor
TMD	Transmembrane Domain
TMD	Transmembrane domain
TNF	Tumor necrosis factor
TPN	Tapasin
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
UD	Unrelated donor
V	Volts
Val (V)	Valine
β_2m	Beta-2 microglobulin

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ABSTRACT

HLA class I molecules are characterized by an extensive polymorphism, making every allele unique in their immunological function to present antigenic peptides to immune effector cells. Genetic diversity of HLA class I molecules offers on one hand protection against pathogens by increasing the repertoire of presented antigenic peptides, since every allele has a distinct peptide pattern. Therefore, this characteristic of HLA class I molecules can be utilized in peptide vaccination strategies or cellular anti-viral therapies. On the other hand, the polymorphic diversity of HLA class I molecules represent the main barrier to hematopoietic stem cell transplantation. Selection of permissive donor:recipient pairs for stem cell transplantation involves meticulous matching of HLA types.

HLA class I polymorphism impacts on the diversity of antigenic peptides and furthermore on the interaction with the antigen presenting pathway of that Tapasin is a dedicated component. Tapasin facilitates peptide loading with high affinity peptides for the presentation of peptide-HLA complexes with a long half-life time. Tapasin-independent peptide loading can result in the selection of an alternate peptide repertoire, consequently those peptide-HLA complexes are characterized by short half-life times, thus circumventing T cell tolerance.

Certain polymorphisms represent non-permissive transplantation scenarios and are associated with acute graft versus host disease. One of the most incompatible mismatches is located on the α2 helix of HLA class I alleles, position 156. The reason for that is that this position directly influences peptide loading, peptide features and thus impacts on the whole peptide-HLA landscape presented to immune effector cells. This could be described previously for the HLA-B*44 and the A*24 group.

Single mismatches at residue 156 also occur within subtypes of the HLA-B*35 group. The aim of this PhD work was to investigate the influences of micropolymorphism at position 156 on the function of three HLA-B*35 variants, HLA-B*35:01^{156Leu}, B*35:08^{156Arg} and B*35:62^{156Trp}.

As a tool to analyze the functional differences distinguishing HLA-B*35/156 variants, their peptide repertoire and its origin was systematically examined. To investigate the mode of peptide recruitment, LCL.TPN- (LCL 721.220, HLA-/TPN-/TAP+), LCL.TPN+ (LCL 721.221, HLA-/TPN+/TAP+) or T2.TAP- (HLA-/TPN+/TAP-) cells were lentivirally transduced with constructs encoding for full length (Exon 1–7) HLA-B*35/156 molecules. All three subtypes showed surface expression even in Tapasin negative cells, thus suggesting a Tapasin-independent mode of peptide acquisition. However, HLA-B*35:08 was found to be relatively dependent on Tapasin compared to HLA-B*35:01 and B*35:62. Moreover, the experimental results revealed that the surface expression of HLA-B*35:62 was comparatively independent of the crucial protein transporter associated with antigen processing (TAP). Immunoprecipitation experiments demonstrated a strong association of HLA-B*35:01 and B*35:08 with TAP in the presence of Tapasin while no such association was observed for HLA-B*35:62. This differential peptide loading complex-independent peptide acquisition of the HLA-B*35/156 subtypes implies an unconventional pathway for peptide selection.

In order to investigate the peptide repertoires of those allelic variants on a high throughput level, lentiviral vectors encoding for soluble HLA-B*35/156 molecules (Exon 1–4) were transduced in LCL.TPN- or LCL.TPN+ cells. Recombinant cells were expanded in bioreactors for large scale sHLA production and the molecules subsequently affinity purified. Trimeric molecules of

differential half-life times were recovered and their respective peptides subdivided into low as well as high affinity peptides. The sequences of peptides were analyzed utilizing mass spectrometry.

Peptides from all three allelic variants acquired in the presence or absence of TPN showed a striking difference in repertoire, origin and features. With the chaperoning of Tapasin HLA-B*35:01 restricted peptides are exclusively anchored with Pro at their p2 position, while B*35:08 restricted peptides are preferentially anchored with Pro or Ala at p2. HLA-B*35:62 restricted peptides however exhibt a high promiscuity in the anchor amino acids at p2. Yet, without the chaperoning of TPN, HLA-B*35:62 restricted peptides are more strictly anchored at p2 (Ala) and pΩ (Trp). The chaperoning or absence of TPN does not change the C-terminally anchor preferences of HLA-B*35:01 and B*35:08 (Tyr, Phe, Leu or Lys). All three HLA-B*35/156 variants were shown to present peptides of non-canonical lengths (>10 amino acids).

These results revealed that a single mismatch at residue 156 impacts on the molecules structure in a way, that the functionality of these allelic variants is notably modified. The systematic characterization of the functional impact of single polymorphism at key positions will be a step further towards intelligent mismatching and histocompatibility prediction.

Furthermore, the observation that HLA-B*35/156 variants were found to present peptides independent of the peptide loading complex indicates a specialized role of these alleles in the presentation of viral epitopes. The knowledge of peptide anchor motifs allows for the prediction of viral peptide epitopes and will be an approach in cellular therapeutics based on viral-specific immune cells.

ZUSAMMENFASSUNG

HLA Klasse I Moleküle weisen einen extrem hohen Polymorphismus auf, diese Eigenschaft macht jede allelische Variante einzigartig in ihrer immunologischen Funktion, die darin besteht, dem Immunsystem antigene Peptide zu präsentieren. Die genetische Diversität von HLA Klasse I Molekülen bietet einerseits einen umfangreichen Schutz vor Pathogenen durch die Erweiterung des Peptiderepertoires, da jede allelische HLA Variante ein individuelles Peptidmuster binden und präsentieren kann. Diese Eigenschaft von HLA Molekülen macht man sich unter Anderem für Peptid-Vakzinierungen oder zelluläre anti-virale Immuntherapien zunutze. Andererseits stellt die ausgeprägte genetische Diversität von HLA die Hauptbarriere der haematopoetischen Stammzell Transplantation dar. Eine sorgfältige Auswahl geeigneter HLA-kompatibler Spender-Empfänger Paare ist somit unumgänglich.

HLA Klasse I Polymorphismus beeinflusst die Diversität der antigenen Peptide sowie die Wechselwirkung zwischen HLA Molekülen und spezifischen Komponenten des Peptidladunskomplexes, insbesondere Tapasin. Tapasin ist ein Molekül, welches die Beladung von HLA Klasse I Molekülen mit hoch-affinen Peptiden unterstützt, wodurch Peptid-HLA Komplexe mit langer Halbwertzeit entstehen. Tapasin-unabhängige Peptid Beladung resultiert dahingegen in der Selektion eines wechselnden Peptidrepertoires, dadurch entstehen Peptid-HLA Komplexe mit geringer Halbwertzeit, die nicht der Immuntoleranz unterliegen.

Einge HLA Klasse I Polymorphismen repräsentieren nicht-kompatible Transplantations Szenarien, welche mit akuter Graft-versus-Host Erkrankung assoziiert sind. Eine der Positionen im HLA Molekül, welche mit Transplant-Inkompatibilität in Verbindung gebracht wird, ist Aminosäure 156, welche auf der α2 Helix lokalisiert ist. Diese Aminosäure beeinflusst durch ihre Position im HLA Molekül direkt die Peptidbeladung, die Eigenschaften der gebundenen Peptide und hat somit eine direkte Auswirkung auf die dem Immunsystem zugängliche Peptid-HLA Struktur. Dieses konnte zuvor für die HLA-B*44 und die HLA-A*24 Gruppe beschrieben werden.

Einzelne Mismatche an Aminosäure Position 156 sind auch in der HLA-B*35 Gruppe zu finden. Der Fokus dieser Doktorarbeit bestand in der funktionellen Analyse des Einflusses der Aminosäure Position 156 am Beispiel der HLA-B*35 Gruppe. Hier wurden drei natürlich vorkommende allelische Varianten untersucht, welche sich ausschließlich an der Position 156 unterscheiden, HLA-B*35:01^{156Leu}, B*35:08^{156Arg} und B*35:62^{156Trp}.

Um die funktionellen Unterschiede zwischen den HLA-B*35/156 Varianten zu analysieren, wurde das jeweilige Peptidrepertoire sowie dessen Herkunft systematisch untersucht. Um die Art der Peptidrekrutierung zu bestimmen, wurden LCL.TPN- (LCL 721.220, HLA-/TPN-/TAP+), LCL.TPN+ (LCL 721.221, HLA-/TPN+/TAP+) sowie T2.TAP- (HLA-/TPN+/TAP-) Zellen lentiviral mit Konstrukten, welche für die jeweiligen HLA-B*35 Varianten kodieren, transduziert. Die drei HLA-B*35 Varianten wiesen sowohl in Tapasin negativen Zellen Oberflächenexpression auf, was ein Hinweis dafür ist, dass diese B*35 Varianten Tapasin-unabhängig Peptide akquirieren können. Dennoch konnte für HLA-B*35:08 eine schwache Tapasin-Abhängigkeit ermittelt werden, welche im Gegensatz zur vollständigen Tapasin-Unabhängigkeit von HLA-B*35:01 und B*35:62 steht. Darüber hinaus konnte gezeigt werden, dass B*35:62 zudem relativ unabhängig vom *transporter associated with antigen processing* (TAP)-Protein Peptide rekrutieren und diese an der Zelloberfläche präsentieren kann. In

Immunpräzipitations Versuchen konnte in Gegenwart von Tapasin eine starke Assoziation von HLA-B*35:01 und B*35:08 mit TAP festgestellt werden, während eine solche Assoziation nicht für das Molekül HLA-B*35:62 gezeigt werden konnte. Diese Unterschiede in der Abhängigkeit vom Peptidladungskomplex der HLA-B*35/156 Varianten implizieren einen unbekannten Peptidladungsweg.

Um die Peptidrepertoires mittels *high thoughput* Analyse untersuchen zu können, wurden LCL.TPN- als auch LCL.TPN+ Zellen lentiviral mit Konstrukten transduziert, welche für trunkiertes (Exon 1–4) HLA kodieren. Die rekombinanten Zellen wurden zur *large scale* sHLA Produktion in Bioreaktoren expandiert und die rekombinanten sHLA Moleküle affinitätschromatographisch aufgereinigt. Aus der Präparation trimerer sHLA-Moleküle mit unterschiedlicher Halbwertszeit konnten schwach-bindende sowie stark-bindende Peptide isoliert und deren Sequenzen mittels massenspektrometischer Analyse bestimmt werden.

Die Peptide, welche aus den untersuchten HLA-B*35 Molekülen isoliert wurden, unterscheiden sich hinsichtlich ihrer Herkunft und ihrer biochemischen Charakteristika. Peptide, welche in Anwesenheit des Chaperons Tapasin an HLA-B-35:01 gebunden wurden, weisen an der p2 Ankerposition die Aminosäure Prolin auf, während B*35:08 gebundene Peptide zusätzlich zu Prolin durch Alanin an p2 verankert werden. Im Gegensatz dazu konnte eine starke Diversität für p2 B*35:62 gebundener Peptide gefunden werden. Wenn Tapasin nicht an der Peptidrekrutierung beteiligt war, wurden vorwiegend Peptide mit einem p2 Anker Alanin und einem pΩ Anker Tryptophan gebunden. Die An- oder Abwesenheit von Tapasin hatte dahingegen keinen Einfluss auf die pΩ Ankerposition der Peptide von B*35:01 oder B*35:08, Tyrosin, Phenylanalin, Leucin oder Lysin. Sämtliche Peptide wiesen eine Länge von >10 Aminosäuren auf.

Diese Ergebnisse zeigen, welche funktionellen Unterschiede durch den Austausch einer einzelnen Aminosäure im HLA Molekül entstehen können. Die systematische Untersuchung von Einzel-Polymorphismen ausgesuchter HLA Varianten ist ein Schritt in Richtung intelligentes Mismatching und Gewebeverträglichkeits-Vorhersage.

Die Beobachtung, dass HLA-B*35/156 Varianten Peptide unabhängig vom Peptidladungskomplex binden können, lässt die Schlussfolgerung zu, dass diese Allele eine Rolle in der viralen Immunität einnehmen. Die Kenntnis der Peptidmotive dieser HLA Varianten ermöglicht nun die Vorhersage viraler Epitope und somit basierend auf Virus-spezifischen Immunzellen den Einsatz zellulärer Immuntherapien.

1 INTRODUCTION

1.1 Overview of the immune system

The immune system is an interactive network of specialized components and processes that provides the host with the ability to defend against infection [1]. Agents that could potentially cause infection include entities such as bacteria, parasites, fungi, viruses and possibly even molecules (prions). The ability of the immune system to recognise self from non-self/foreign makes it more dynamic in terms of specific recognition and elimination of invasive agents. This property of the immune system, to recognise self antigens and prevent them from immune destruction, is termed as self tolerance [2]. In vertebrates, the immune system consists of lymphoid organs (bone marrow, thymus, spleen, lymph nodes), cells (myeloid cells, lymphocytes), antibodies, cytokines and complements. The interplay between these immune system components is required to achieve basic functions such as immune recognition, effector cell response, immune regulation or immunological memory.

The immune system can be discriminated in the innate (natural) immune system and the adaptive (acquired) immune system [3]. The innate immune system is the first line of host defence against infection and provides non-specific but immediate response against infectious agents. It comprises of anatomical barriers (skin, epithelial layers, mucous membranes and epithelial cilia) and various antibacterial substances in biological secretions (lysozyme, lactoferrin) that prevent the entry of agents into the host body [4]. Cuts, abrasions, wounds and any sort of breaching of these barriers would provide easy access for pathogens to get through the body. When the mechanical barriers are broken, the cellular and molecular components of the innate immunity come into action to serve the function of non-specific and rapid elimination of the pathogens by the processes of opsonisation and phagocytosis [5]. The cellular components of the innate immune system involve phagocytes (neutrophils, monocytes/macrophages and dendritic cells), natural killer cells (NK cells), basophils, mast cells and eosinophils. Several humoral factors that contribute to innate immunity include complement proteins, defensins, ficolins, cytokines and chemokines. Activation of the complement cascade system is an important function of innate immunity that augments opsonization and lysis of pathogens [6].

The adaptive immune system is as a second line of defence against infection. Unlike the innate immunity, the adaptive immunity provides an acquired and more specific immune response. The

adaptive response takes a long time but builds immunological memory that provides efficient and quick response to future exposure against specific antigen. The adaptive immune response is primarily based on the clonal proliferation of T or B lymphocytes that possess antigen specific receptors on their surface. T and B cells develop from the common lymphoid progenitor cells in the bone marrow. B cells remain in the bone marrow until they mature while T cells migrate to the thymus where they develop into different T cell subsets (CD4+ or CD8+ T cells). The adaptive immunity can be classified into two classes: cell mediated adaptive immunity and humoral adaptive immunity. Humoral immunity is mediated via antibodies produced by B lymphocytes in response to free extracellular antigens. The extracellular antigens are recognised by B cell antigen receptors (BCRs) located on the surface of B cells that trigger the transformation and proliferation of naïve B cells into antibody producing plasma cells. Antibodies mediate the inactivation of infectious agents by binding to specific epitopes that activated their production. Cellular adaptive immunity is mediated by T lymphocytes that recognise antigens presented either by major histocompatibility complex (MHC) class I or class II molecules on the surface of antigen-presenting cells (APCs). Antigens presented by MHC molecules on APCs are recognised by antigen-specific T cell receptors (TCRs) on T cells that leads to diverse signalling immune pathways [7]. Antigens presented by the MHC class I molecules trigger CD8+ T cell activation that leads to elimination of infected or cancer cells. Furthermore, antigens presented by MHC class II molecules are recognised by CD4+ T cells (helper T cells) and initiate T cell-dependent immunoglobulin production by B lymphocytes.

1.2 Introduction to Human Leukocyte Antigen (HLA)

1.2.1 Discovery of HLA

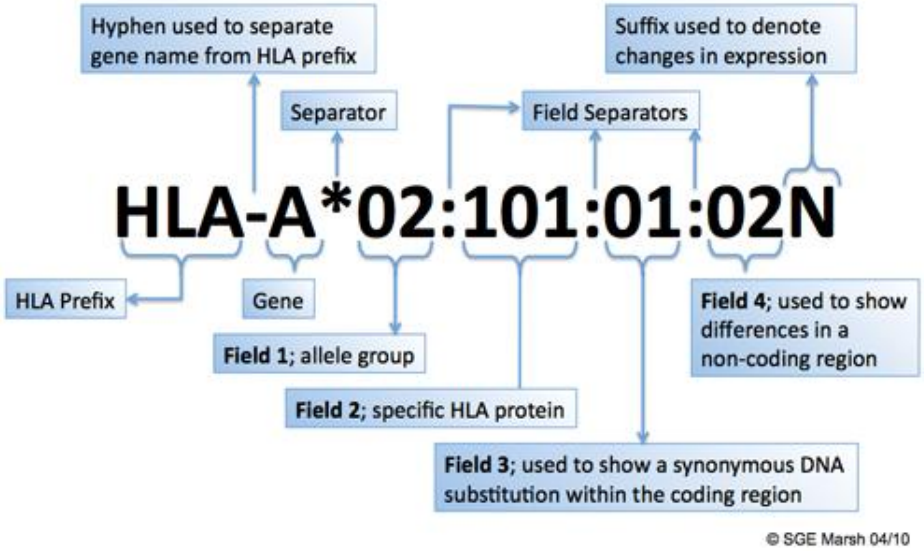
The discovery of MHC originated in investigations of murine allogenic tissue transplantation in 1940s [8]. In 1948, Gorer and Snell [9] described the role of certain group of genes, located on the H-2 locus, in mediating graft rejection in mice. This murine transplantation antigen (H-2) was called major histocompatibility complex (MHC) antigen. Similar serological scheme was attempted to characterize the histocompatibility antigens in human sera from patients who had received multiple blood transfusions [8]. The majority of these histocompatibility antigens were not detected in erythrocytes but were present ubiquitously in leukocytes. The human version of

MHC was termed as human leukocyte antigen (HLA). In 1958, a first HLA was described by Dausset [10] as ‘MAC’ (named after the initials of the names of important volunteers for his study) and later renamed as HLA-A2.

1.2.2 Nomenclature of HLA

The label of each HLA variant is unique, represented by an identification name that starts with the gene name followed by the pairs of digits separated by colons (Figure 1.1) [11]. Each pair of digits is referred to as a ‘field’. The first field (digits before the first colon) represents the serological type of the HLA allele. The second field indicates the allele subtypes, the numbers of which are assigned in a chronological order of determination of DNA sequences. The third field represents silent nucleotide substitutions within the coding region and the fourth field refers the differences in non-coding regions. In addition to these four fields, suffixes 'N', L', 'S', 'C', 'A' or 'Q' are optionally used to describe the expression status of HLA.

Figure 1.1 HLA Nomenclature

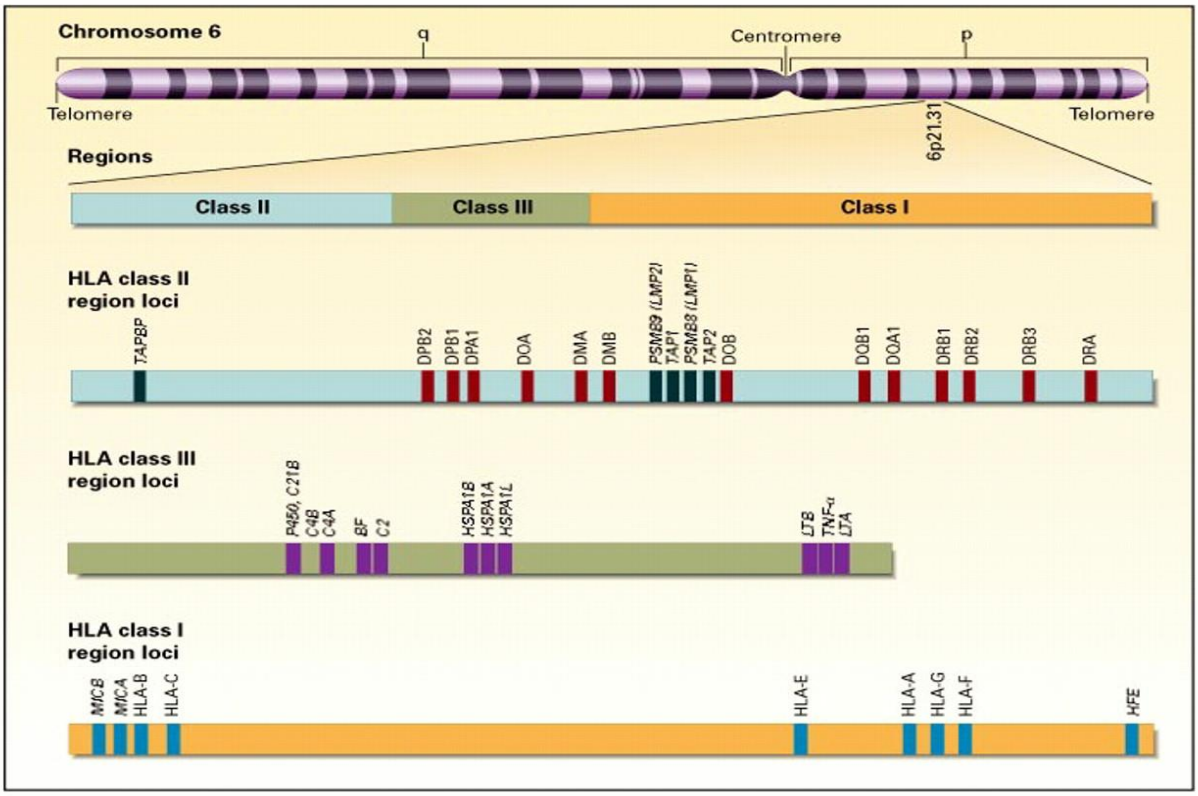


Annotation of additional suffix: 'N' - no expression or Null' alleles; 'L' - low cell surface expression; 'S '- expression as a soluble/secreted molecule; 'C '- expression in cytoplasm; 'A' - Aberrant expression;' Q' - questionable expression [11].

1.2.3 Genomic organization of HLA

The HLA complex is located on the short arm of chromosome 6 (6p21.3 region) (Figure 1.2) and spans over 4000 kilo bases (kb). It encodes more than 220 genes of diverse function, 40 of them belong to leukocyte antigens or other functions of the immune system [12]. The genomic locus of the HLA complex is divided into three major sub-regions: the class I region encoding HLA class I molecules, the class II region encoding HLA class II molecules and the class III region encoding genes that are not related to structure and function of HLA class I and class II genes. HLA class I region covers around 1800 kb and is located near the telomeric end of chromosome 6. It comprises of around 20 HLA class I genes, among which HLA-A, -B, and -C are known as classical class Ia genes, and HLA-E, -F, and -G are termed as the non-classical HLA-Ib genes. The class II sub-region spans 800 kb and is located near the centromere. This sub-region encodes major HLA class II genes: HLA-DRA, HLA-DRB, HLA-DQA, HLA-DQB, HLA-DPA, HLA-DPB, HLA-DNA, HLA-DMA and HLA-DOB. In addition to major HLA class II genes, this region also encodes several other genes involved in antigen presentation such as LMP1, LMP2, TAP1, TAP2 and TPN [13]. The class III region is a highly gene dense region, located between the HLA-B and HLA-DRB region and spans over 1100 kb of DNA. The class III region includes genes related to immune function such as complement factor genes C2, C4 and Bf, the cytokine genes TNF (Tumor Necrosis Factor), LTA (Lymphotoxin A) and LTB (Lymphotoxin B) [13].

Figure 1.2 Arrangement of the HLA complex on chromosome 6



Adapted from Klein and Sato (2000) [14]. Annotation of genes: BF - Complement factor B; C2, C4A and C4B - Complement component 2, 4A and 4B; C21B - Cytochrome P-450, subfamily XXI; HFE - Hemochromatosis; HSP – Heat shock protein; LMP - Large Multifunctional Protease; LTA and LTB - Lymphotoxins A and B; MICA and MICB - Major histocompatibility complex class I chain genes A and B; P450-Cytochrome P-450; PSMB8 and 9 - Proteasome β 8 and 9; TAP1 and TAP2 - Transporter associated with antigen processing 1 and 2; TAPBP - TAP-binding protein (tapasin); TNF-α - Tumor necrosis factor α; HSPA1A, HSPA1B, and HSPA1L - Heat shock protein.

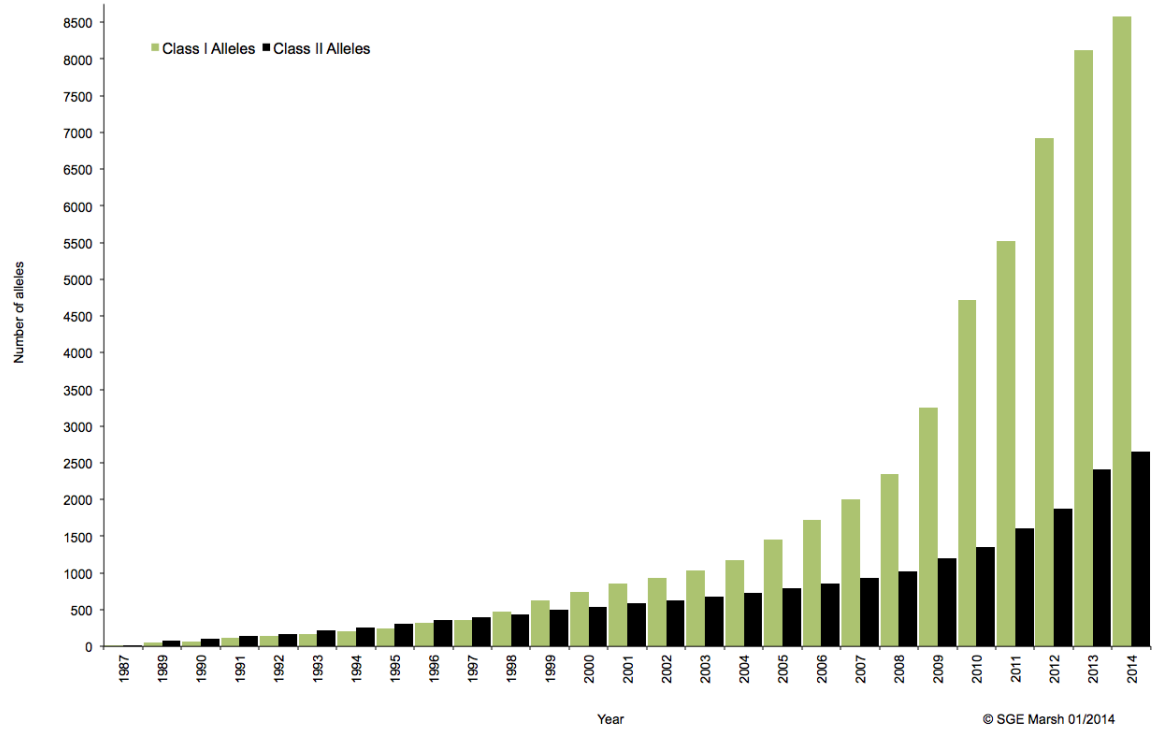
1.2.4 HLA polymorphism

The genes encoding for HLA molecules are the most polymorphic genes within the human genome [15]. Most polymorphisms in HLA class I allelic variants are located in the α1 and/or α2 domains, the peptide binding region (PBR) [16]. Depending on the position and nature of the polymorphism, those AA exchanges can cause alterations in structural conformation of the PBR, hence resulting in an alteration of specificities and features of bound peptides [17]. These AA exchanges within the PBR result in differential allorecognition by T cell receptors (TCR) and can trigger immunological episodes when mismatched in a transplantation setting.

Since the discovery of the first HLA by Dausset in 1958, tremendous advancement has been achieved in the field of HLA research. The discovery of new alleles by high resolution

sequencing provides updated information on the diversity of HLA molecules. Currently 10,533 HLA and related alleles are recorded in the IMGT/HLA database, out of which 8,124 alleles belong to class I and 2,409 to class II (Figure 1.3) [18]. The excessive polymorphism within HLA molecules generates a huge variance in epitope specificity. The frequency of HLA polymorphisms in terms of evolution is maintained in distinct populations as a consequence of natural selection following pathogen invasion [19-21]. In the process of host pathogen co-evolution, pathogens constantly evolve to mask the host HLA presentation pathway and on the other way around, HLA molecules consecutively evolve to adapt the pathogen immune evasion strategies [22-24].

Figure 1.3 HLA alleles included in the IMGT/HLA Database from 1987 to 2014



Green bars represent class I alleles and black bars represent class II alleles [25].

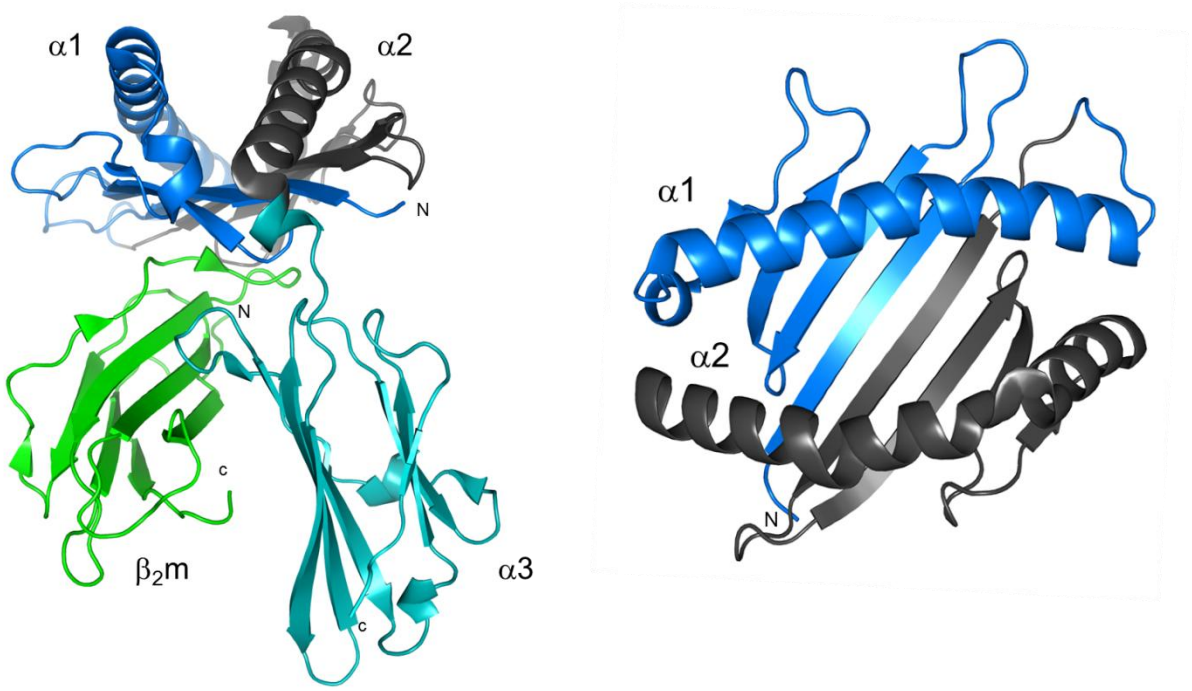
1.3 Structure of HLA class I molecules

The HLA class I molecule is a heterodimer (Figure 1.4) comprising of two chains, the α or heavy chain (hc), encoded by the HLA class I gene on chromosome 6 and beta-2 microglobulin (β_2m), encoded by the β_2m gene located on chromosome 15. The hc of class I molecules consists of three extracellular domains (α1, α2 and α3), a transmembrane region, and a cytoplasmic domain [26]. The α1 and α2 domains of the HLA class I molecule, encoded by exon 2 and 3 respectively [27], are highly polymorphic and fold to make up a scaffold domain, the PBR.

Structural analysis shows that the PBR is composed of two side walls of α helices supported on the platform of eight β-pleated sheets (Figure 1.4B) [28-29]. Structural investigation of peptide HLA (pHLA) complexes by high resolution X-ray crystallography facilitated the classification of the PBR into six "specificity pockets" A–F [30]. An updated pocket definition based on more crystallographic structures available at that time was described by Chelvanayagam in 1996 [31]. In 2007, DeLuca *et al.* [32] described HLA molecules as mosaiques based on the classical pocket definition and developed the "modular concept" (http://www.peptidecheck.de) for the prediction of peptide binding motifs. The term motif for a HLA allele describes the pattern of AA residues occurring at anchor positions of a peptide of defined length, which is recognized by a particular HLA molecule [33]. This anchor motif data characterize HLA-peptide specificity in terms of dominant anchor positions with strong preferences for certain AA residues. The residues which contribute significantly to the peptide binding are called primary anchor residues and the positions they occur are called primary anchor positions [34-35]. In 2012, Huyton *et al.* [36] described a broader "dynamic pocket" definition based on more recent crystallographic data (>100 pHLA structures) to increase the precision of peptide prediction. These studies highlight the importance of residues within the HLA molecule that are in contact with distinct positions of the bound peptide. The conformation of a certain pocket can be influenced by an AA exchange between two mismatched alleles. The impact of each polymorphism varies from alteration of the peptide binding motif or the peptide length and might also influence the interaction with neighbouring AAs within the PBR.

Figure 1.4 Schematic representation of an HLA class I molecule

A B



A) Extracellular domains of HLA class I molecules. Domain α1 (blue) and α2 (grey) form the PBR; domain α3 is given in cyan while β_2m is given in green. B) PBR formed by two α helices and eight β sheets. PyMOL computer simulation of HLA class I molecule based on X-ray crystallography (PDB 2AXF) [37]. N and C denotes N- and C-terminus of polypeptides respectively.

1.4 Roles of HLA class I molecules

1.4.1 Presentation of self and non-self peptides

The vertebrate immune system has the dynamic potential to discriminate between peptides derived from their endogenous source (self) and those originated from foreign sources (non-self). The ability of the adaptive immune system to perform self/non-self discrimination leads to specific recognition and elimination of invasive agents, while preventing the destruction of cells presenting self peptides. The property of the immune system to recognise self antigens and prevent them from immune lysis is termed as self tolerance [38].

Tolerance to self peptides is achieved by central thymic selection via central tolerance [39] and/or by regulatory T cells mediated peripheral tolerance [40]. Immunological tolerance both in

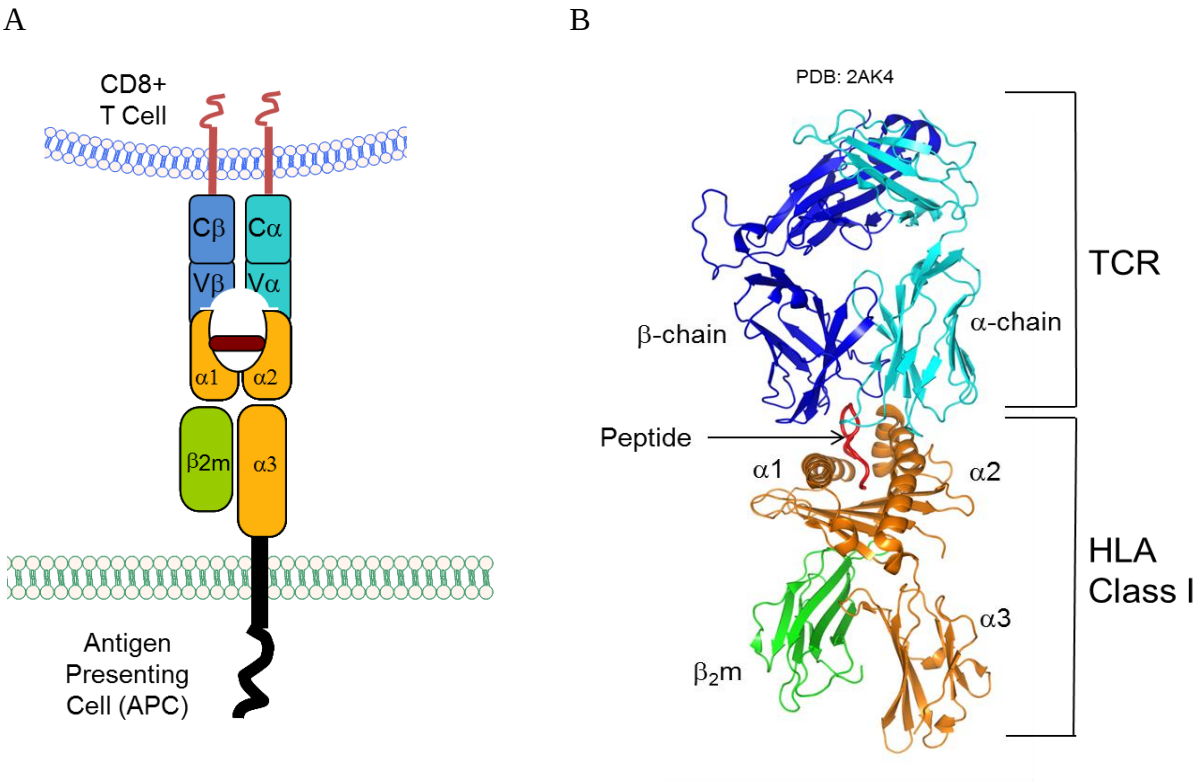
the thymus and the peripheral immune system is conferred by the priming and half life time of T cells selected against distinct pHLA molecules. Thymic assortment of self T cell repertoire via negative and positive selection in the course of T cell development and receptor rearrangements results in the adaptive discrimination between self and non-self. The immune balance of positive and negative selection of thymocytes is essential to create the mature T cell repertoire that renders specificity for self and non-self antigens.

The specific role of HLA molecules is to scan the intracellular proteome and present peptides of foreign origin (immunogenic, non-self) on the cell surface, that can be recognised by cytotoxic CD8+ T cells [41]. Here it has to be considered that every single HLA molecule with a given peptide constitute a unique surface that can be recognised by a unique T cell receptor expressed on CD8+ T cells.

1.4.2 T cell recognition

pHLA complexes are recognized by T cell receptors (TCRs) located on the surface of CD8+ T cells [42]. The TCR is a heterodimer that consists of two polypeptide chains, α-(TCRα) and β-chain (TCRβ) [43]. The extracellular region of the TCRα and TCRβ consists of a variable (V) region and and a constant (C) region connected by a disulfide bond (Figure 1.5) [43-44].

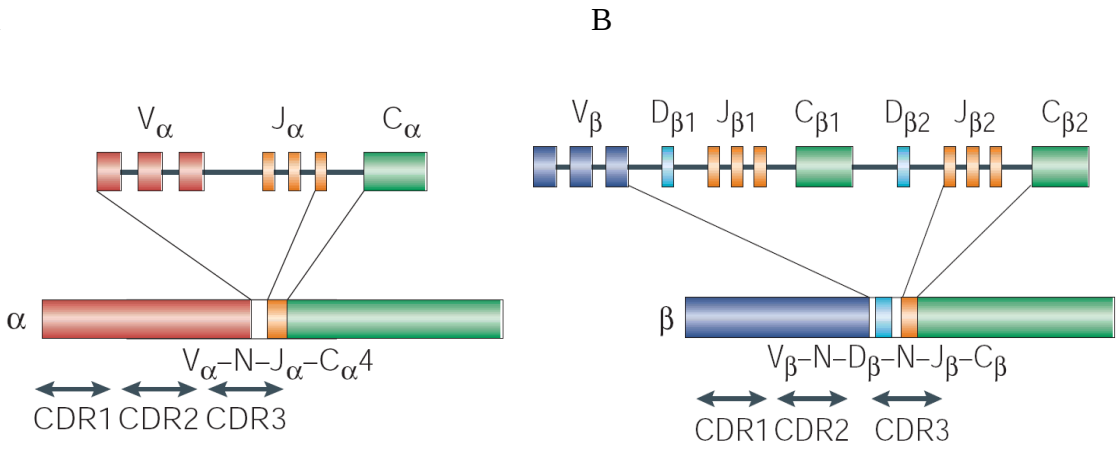
Figure 1.5 Schematic representation of T cell recognition



A) Peptide (in red) presentation by HLA class I molecule (extracellular domains represented in yellow) and recognition by TCR (extracellular domains represented in blue and cyan). B) PyMOL computer simulation of TCR/pHLA complex (SB27 TCR in complex with HLA-B*35:08-13-mer EBV BZLF1 LPEPLPQGQLTAY peptide) based on X-ray crystallography (PDB 2AK4) [45].

The variable region of each chain is assembled of complementarity determining regions (CDRs) that contact pHLA complexes. The immense diversity of TCRs is generated during thymocyte development via somatic rearrangement of variable (V), diversity (D), and joining (J) gene segments in the CDR3 of TCR (Figure 1.6) [44]. The diversity in TCRs enables them to recognize the vast amounts of peptides presented by HLA molecules. Since a single TCR might recognize more than $1x10^6$ pHLA ligands [46], the functional relevance for the immune defence to recruit a sufficient number of T cells becomes clear. The TCR diversity contributes to the immune defence against pathogens in two ways, first it provides the pool of specific T cells against immunogenic epitopes and secondly it provides flexible TCR repertoires [46].

Figure 1.6 Structural diversity of TCR



Adapted from Nikolich-Zugich *et al.* (2004) [46]. T cell receptor diversity in TCR αβ chains are generated through the recombination events of a variable (V), diversity (D) and joining (J) segment to a constant region (C). A) V-J-C joining in TCR α chain. B) V-D-J-C joining in TCR β chain.

1.4.3 HLA and transplantation

Hematopoietic stem cell transplantation (HSCT) is a proven efficient curative therapy for various hematological malignancies and immune disorders [47]. Matching between donor and recipient HLA is one of the important criteria to be considered in transplantation, since mismatching could result in differential T cell allorecognition and could lead to serious transplantation outcomes including graft-versus-host disease (GvHD), graft rejection and/or mortality [48-49]. Rejection episodes occur when the transplanted donor-derived T cells trigger allorecognition of incompatible recipient antigens following HSCT [50-51].

The chances of finding an identical donor for HSCT is still only 30 % in the Caucasian population [52], hence in most cases HLA mismatched transplants from related or unrelated donors cannot be avoided. Therefore it is important to identify the impact of AA exchanges to mismatch patients and still achieve successful transplantation outcomes when no compatible donor is available.

1.5 HLA class I antigen presentation pathway

1.5.1 Peptide loading complex

The process of peptide presentation through HLA molecules is carried out by a highly specialized antigen presentation machinery that involves the so called peptide loading complex (PLC). This macromolecular assembly of proteins consist of general housekeeping proteins such as the chaperone calreticulin (CRT), thiol-depenent ERp57 (PDIA3), the two subunits of the transporter associated with antigen processing (TAP) and the HLA class I dedicated tapasin (TPN), a protein that helps in loading optimized peptide ligands.

In detail, proteins are proteolytically degraded by the proteasomes in the cytosol [53], these synthesized peptides are translocated from the cytosol into the endoplasmic reticulum (ER) lumen by TAP [54-55]. Independent of these events, nascent HLA class I molecules fold and assemble in the ER with the help of the ER chaperones calnexin (CNX), CRT and ERp57 [56-57]. By transient interaction with the proteins of the PLC, the HLA hc and β_2m attain a peptide receptive form, peptides are than loaded into the PBR with the assistance of TPN. Following peptide loading, pHLA complexes release from the PLC, selectively trafficked through the golgi apparatus and delivered on the cell surface (Figure 1.7), where pHLA molecules are monitored by CD8+ T cells [57-58].

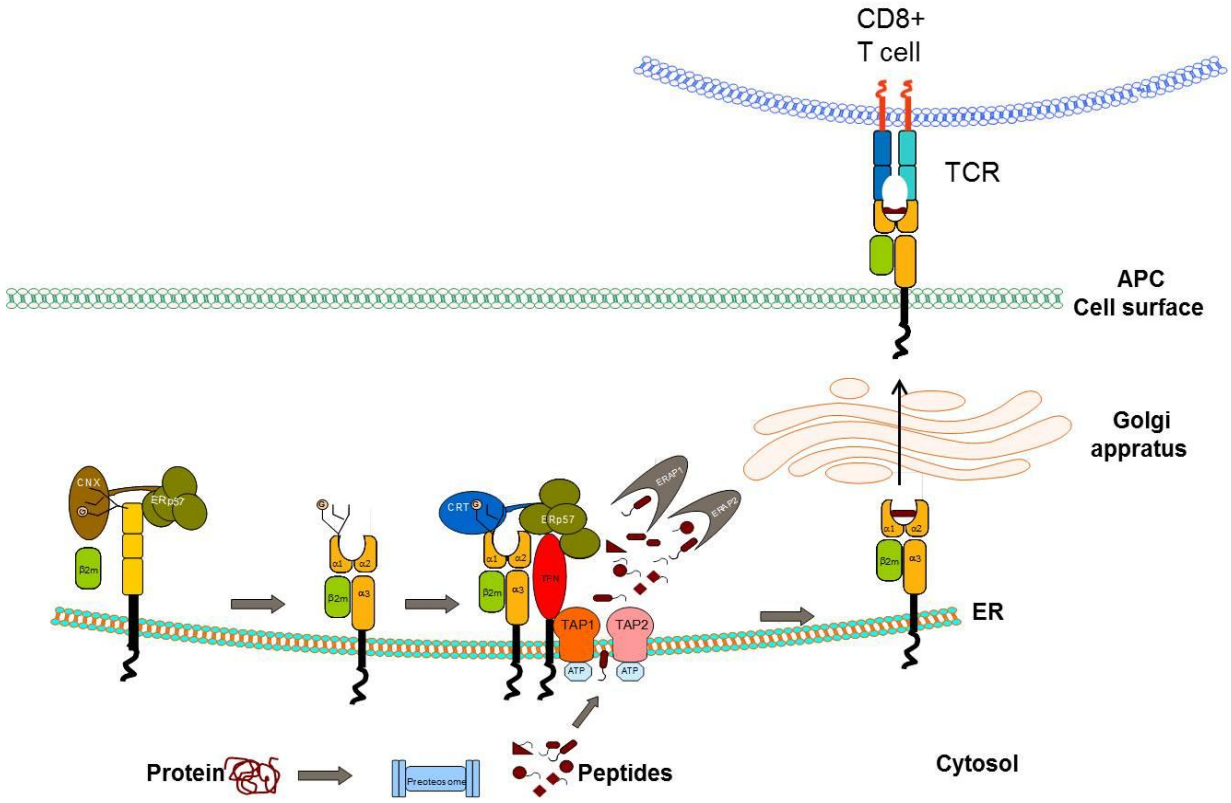
1.5.2 Dynamics of the HLA class I antigen presentation pathway

1.5.2.1 Generation of antigenic peptides in the cytosol

The majority of antigenic peptides that would be available for the HLA class I antigen presentation pathway are generated by the proteasomal degradation of intracellular proteins derived from an endogenous (self) or an exogenous (pathogen derived) source. Defective ribosomal products (DRiPs) [59] and as well as mature redundant proteins [60] could also serve as a source of peptide ligands for HLA class I molecules. Proteins are degraded by proteases in the proteasome, being either the housekeeping proteasome or the interferon-γ (IFN-γ) induced immunoproteasome. The proteases in these proteasomes generate the majority of peptides with correct C-termini [61-62] and to certain extent some peptides with correct N-termini [63]. The larger precursor peptides are trimmed at the N-termini by cytosolic aminopeptidases including

leucine aminopeptidase [64], bleomycin hydrolase, puromycin-sensitive aminopeptidase and tripeptidyl peptidase-II (TPP-II) [65]. However, the proteasomal enzymes of the immunoproteasome generate peptides that are extended at their N-termini by few residues [66].

Figure 1.7 HLA class I antigen presentation pathway



Adapted from Wearsch and Cresswell (2008) [67]. The folding of the HLA class I hc is assisted by CNX and ERp57. The hc assembles with β_2m and forms an empty unstable HLA class I heterodimer in the ER. The hc/β_2m dimer is stabilized by components of the PLC: TPN, ERp57, CRT, and TAP. Proteins are proteolytically degraded in the proteasome and the generated peptides are transported from the cytosol to the ER by TAP. These peptides are further processed by ERAP1/2 to generate optimized high affinity peptides that would fit into the PBR of the respective HLA class I molecule. Those peptides are loaded onto the empty class I molecules with the help of TPN and components of PLC. Finally, pHLA complexes disintegrate from the PLC and translocate to the cell surface where they are available for the survey by immune effector cells.

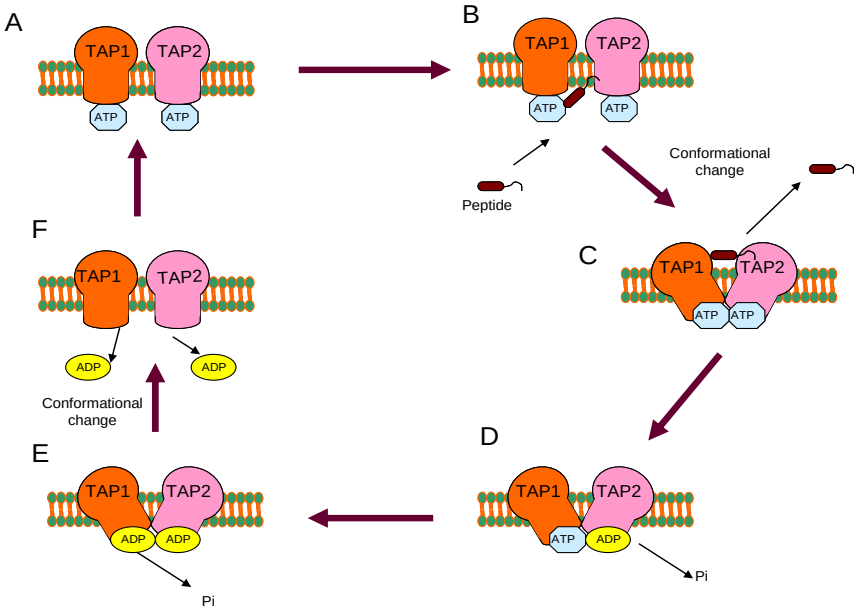
1.5.2.2 Translocation of peptides to the ER

The processed peptides are transported to the ER lumen by the TAP complex (Figure 1.8). TAP belongs to members of ATP-binding cassette (ABC) transporters that utilize the energy of adenosine triphosphate (ATP) binding and hydrolysis for translocation of peptides from the

cytosol to the ER [68]. The TAP complex is a heterodimer composing of two subunits, TAP1 (ABCB2) and TAP2 (ABCB3) [69].

Both TAP1 and TAP2 consist of an amino terminal transmembrane domain (TMD) and a cytosolic carboxyl terminal nucleotide binding domain (NBD). Each TMD can be subdivided into an N-terminal region and a core complex of 6 transmembrane core helices. The N-terminal region consists of 4 and 3 helices for TAP1 and TAP2 respectively. The core complex is involved in the heterodimer assembly, peptide binding and transport while the N-terminal extensions of the TMD is essential for TPN binding [70]. Crystallographic structures revealed the presence of highly conserved walker A and walker B motifs in the NBDs of TAP1 and TAP2. These motifs have differential ATP binding specificities and are involved in the generation of energy for peptide translocation via ATP hydrolysis [71]. The consecutive phenomenon of dimerisation and disintegration of the two NBDs coupled with the process of ATP hydrolysis in these domains generate the conformational changes in the TMD that finally facilitate the transport of cytosolic peptides across the membrane (Figure 1.8) [71-72].

Figure 1.8 Mechanism of peptide transport by TAP



Adapted from Schölz and Tampé (2009) [71]. A) Initially in the starting phase the NBDs of TAP are separated and loaded with ATP. B) and C) Dimerisation of the NBD is triggered by entry of peptide and it induces the conformational change of the TMD. C) The peptide is translocated across the membrane from the cytosol into the ER lumen. D) - F) Subsequent ATP hydrolysis cause separation of the NBD dimer and return of TAP into the original resting conformation.

1.5.2.3 Customised peptide trimming by ERAAP

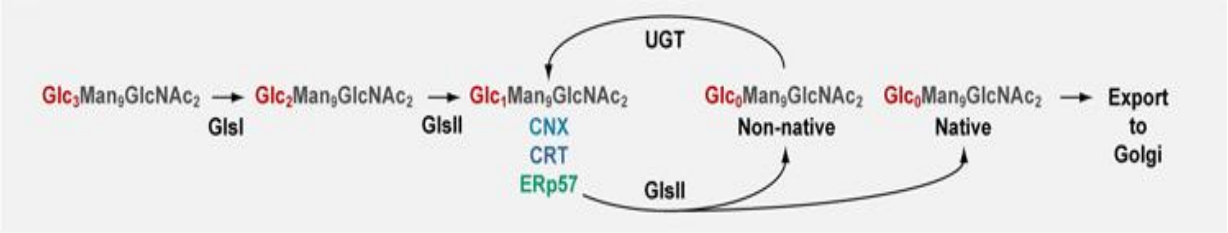
Certain proteasomally processed peptides transported into the ER have extended residues at the NH_2 termini. Compared to NH_2 terminally trimmed mature peptides, the larger NH_2 termini extended peptide precursors have a longer half-life in the cytosol [73] and higher affinity to the TAP complex [74]. However the peptides should be of "optimal" length and chemical conformation to meet the requirements of allele specific binding pockets within the PBR. Hence, after the transport of precursor peptides from the cytosol to the ER, the extended NH_2 termini of the precursor peptides are further processed and trimmed by the ER aminopeptidase associated with antigen processing ERAAP (ERAP1/2 in humans) [75-77].

1.5.2.4 Folding of HLA class I molecules

Independent of the events for peptide processing, nascent HLA class I molecules fold and mature in the ER. Newly synthesized components of class I hc and β_2m are translocated into the ER by their NH_2 terminal signal sequences. With the assistance of the lectin-like chaperones CNX and CRT, nascent HLA hc folds to a mature conformation. CNX and CRT interact specifically with monoglucosylated N-glycosylated class I hc and promote its proper folding and association with ß2m.

The class I hc is initially glycosylated by triglucose N-linked glycan ($Glc_3Man_9GlcNAc_{2,}$) (Figure 1.9) at AA position 86. The first two terminal glucose residues are then sequentially removed by glucosidases I (GlsI) and II (GlsII). The chaperone CNX connects monoglucosylated glycans ($Glc_1Man_9GlcNAc_2$) to the hc supporting the formation of stable class I hc-ß$_2$m heterodimers.

Figure 1.9 N-linked glycosylation pathway



Adapted from Wearsch and Cresswell (2008) [67]. N-linked glycan $Glc_3Man_9GlcNAc_2$ is transferred to newly synthesized HLA class I hc. After the removal of two terminal glucose residues by GlsI and GlsII, the monoglucose N-linked glycan, $Glc_1Man_9GlcNAc_2$, enters the CNX/CRT cycle. Following the release of the substrate from CNX, CRT and ERp57, the glycan is completely deglucosylated by GlsII and exported to the Golgi.

1.5.2.5 Peptide loading and surface expression

Following the formation of class I hc-ß$_2$m heterodimers in the ER, CNX is replaced by CRT and the proper folding of HLA class I molecules induce the recruitment of other components of the PLC: ERp57 and TPN. With the assembly of a stable PLC, peptides are loaded onto HLA class I molecules.

ERp57, a member of the protein disulphide isomerase (PDI), is covalently associated with TPN by a disulfide (S-S) bond located between cysteine 95 of TPN and cysteine 57 of ERp57 [78-79]. Erp57 catalyse both reduction and oxidation of S-S bonds located at the N-terminus of TPN that helps in stabilization of HLA class I molecules in the ER [80]. However, the redox activity of ERp57 is shown to be dispensable for the basic function of the PLC [81]. However, the experimental results show decreased MHC class I surface expression and sub-optimally loaded MHC class I in ERp57 deletion mouse models [82] and human cells [78], respectively, highlighting its role in antigen presentation. Moreover, ERp57 interacts with CNX/CRT and helps in folding of N-glycosylated molecules [83] and assists in the formation of disulfide bonds in HLA class I molecules [84].

Unlike other housekeeping proteins that facilitate the folding of various proteins, TPN is solely dedicated to MHC class I molecules. TPN forms an interacting bridge between TAP and the class I molecule to support peptide loading. Surface expression of peptide loaded HLA class I molecules [85] and cell surface stability of pHLA class I molecules [86] was found to be reduced in TPN deficient LCL.TPN- cells [85]. This observation is supported by functional studies in TPN knockout mice, that also revealed impaired MHC class I assembly, reduced class I cell

surface expression and poor CTL cell responses [87-88]. TPN assists peptide loading in two different ways. The N-terminal part of TPN, that interacts with HLA class I and ERp57, directly assists peptide loading through stabilization of the class I molecules in the ER [89-90]. Furthermore, the C-terminal part of TPN stabilizes the TAP complex and thus indirectly promotes peptide accessibility. Besides peptide loading, TPN also functions in peptide editing and peptide optimisation [91]. TPN prevents the release of empty class I molecules in the ER [92] and retains empty class I molecules in the ER until they are bound to "optimized" peptides [92-94]. The comparative analysis of bound peptides on TPN-deficient and TPN-competent cells revealed association of "non-optimized" peptides in a TPN deficient state [95]. This result supports the understanding of TPN as a chaperone for peptide selection. Moreover, TPN was shown to play an important role in stabilization, increasing the half-life time and promoting the steady state expression of TAP [85, 96].

pHLA class I complexes dissociate from the PLC and traffic through the golgi apparatus after which they are delivered to the cell surface. Recent investigations also highlight the role of the tapasin-related protein (TAPBPR) in post ER steps, as a chaperone for mature pHLA class I complexes through the golgi complex [97].

1.6 Down-regulation of HLA class I presentation

Down-regulation of HLA class I expression is a strategy used by cancer cells and certain pathogens such as viruses to escape immune responses. Many viral proteins for example target distinct proteins of the HLA class I antigen presentation pathway to prevent the presentation of viral peptides through HLA class I molecules and thus escape the host immune-recognition [98]. Here, TPN as a dedicated component of the class I antigen presentation pathway is a key target for certain viral proteins to escape the host immune system. One of the viral immune evasions, that inhibit TPN, is the human cytomegalovirus (HCMV) derived protein US3 [99]. Other viral immune evasions are the HCMV derived US6 protein [100-103] or the herpes simplex virus derived protein ICP47 [104], both of them inhibit the function of TAP. The adenovirus derived protein E19 has shown to block the process of peptide loading by inhibiting the interaction between TPN and TAP [105].

Molecular mechanisms underlying the down-regulation of HLA class I presentation pathway in tumour cells include incorporation of gene mutations in the HLA hc and β_2m [106]; alteration in transcriptional regulators that drive the transcription of HLA molecules and components of the PLC [107-108] and deletions of genes related to loss of heterozygosity [109]. Besides the mutations in the HLA hc and β_2m, TAP and TPN are also found to be frequently mutated in tumour cells [110]. Moreover, reduced expression of the proteasomal subunits low molecular mass polypeptide 2 (LMP2) and LMP7 was found to alter the spectrum of peptides presented by HLA molecules in cancer cells [111].

1.7 HLA class I polymorphism and TPN dependence

Greenwood *et al*. (1994) [112] demonstrated for the first time allele specific differential expression of recombinant HLA-A or -B molecules on the surface of TPN-deficient cell lines (LCL 721.220), these studies have been completed later by the observation that a differential dependence of TPN is responsible for effective peptide loading [86, 112-114] and thus the surface expression of pHLA molecules.

Distinct HLA class I alleles show a unique profile of TPN dependence for peptide loading and cell surface expression, depending on the nature of AAs located on certain polymorphic sites within or outside the PBR [99, 115]. Park *et al.* (2003) [90], showed that TPN dependence of HLA class I alleles directly correlates with the acidic property of the AA located at position 114, a residue that is characterized by a high polymorphism. This study demonstrated that HLA class I molecules with acidic AAs at position 114 such as HLA-B*44:02^{114Asp} or HLA-A*30:01^{114Glu} were highly dependent on TPN compared to those with basic AAs such as HLA-B*27:02^{114His}, HLA-B*27:05^{114His}, or HLA-A*24:01^{114His} [90]. Since every AA at a given position located in the PBR is affected by proximate AAs, it becomes clear that differences in TPN interaction cannot exclusively be dedicated to one single AA residue. HLA-B*44:02 and HLA-B*44:05, both of which have acidic AAs at position 114, showed contrasting TPN dependency. These two alleles differ by a single AA at position 116 which is located in the F pocket of the peptide binding groove and contacts C-terminus of the bound peptide. While HLA-B*44:05^{116Tyr} shows efficient peptide loading both in the presence and absence of TPN, HLA-B*44:02^{116Asp} was highly dependent upon TPN for peptide optimisation and surface expression [91]. Recent

findings by Badrinath *et al.* (2013) [115] demonstrated the impact of micropolymorphism at position 156 on TPN dependence in HLA-B*44 allotypes (Table 1.1).

Table 1.1 Effect of polymorphism on TPN dependence

TPN-dependent	TPN-independent	Reference
HLA-B*44:02^{116Asp}	HLA-B*44:05^{116Tyr}	Williams *et al*., 2002 [91]
HLA-B*44:02^{156Asp}	HLA-B*44:28^{156Arg}	Badrinath *et al.*, 2012 [115]
HLA-A*24:02^{156Gln}	HLA-A*24:06^{156Trp} HLA-A*24:13^{156Leu}	Badrinath *et al.*, 2014 [116]

The table shows impact of single AA mismatches in determining the dependence of TPN in various HLA-A and HLA-B allotypes.

1.8 Effect of polymorphism at AA position 156 within the PBR

AA residues at position 156, located in the center of the PBR [30-31] play an influential role in determining the peptide binding motif of an HLA allele, since residue 156 contact a peptide at p3, p4, p5, p6, and/or p7 [36]. Position 156 could be described [117] to play a significant role in manipulating peptide binding motif, peptide repertoire, and T cell responses when mismatched in B*44 subtypes [117-118]. Furthermore, a micropolymorphism at 156 in HLA-B*44 subtypes alters the mode of peptide loading including its dependence on TPN [115, 118] and triggers strong T cell alloresponses [119-120] and graft rejection [121].

HLA-B*35 is one of the most polymorphic serotype with more than 180 alleles (B*35:01-B*35:186) so far detected by molecular typing methods in different populations [122]. Within the HLA-B*35 allelic group three allotypes can be distinguished by a single AA exchange at position 156 (HLA-B*35:01^{156Leu}, B*35:08^{156Arg} and B*35:62^{156Trp}). HLA-B*35 molecules are expressed by approximately 20 % of Caucasians with the most frequent one being HLA-B*35:01^{156Leu} (9 % of Caucasians) [123-124]. Among HLA-B*35 alleles, HLA-B*35:01 and B*35:08 was found to be associated with decreased survival rate following AIDS [125-126]. HLA-B*35 alleles, including HLA-B*35:01, have been described to be a risk factor for the progression of certain pathological conditions such as ER stress-mediated endothelial cell dysfunction [127-129]. Moreover, HLA-B*35:01 and B*35:08 allotypes have been demonstrated

to present peptides of viral origin, indicating their role in viral immunity. The peptides presented by HLA-B*35:01 include EBV EBNA1 11-mer (HPVGEADYFEY) [130], EBV BZLF1 13-mer (LPEPLPQGQLTAY) [131] and EBV BZLF1 11-mer (EPLPQGQLTAY) [132]. HLA-B*35:08 presents EBV BZLF1 13-mer (LPEPLPQGQLTAY) [131], HCMV pp65 12-mer (CPSQEPMSTYVY) [133] and HCMV pp65 octamer (FPTKDVAL) [133] peptides. The ability of these HLA-B*35 alleles to present those viral peptides and trigger T cell responses could be suggestive of their possible roles in overcoming the dependence on classical HLA class I presentation pathway and viral immune evasion strategies.

Crystallographic studies have been carried out to understand the structural implication of AA residue 156 on peptide binding and interaction with their cognate TCRs. Macdonald *et al.* (2003) [117] demonstrated the structural impact of AA position 156 in HLA-B*44 allotypes by comparing the crystal structures of HLA-B*44:02^{156Asp} and B*44:03^{156Leu} bound to the same high affinity ligand. Several studies highlight the structural role of position 156 in altering the conformation of the PBR in HLA-B*35 allotypes. HLA-B*35:01^{156Leu} and B*35:08^{156Arg} were found to respond differentially to three overlapping peptide sequences of the EBV BZLF1 antigen: nonamer ($_{56}$LPQGQLTAY$_{64}$), 11-mer ($_{54}$EPLPQGQLTAY$_{64}$) and 13-mer ($_{52}$LPEPLPQGQLTAY$_{64}$) [134]. The study demonstrated that both HLA-B*35:01^{156Leu} and B*35:08^{156Arg} were able to bind all three peptides in-vitro. However, it could be demonstrated that CTLs from HLA-B*35:01 positive individuals responded exclusively to the 11-mer peptide ($_{54}$EPLPQGQLTAY$_{64}$), while the CTLs from HLA-B*35:08 positive individuals recognized exclusively the 13-mer peptide ($_{52}$LPEPLPQGQLTAY$_{64}$). This phenomenon could be explained by high resolution X-ray crystallography of the HLA-B*35:08^{LPEPLPQGQLTAY} complex, where the network between residues within the peptide and residue 156 within the HLA hc resulted in an altered landscape accessible for the TCR and subsequently CTL responses [131]. To understand the magnitude of residue 156 alterations on immunogenicity, crystal structures of HLA-B*35:01^{156Leu} and B*35:08^{156Arg} bound to the same ligand have been investigated [37] and provided an explanation for the alteration of allele specific immunogenicity.

1.9 Peptide prediction tools

Based on available peptide data, numerous prediction tools have been developed, that can be categorized into motif and scoring matrix-based methods, hidden Markov models, and artificial neural networks [135-139]. Allele specific peptide data facilitate the prediction and ranking of T cell epitopes by SYFPETHI [140-141], NetMHC [142], RANKPEP [143], PeptideCheck [144], BIMAS [145] or IEDB [146]. The IEDB prediction tool additionally provides information on proteasomal cleavage and TAP transport efficiency of the peptides. Proteasomal cleavage prediction tools evaluate how efficiently the C-terminally chopped peptide would be cleaved from source peptide and TAP transport predictions tool estimates the efficiency of the translocation of chopped peptide or its N-terminally prolonged precursors into the ER by TAP [147]. This tool processes these information together with HLA class I binding predictions to provide an accurate prediction of the binding efficiency for a given peptide to a defined HLA molecule.

In the present study, a proteasomal cleavage prediction tool to predict the set of peptides that would be generated by proteasomal peptidases was used. Proteasomal cleavage prediction tools generate peptides trimmed correctly at the C-terminus [148], since the N-terminus of peptides are always produced with an extension and later trimmed by aminopeptidases of the ERAAP complex [149]. The proteasomal cleavage prediction tools estimate the efficiency of a peptide or its N-terminally prolonged precursors to be liberated from its source protein. Currently, there are three methods, PAProc, MAPPP and NetChop [150], available for prediction of proteasomal cleavage sites of the proteins. Among these three tools, NetChop [151-152] is described to be the most specific for predicting the constitutive or immunoproteasome cleavage sites on the basis of multi-layered artificial neural network [150]. The method is based on the *in-vitro* digestion data and sequence signal from the boundaries of naturally processed MHC class I ligands. PAProC server [153-154] can be used to predict potential cleavage sites of the human proteasomes based on experimental cleavage data. And PAProC can be useful in assessment of the cleavability of disease-linked proteins. FragPredict developed by Holzhütter *et al.* (2000) [155] is the part of the MHC I Antigenic Peptide Processing Prediction (MAPPP) [156] package that deals with proteasomal cleavage prediction. The method uses the kinetic model of the 20S proteasome to incorporate time dependent degradation of peptides into consideration. The MAPPP

(FragPredict) tool was developed to generate an assortment of peptides that are most likely to be produced by proteasomal cleavage.

The knowledge of the HLA-B*35 specific self peptidome will help to update current peptide prediction algorithms. The peptide anchor motifs obtained in this study enabled the prediction of potential viral epitopes to be presented by HLA-B*35 allotypes.

1.10 Aim of the study

Matching between donor and recipient HLA is crucial to reduce the transplantation related outcomes. Due to the polymorphic nature of HLA molecules, in most of the cases mismatches cannot be avoided, but these mismatched transplants are associated with significant risks of GvHD, graft failure or transplant related mortality. Moreover, the nature and position of mismatches within the HLA molecule impacts on the severity of T cell allorecognition and subsequently clinical outcomes following transplantation. The challenge is to determine permissive and non-permissive mismatches and to determine the best possible mismatch when a matched donor is not available. Even a single AA exchange within the HLA heavy chain might impact the peptides that are selected and presented, thereafter altering the conformation of HLA molecules. A measurement of differential alloreactivity can only take place as a systematic *in-vitro* study on the most variable part of an HLA molecule, the bound peptide.

A mismatch cannot only have an effect on the sequence and/or repertoire of bound peptides, but also on the mode of peptide loading. Viruses evolved various strategies to evade antigen presentation through HLA molecules. An example is the HCMV protein US3, that blocks TPN and thus circumvent selection and loading of HCMV derived peptides on HLA class I molecules. The ability of HLA-B*35 molecules to present viral peptides led to the assumption, that B*35 allotypes are loading their peptides TPN-independently and therefore through a different pathway. Hence, the aim was to investigate the effect of micropolymorphism at position 156 on the property of the HLA*B35 PBR and the peptide repertoire, that might be altered from TPN dependent HLA allotypes. Utilizing soluble HLA technology the aim was to determine the mode of peptide loading and the peptide binding features of HLA-B*35 allotypes.

The knowledge of allele specific peptide binding motifs facilitates peptide prediction for vaccination purposes. Therefore, the intention was to use the data obtained in this study to predict peptides for B*35 allotypes.

2. OBJECTIVES

The study was carried out to understand the impact of micropolymorphism at position 156 in HLA-B*35 variants (HLA-B*35:01^{156Leu}, B*35:08^{156Arg} and B*35:62^{156Trp}) with the following objectives.

Part I. Surface expression of HLA-B*35/156 variants

1. To construct lentiviral vectors encoding for full length (m)HLA-B*35/156 hc
2. To transduce the lentiviral vectors encoding for mHLA-B*35/156 hc in target cells (LCL.TPN-, LCL.TPN+ and T2.TAP- cells) by lipofectamine method
3. To analyze the surface expression of HLA-B*35/156 molecules by flow cytometry

Part II. Interaction of HLA-B*35/156 variants with the components of PLC

1. To investigate the interaction of HLA-B*35/156 molecules with components of PLC by immunoprecipitation experiments

Part III. Peptide profiling

1. To construct lentiviral vectors encoding for soluble (s)HLA-B*35/156 hc
2. To transduce the lentiviral vectors encoding for sHLA-B*35/156 molecules in target cells (LCL.TPN- and LCL.TPN+) by lipofectamine method
3. To produce sHLA-peptide trimeric complexes in bioreactors
4. To determine the production of sHLA-peptide trimeric complexes by sandwich ELISA
5. To isolate low binding (LB) and high binding (HB) peptides from supernatants containing sHLA-peptide trimeric complexes
6. To understand the repertoire of presented peptides using mass spectrometry (MALDI-TOF-MS and nano-LC-ESI-MS/MS)

Part IV. Prediction of viral epitopes

1. To predict the probable viral epitopes presented by HLA-B*35/156 variants using SYFPEITHI and IEDB prediction tools
2. To predict the probable viral epitopes presented by HLA-B*35:62 variants using proteasomal cleavage prediction tools and anchor motif data from the experimental observation

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, solvents and buffers

Table 3.1 List of chemicals

Name	Source
Ampicillin ($C_{16}H_{19}N_3O_4S$)	Sigma Aldrich GmbH, Hamburg, Germany
Digitonin ($C_{56}H_{92}O_{29}$)	Sigma Aldrich GmbH, Hamburg, Germany
Disodium hydrogenphosphate (Na_2HPO_4)	Sigma Aldrich GmbH, Hamburg, Germany
G418 sulphate (Geneticin®) ($C_{20}H_{40}N_4O_{10X}$ $2H_2SO$)	Life Technologies GmbH, Darmstadt, Germany
Glycine ($C_2H_5NO_2$)	Sigma Aldrich GmbH, Hamburg, Germany
Sodium acetate ($NaCH_3COO$)	Sigma Aldrich GmbH, Hamburg, Germany
Sodiumazide (NaN_3)	Merck KGaA, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	Sigma Aldrich GmbH, Hamburg, Germany
Sodium chloride (NaCl)	Sigma Aldrich GmbH, Hamburg, Germany
Sodium hydrogencarbonate ($NaHCO_3$)	Sigma Aldrich GmbH, Hamburg, Germany
Tris ($C_4H_{11}NO_3$)	Sigma Aldrich GmbH, Hamburg, Germany

Table 3.2 List of solvents

Name	Source
Dimethylsulfoxide (DMSO) (C_2H_6OS)	Sigma Aldrich GmbH, Hamburg, Germany
Ethanol (J.T. Baker®) (CH_3CH_2OH)	Avantor Performance BV, Center Valley, USA
Glycerol ($C_{31}H_{65}NO_{15}$)	Carl Roth GmbH, Karlsruhe, Germany
Hydrochloric acid (HCl)	Sigma Aldrich GmbH, Hamburg, Germany
Isopropanol (J.T. Baker®) (C_3H_8O)	Avantor Performance Materials, Center Valley, USA
Methanol (J.T. Baker®) (CH_3OH)	Avantor Performance Materials, Center Valley, USA
Sulphuric acid (H_2SO_4)	Sigma Aldrich GmbH, Hamburg, Germany
Tween® 20 ($C_{58}H_{114}O_{26}$)	Applichem GmbH, Darmstadt, Germany
β-mercaptoethanol (C_6H_6OS)	Merck KGaA, Darmstadt, Germany
Water (J.T. Baker®) (HPLC grade) (H_2O)	Avantor Performance Materials, Center Valley, USA

Table 3.3 List of buffers and solutions (commercial)

Name	Conc.	Source
Antarctic phosphatase reaction buffer	10x	New England Biolabs GmbH, Frankfurt am Main, Germany
Bacillol	1x	Bode Chemie GmbH, Hamburg, Germany
BD FACS Clean solution	1x	Becton Dickinson GmbH, Heidelberg, Germany
BD FACS Rinse solution	1x	Becton Dickinson GmbH, Heidelberg, Germany
dNTP mix	100 mM	Agilent Technologies, Waldbronn, Germany
FastRuler DNA ladder (high range, middle range and low range)		Thermo Fisher Scientific GmbH, Schwerte, Germany
Fetal bovine serum (FBS)		Lonza, Verviers, Belgium
GelStar™ nucleic acid gel stain	10000x	Lonza, Verviers, Belgium
Korsolex		Bode Chemie GmbH; Hamburg, Germany
L-Glutamine	200 mM	c.c.pro GmbH, Karlsruhe, Germany
Ligase buffer	10x	New England Biolabs GmbH, Frankfurt am Main, Germany
Lipofectamine®2000 Reagent		Invitrogen/Life Technologies GmbH, Darmstadt, Germany
Mass ruler DNA loading dye	6x	Thermo Fisher Scientific GmbH, Schwerte, Germany
NEBuffers	10x	New England Biolabs GmbH, Frankfurt am Main, Germany
NuPAGE® LDS sample buffer	4x	Novex/Life Technologies GmbH, Darmstadt, Germany
NuPAGE® MES SDS running buffer	20x	Novex/Life Technologies GmbH, Darmstadt, Germany
NuPAGE® MOPS SDS running buffer	20x	Novex/Life Technologies GmbH, Darmstadt, Germany
NuPAGE® reducing agent	10x	Novex/Life Technologies GmbH, Darmstadt, Germany
Penicillin-Streptomycin	10 mg/ml	c.c.pro GmbH, Karlsruhe, Germany
Phosphate buffered saline (PBS)	1x	Zentralapotheke, Medizinische Hochschule Hannover, Germany
Quenching solution	1x	Thermo Fisher Scientific GmbH, Schwerte, Germany

Roti®-Lumin substrate		Carl Roth GmbH, Karlsruhe, Germany
SimplyBlue™ SafeStain (Coomassie® G-250)	1x	Life Technologies GmbH, Darmstadt, Germany
Sodium cyanoborohydride solution	5 M	Thermo Fisher Scientific GmbH, Schwerte, Germany
Spectra™ multicolour protein marker (high range, middle range and low range)		Thermo Fisher Scientific GmbH, Schwerte, Germany
Tris borate EDTA (TBE) buffer	10x	Carl Roth GmbH, Karlsruhe, Germany
TMB ONE™ substrate		Kem-en-Tec Diagnostics, Taastrup, Denmark
Trypan blue solution	0.4 %	Sigma Aldrich GmbH, Hamburg, Germany
Trypsin-EDTA	10x	Life Technologies GmbH, Darmstadt, Germany

Table 3.4 List of buffers and solutions (working)

Name	Conc.	Composition	
Affinity Chromatography			
Start buffer	75 mM	Tris	4.543 g
		ddH_2O	Final vol. 500 ml
			pH 8.0
Elution buffer	0.1 M	Glycine	3.754 g
		ddH_2O	Final vol. 500 ml
			pH 2.7
Storage buffer	0.1 %	NaN_3	0.500 g
	0.05 M	Na_2HPO_4	3.549 g
		ddH_2O	Final vol. 500 ml
			pH 7.0
Immunoprecipitation			
Digitonin stock	20 mg/ml	Digitonin	200 mg
		ddH_2O	Final vol. 10 ml
Protease inhibitor cocktail stock	25x	Protease inhibitor cocktail	One tablet
		ddH_2O	Final vol. 2 ml
Tris-buffered saline (TBS)	2x	1 M TrisHCl (pH 7.4)	5 ml
		5 M NaCl	3 ml
		ddH_2O	Final vol. 50 ml

NaCl	5 M	NaCl	292.50 g
		ddH_2O	Final vol. 1000 ml
Tris-HCl	1 M	Tris	121.14 g
		ddH_2O	Final vol. 1000 ml
			pH 7.4
Digitonin lysis buffer	0.2 mg/ml	Digitonin stock (20 mg/ml)	2.5 ml
		2x TBS	5.0 ml
		25x protease inhibitor	400 µl
		ddH_2O	Final vol. 10 ml
Wash buffer 1	0.1 %	Digitonin stock (20 mg/ml)	0.5 ml
		ddH_2O	4.5 ml
		2x TBS	Final vol. 10 ml
Wash buffer 2	0.1 %	Digitonin stock (20 mg/ml)	0.5 ml
	450 mM	5 M NaCl	0.9 ml
	10 mM	1 M Tris-HCl (pH 7.4)	100 µl
		ddH_2O	Final vol. 10 ml
			pH 7.4
Wash buffer 3	10 mM	1 M Tris-HCl (pH 7.4)	100 µl
		ddH_2O	Final vol. 10 ml
			pH 7.4
ELISA			
Phosphate buffered saline with Tween (PBST)	0.05 %	Tween® 20	0.5 ml
		PBST	Final vol. 1000 ml
Blocking solution	2 %	BSA	2 g
		PBST	100 ml
Stop solution	3 M	5 M H_2SO_4	60 ml
	1 M	10 M HCl	10 ml
		ddH_2O	Final vol. 100 ml
Western blotting			
Blocking solution (in PBS)	3 %	Milk powder	3 g
		PBS	Final vol. 100 ml
NuPAGE® MES running buffer	1x	20x NuPAGE® MES buffer	50 ml
		ddH_2O	Final vol. 1000 ml
NuPAGE® MOPS running buffer	1x	20x NuPAGE® MOPS buffer	50 ml
		ddH_2O	Final vol. 1000 ml

Others			
Carbonate buffer	0.2 M	Na_2CO_3	2.2 g
		ddH_2O	Final vol. 100 ml
Bicarbonate buffer	0.2 M	$NaHCO_3$	1.68 g
		ddH_2O	Final vol. 100 ml
Carbonate-bicarbonate buffer		Carbonate buffer	4 ml
		Bicarbonate buffer	46ml
		ddH_2O	Final vol. 200 ml
			pH 9.2
G418 (Geneticin®) stock solution	5 mg/ml	Geneticin®	2.5 g
		Hepes buffer	Final vol. 500 ml
			pH 7.3

Table 3.5 List of miscellaneous items

Name	Source
Agar-agar	Carl Roth GmbH, Karlsruhe, Germany
BSA (Albumin fraction-V)	Carl Roth GmbH, Karlsruhe, Germany
Hepes	Sigma Aldrich GmbH, Hamburg, Germany
Milk powder (blotting grade)	Carl Roth GmbH, Karlsruhe, Germany
Protamine sulphate	Life Technologies GmbH, Darmstadt, Germany
Protease inhibitor	Roche Diagnostics, Hvidovre, Denmark
Protein A-sepharose beads CL-4B	GE Healthcare Europe GmbH, Freiburg, Germany
SeaKem® LE Agarose	Lonza, Allendale, USA
Sephadex™ G-50 Fine	GE Healthcare Europe GmbH, Freiburg, Germany
Tryptone	Carl Roth GmbH, Karlsruhe, Germany
Yeast extract	Carl Roth GmbH, Karlsruhe, Germany

3.1.2 Kits

Table 3.6 List of kits

Name	Source
BigDye® Terminator v.1.1 cycle sequencing kit	Perkin Elmer/Applied Biosystems, Darmstadt, Germany
BIO-X-ACT™ short mix	BiolineGmbH, Luckenwalde, Germany
EZ-Link™ plus activated peroxidase kit	Thermo Fisher Scientific GmbH, Schwerte, Germany
pcDNA™3.1/V5-His TOPO® TA expression kit	Invitrogen/Life Technologies GmbH, Darmstadt, Germanny
EndoFree® plasmid maxi kit	Qiagen GmbH, Hilden, Germany
QIAprep® spin miniprep kit	Qiagen GmbH, Hilden, Germany
QIAquick® gel extraction kit	Qiagen GmbH, Hilden, Germany
QIAquick® PCR purification kit	Qiagen GmbH, Hilden, Germany
QuikChange® II XL site-directed mutagenesis kit	Agilent Technologies, Waldbronn, Germany

3.1.3 Laboratory consumables

Table 3.7 List of laboratory consumables

Name	Source
Adherent foil	Kisker Biotech GmbH, Steinfurt, Germany
Automatic pipette (Akku Jet)	Brand GmbH + Co KG, Wertheim, Germany
Cell culture plates (for suspension cells)	Sarstedt AG, Nümbrecht, Germany
Cell culture flasks	TPP, Trasadingen, Switzerland
CELLine classic 1000	Integra Biosciences GmbH, Fernwald, Germany,
Centrifuge tubes (15 ml, 50 ml)	Sarstedt AG, Nümbrecht, Germany
Cryogenic storage vials (Cryo.s™)	Greiner Bio-one GmbH, Frickenhausen, Germany
Disposable pipette tips(2.5µl, 5µl, 100 µl, 200 µl, 1000 µl)	Sarstedt AG, Nümbrecht, Germany
Gloves	Kimberly Clark, Rosewell, USA
MicroAmp® 96-well reactionplate	Life Technologies GmbH, Darmstadt, Germany
MicroAmp® optical adhesion film	Life Technologies GmbH, Darmstadt, Germany
Microcentrifuge tubes (1.5 ml)	Eppendorf, Hamburg, Germany

Micro-spin™ G-50 columns	GE Healthcare Europe GmbH, Freiburg, Germany
Nalgene® Cryo 1°C freezing container	Thermo Fisher Scientific GmbH, Schwerte, Germany
Neubauerchamber	Glaswaren Fabrik Karl Hecht GmbH, Gemany
NHS-activated HiTrap columns	GE Healthcare Europe GmbH, Freiburg, Germany
Nunc Maxisorp™ plate C96	Thermo Fisher Scientific GmbH, Schwerte, Germany
NuPAGE Novex® 4 –12 % Bis-Tris protein gels	Life Technologies GmbH, Darmstadt, Germany
Parafilm®sealing film	Labor-Brand, Gießen, Germany
PCR tubes	Life Technologies GmbH, Darmstadt, Germany
Polycarbonate ultracentrifugation bottle assembly (70 ml)	Beckman Coulter GmbH, Sinsheim, Germany
Polystyrene flow cytometry tubes (5 ml)	Sarstedt AG, Nümbrecht, Germany
PVDF membranes (iBlot® transfer stack)	Life Technologies GmbH, Darmstadt, Germany
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt AG, Nümbrecht, Germany
Millex® syringe filter units (PVDF) (poresize 0.22 µm, 0.45 µm)	EMD Millipore/Merck KgaA, Darmstadt, Germany
Perfusor® syringe (50 ml)	B. Braun Melsungen AG, Melsungen, Germany
Tissue culture plates	TPP, Trasadingen, Switzerland

3.1.4 Laboratory appliances

Table 3.8 List of laboratory appliances

Name	Source
Autoclave (Systec EL-5050)	Systec GmbH, Wettenberg, Germany
Autoclave (Systec VX-150)	Systec GmbH, Wettenberg, Germany
BioLogic DuoFlow system	Bio-Rad Laboratories GmbH, München, Germany
Biosafety cabinet (Biowizard SL130)	Kojar Sales, Vught, The Netherlands
Centrifuge (Heraeus Megafuge 16R)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Centrifuge (Heraeus Fresco 17)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Centrifuge (Sigma 6K15)	Sartorius Group, Göttingen, Germany
CO_2 incubator (Heracell 2420)	Thermo Fisher Scientific GmbH, Schwerte, Germany
ABI Prism 3730 DNA analyzer	Applied Biosystems GmbH, Darmstadt, Germany
Flow cytometer (BD FACSCanto™ II)	Becton Dickinson GmbH, Heidelberg, Germany

FluorChem® imager(FC2)	AlphaInnotech GmbH, Kasendorf, Germany
Freezer (Profiline -20°C)	Liebherr Firmengruppe, Biberach, Germany
Freezers (UltraLow -150°C, -80°C):	Sanyo, Osaka, Japan
Horizontal electrophoresis chamber	VWR International, Darmstadt, Germany
HydroFlex™ microplate washer	Tecan GmbH, Crailsheim, Germany
iBlot®Gel Transfer Device (IB001)	Life Technologies GmbH, Darmstadt, Germany
Inolab® pH meter (pH level 1)	Wissenschaftliche Tech. Werkstätten, Weilheim,Germany
Laboratory waterbath	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
MACSmix™ tuberotator	Miltenyl Biotech GmbH, Bergisch Gladbach,Germany
Magnetic stirrer (MR 2002)	Heidolph Instruments GmbH, Schwabach, Germany
Microcentrifuge (Heraeus Fresco17)	Thermo Fisher Scientific GmbH, Schwerte, Germany
Microscope (Olympus IMT2)	Olympus GmbH, Hamburg, Germany
Microwave (Alaska CRS)	Alaska e.K.,Viernheim, Germany
Multi detection reader for microplate (Synergy 2)	Biotek Inc., Bad Friedrichshall, Germany
NanoDrop® photometer (ND-1000)	Peqlab Biotechnologie GmbH, Erlangen, Germany
Power supply equipment (Consort E865)	Consort N.V., Turnhout, Belgium
Precison balance (KERN PLJ-N)	Kern and Sohn GmbH, Balingen, Germany
Refrigerator (Profiline 4°C)	Liebherr Firmengruppe, Germany
Shaking incubator (SS15)	Omnilab Laborzentrum, Gehrden, Germany
Thermoblock (Thermostat 5320)	Eppindorf GmbH, Hamburg, Germany
Thermocyclers (Gene Amp® PCR system 2700)	Life Technologies GmbH, Darmstadt, Germany
Thermomixer	Eppendorf GmbH, Hamburg, Germany
Ultra pure water systems (Arium® 611UF)	Sartorius AG, Göttingen, Germany
Ultracentrifuge (Optima™ L-100 XP)	Beckman Coulter GmbH, Sinsheim, Germany
UV illuminator and gel documentation system (Intas MW312nm)	Intas Science Imaging Instruments GmbH, Göttingen, Germany
VACUSAFE vacuum pump (comfort)	Integra Biosciences GmbH, Fernwald, Germany, Switzerland
Vortex (REAX2000)	Heidolph Instruments GmbH, Schwabach, Germany
Xcell Sure Lock®mini-cell (E1001)	Life Technologies GmbH, Darmstadt, Germany

3.1.5 Instruments and consumables used for MS

Table 3.9 Instruments and consumables used for MS by TopLab GmbH

Name	Source
Eksigent nano-LC Ultra 2D HPLC	Eksigent/AB SCIEX GmbH, Darmstadt, Germany
LTQ Orbitrap ion trap mass spectrometry	Thermo Fisher Scientific GmbH, Schwerte, Germany
Acclaim® PepMap100 C18 column	Dionex Corporation/Thermo Fisher Scientific GmbH, Schwerte, Germany
Acclaim® PepMap100 C18 trap column	Dionex Corporation/Thermo Fisher Scientific GmbH, Schwerte, Germany
ZipTip® pipette tip device	EMD Millipore/Merck KgaA, Darmstadt, Germany

3.1.6 Antibodies

Table 3.10 List of antibodies

Name	Description	Source
Anti-goat IgG-HRP	Rabbit polyclonal	Dako GmbH, Hamburg, Germany
Anti-HLA-A/B/C (Clone LY-5.1)	Mouse monoclonal	Santacruz Biotechnology Inc, Heidelberg, Germany
Anti-mouse IgG-HRP	Goat polyclonal	Dako GmbH, Hamburg, Germany
Anti-mouse IgG-HRP	Rabbit polyclonal	Dako GmbH, Hamburg, Germany
Anti-rabbit IgG-HRP	Goat polyclonal	Dako GmbH, Hamburg, Germany
Ant -β2m-HRP	Rabbit polyclonal	Dako GmbH, Hamburg, Germany
Anti-bw6-FITC (Clone H0038)	Mouse monoclonal	One lambda, Meerbusch-Osterath, Germany
Anti-CRT	Rabbit polyclonal	Dianova GmbH, Hamburg, Germany
Anti-Erp57 (Clone MaP.Erp57)	Mouse monoclonal	Enzo Lifesciences GmbH, Lörrach, Germany
Anti-HLA-B (N-20)	Goat polyclonal	Santacruz Biotechnology Inc, Heidelberg, Germany
Anti-TAP1	Rabbit polyclonal	Enzo Lifesciences GmbH, Lörrach, Germany
Anti-TPN (Article N-17)	Goat polyclonal	Santacruz Biotechnology Inc, Heidelberg, Germany
Anti-HLA-A/B/C (Clone W6/32)	Mouse monoclonal	Bio-Rad AbD Serotec GmbH, Puchheim, Germany
Anti-HLA-A/B/C-PE (Clone W6/32)	Mouse monoclonal	Biolegend, Fell, Germany

3.1.7 Softwares

Table 3.11 List of softwares

Name	Source	Utility
BD FACSDIVA™ software	Becton Dickinson GmbH, Heidelberg, Germany	Acquisition and analysis of data from flow cytometry workflow
BioLogic DuoFlow software	Bio-Rad Laboratories GmbH, München, Germany	Peak recovery control and data review
Data collection software v2.0.	Applied Biosystems/Life Technologies GmbH, Darmstadt, Germany	Collection of the sequencing data from ABI Prism 3730 DNA Analyzer
DNASTAR software	DNASTAR Inc., Madison, USA	Analysis and alignment of nucleotide sequences
Endnote 9	Thomson Reuters, Philadelphia, USA	Reference managing
FlowJo software v7.6.5	Tree Star Inc., Ashland, USA	Analysis of flow cytometry data
Fluorchem FC2 software	Alpha Innotech GmbH, Kasendorf, Germany	Acquisition and analysis of chemiluminiscent digital images
Gen5™ data analysis software	BioTek Inc., Winooski, USA	Analysis of spectrophotometric data for ELISA
GraphPad Prism 5	GraphPad Software Inc., La Jolla, USA	Data analysis, statistics and graphing
Intas GDS software	INTAS Science Imaging Instruments GmbH, Göttingen, Germany	Documentation and analysis of agarose gels
Mascot Server and Mascot Daemon	Matrix Sciences Inc., Boston, USA (http://www.matrixscience.com)	Identification and analysis of peptides using MS data
NanoDropND-1000 software	Peqlab Biotechnologie GmbH, Erlangen, Germany	Documentation of spectrophotometric analysis of samples
PyMOL	Schrodinger Sales Center, Manheim, Germany (http://www.pymol.org)	Visualization of 3D structures of macromolecules
YASARA/FoldX	Vrije Universiteit Brussel, Belgium (http://www.yasara.org)	Modelling of the macromolecular structures; prediction of the effect of mutations on the stability of a protein

3.1.8 Organisms

Table 3.12 List of bacterial strains

Bacterial strain	Genotype	Source
Escherichia coli (One Shot® TOP10 cells)	F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG	Invitrogen/Life Technologies GmbH, Darmstadt, Germany
Escherichia coli (One Shot® Stbl3 cells)	F^- mcrB mrr hsdS20 (rB^-, mB^-) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(Str^R) xyl-5 λ^- leu mtl-1 endA1+	Invitrogen/Life Technologies GmbH, Darmstadt, Germany

Table 3.13 List of human cell lines

Cell line	Description	Reference	Source
HEK293T	Human embryonic kidney cells; adenovirus type 5transformed; adherent fibroblastoid cells, monolayer; express the simian virus 40 (SV40) large T antigens	Graham *et al*., 1977 [157]	DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany
LCL.TPN-	Human B lymphoblastoid cell line LCL 721.220; derived from LCL 721 cells following selection by γ-ray-induced mutations; lacks expression of HLA class I molecules and functional TPN	Greenwood *et al*., 1994 [112]	LGC Standards GmbH, Wessel, Germany
LCL.TPN+	Human B-lymphoblastoid cell line LCL 721.221; derived from LCL 721 cells following selection by γ-ray-induced mutations; lacks expression of HLA class I molecules	Kavathas *et al*., 1980 [158]	LGC Standards GmbH, Wessel, Germany
T2.TAP-	Human lymphoblastoid hybrid cell line (B-LCL721.174 with an 8-azaguanine and ouabain-resistant variant of the T-LCL CEM (CEM R.3)), no expression of functional TAP; surface expression of low amounts of HLA class I molecules	Salter *et al*., 1985 [159] Salter and Cresswell, 1986 [160]	Institute for Transfusion Medicine, MHH, Hannover, Germany

3.1.9 Plasmids

Table 3.14 List of plasmids

Plasmids	Genotype, relevant characteristics	Source
pcDNA™ 3.1/V5-His	Mammalian expression; CMV promoter for high-level constitutive expression; C-terminal V5 epitope and polyhistidine (6x His) tag; Amp resistance	Life Technologies GmbH, Darmstadt, Germany
pRRL.PPT.SF.pre.V5-His	Lentiviral expression; lac promoter; central polypurine tract (PPT) and post-transcriptional regulatory element (pre) for increased transduction efficiency; C-terminal V5 epitope and polyhistidine (6x His) tag; Amp resistance	Institute for Transfusion Medicine, MHH, Hannover
pMD2G (Plasmid 12259)	Envelope expression plasmid; CMV promoter; vesicular stomatitis virus-G (VSV-G) envelope glycoprotein expression; Amp resistance	Addgene, Cambridge, USA
psPAX2 (Plasmid 12260)	Second generation lentiviral packaging plasmid; lac promoter; codes for gag (virion main structural proteins) and RRE (binding site for the Rev protein which facilitates export of the RNA from the nucleus) and central polypurine tract (PPT); Amp resistance	Addgene, Cambridge, USA
pcDNA.mHLA-B*3501	Derivate of pcDNA™ 3.1/V5-His; Expression of full length HLA-B*35:01 (Exon 1–7)	This work
pRRL.mHLA-B*3501	Derivate of pRRL.PPT.SF.pre.V5-His; Expression of full length HLA-B*35:01 (Exon 1–7)	This work
pRRL.mHLA-B*3508	Derivate of pRRL.PPT.SF.pre.V5-His; Expresison of full length HLA-B*35:08 (Exon 1–7)	This work
pRRL.mHLA-B*3562	Derivate of pRRL.PPT.SF.pre.V5-His; Expression of full length HLA-B*35:62 (Exon 1–7)	This work
pRRL.sHLA-B*3501	Derivate of pRRL.PPT.SF.pre.V5-His; Expression of soluble HLA-B*35:01 (Exon 1–4)	This work
pRRL.sHLA-B*3508	Derivate of pRRL.PPT.SF.pre.V5-His; Expression of soluble HLA-B*35:08 (Exon 1–4)	This work
pRRL.sHLA-B*3562	Derivate of pRRL.PPT.SF.pre.V5-His; Expression of soluble HLA-B*35:62 (Exon 1–4)	This work

3.1.10 Oligonucleotides

Table 3.15 List of oligonucleotides

Name	Sequence	T_m	Length
HLA-B1-TAS	5′- GAGATGCGGGTCACGGCG -3′	64.4°C	18
HLA-B-TAAS-E7	5′- TCAAGCTGTGAGAGACACATCAG -3′	55.7 °C	23
Sdm_B35_sE4_F	5′- CCTCACCCTGAGATGAGAGCCATCTTCCCAGTC -3′	72.5 °C	33
Sdm_B35_sE4_R	5′- GACTGGGAAGATGGCTCTCATCTCAGGGTGAGG -3′	72.5°C	33
Sdm_B3508_156_F	5'- GTGGCGGAGCAGCGGAGAGCCTACC -3'	73.4 °C	25
Sdm_B3508_156_R	5'- GGTAGGCTCTCCGCTGCTCCGCCAC -3'	73.4°C	25
Sdm_B3562_156_F	5'- GTGGCGGAGCAGTGGAGAGCCTACCTG -3'	72.3°C	27
Sdm_B3562_156_R	5'- CAGGTAGGCTCTCCACTGCTCCGCCAC -3'	72.3°C	27
Seq-pRRL_S	5´- AGTCCTCCGACAGACTGAG -3´	52.1°C	19
Seq-pRRL_AS	5´- AGCAGCGTATCCACATAGCG -3´	57.7°C	20
pcDNA-3.1_S	5´- TAATACGACTCACTATAGGG -3´	43.4°C	20
pcDNA-3.1_AS	5´- TAGAAGGCACAGTCGAGG -3´	51.4°C	18

All the primers were custom designed and ordered from TIB MOLBIOL Syntheselabor GmbH, Germany. Melting temperature (Tm) was calculated using Breslauer thermodynamic parameters, as recommended by the manufacturer.

3.1.11 Enzymes

Table 3.16 List of enzymes

Restriction enzymes

Name	Restriction sites	Source
*Bam*HI	5'- G$^{\downarrow}$GATCC -3' 3'- CCTAG$_{\uparrow}$G -5'	New England Biolabs GmbH, Frankfurt am Main, Germany
*Dpn*I	CH_3 | 5'- GA$^{\downarrow}$TC -3' 3'- CT$_{\uparrow}$AG -5' | CH_3	New England Biolabs GmbH, Frankfurt am Main, Germany
*Xba*I	5'- T$^{\downarrow}$CTAGA -3' 3'- AGATC$_{\uparrow}$T -5'	New England Biolabs GmbH, Frankfurt am Main, Germany

Modifying enzymes

Name	Source
Antarctic phosphatase	New England Biolabs GmbH, Frankfurt am Main, Germany
T4 DNA ligase	New England Biolabs GmbH, Frankfurt am Main, Germany

3.1.12 Culture media

Table 3.17 List of culture media

Name	Source
Ready to use media	
DMEM	Lonza, Verviers, Belgium
RPMI 1640	Lonza, Verviers, Belgium
S.O.C. medium	Life Technologies GmbH, Darmstadt, Germany
Media for *E. Coli*	
2x YT-Ampicillin medium	Inhouse prepared
2x YT-Ampicillin agar	Inhouse prepared
Media for human cell lines	
10 % medium for LCL cells	Inhouse prepared
5 % medium for LCL cells	Inhouse prepared
10 % medium for HEK293T cells	Inhouse prepared
10 % DMSO cryopreservation medium	Inhouse prepared

2x YT-Ampicillin medium

Ingredients	Amount
Yeast extract	10 g
NaCl	5 g
Tryptone	16 g

The ingredients were dissolved in 1000 ml ddH_2O, pH was adjusted to 7.0 and the solution autoclaved at 121°C for 15 min. The prepared medium was stored at 4°C. The required antibiotics were added prior to use (100 µg/ml Ampicillin).

2x YT-Ampicillin agar

Ingredients	Amount
Agar	25 g
Yeast extract	10 g
NaCl	5 g
Tryptone	16 g

The ingredients were dissolved in 1000 ml ddH_2O, pH was adjusted to 7.0 and the solution autoclaved at 121°C for 15 min. The prepared medium was cooled down to around 55°C, finally the required antibiotics were added and plated out in the petri dishes (25 ml/90 mm dish).

10 % medium for LCL cells (0.5 Liter)

Ingredients	Volume
RPMI 1640	445 ml
FBS (heat inactivated)	50 ml
L-Glutamine (200 mM)	5 ml

Heat inactivation of FBS was conducted at 56°C for 20 min in the water bath.

5 % medium for LCL cells (0.5 Liter)

Ingredients	Volume
RPMI 1640	460 ml
FBS (heat inactivated)	25 ml
L-Glutamine (200 mM)	5 ml
Penicillin-Streptomycin (10 mg/ml)	10 ml

Heat inactivation of FBS was conducted at 56°C for 20 min in the water bath.

10 % Medium for HEK293T cells (0.5 Liter)

Ingredients	Volume
DMEM	425 ml
FBS (heat inactivated)	50 ml
G418 (50 mg/ml)	10 ml
Penicillin-Streptomycin (10 mg/ml)	10 ml
L-Glutamine (200 mM)	5 ml

Heat inactivation of FBS was conducted at 56°C for 20 min in the water bath.

Cryopreservation media

10 % DMSO cryopreservation medium (10 ml)

Ingredients	Volume
DMSO	1 ml (10 %)
FBS (heat inactivated)	5 ml (50 %)
Sterile PBS or RPMI	4 ml

Heat inactivation of FBS was conducted at 56°C for 20 min in the water bath.

3.2 Methods

3.2.1 Construction of eukaryotic and lentiviral expression vectors

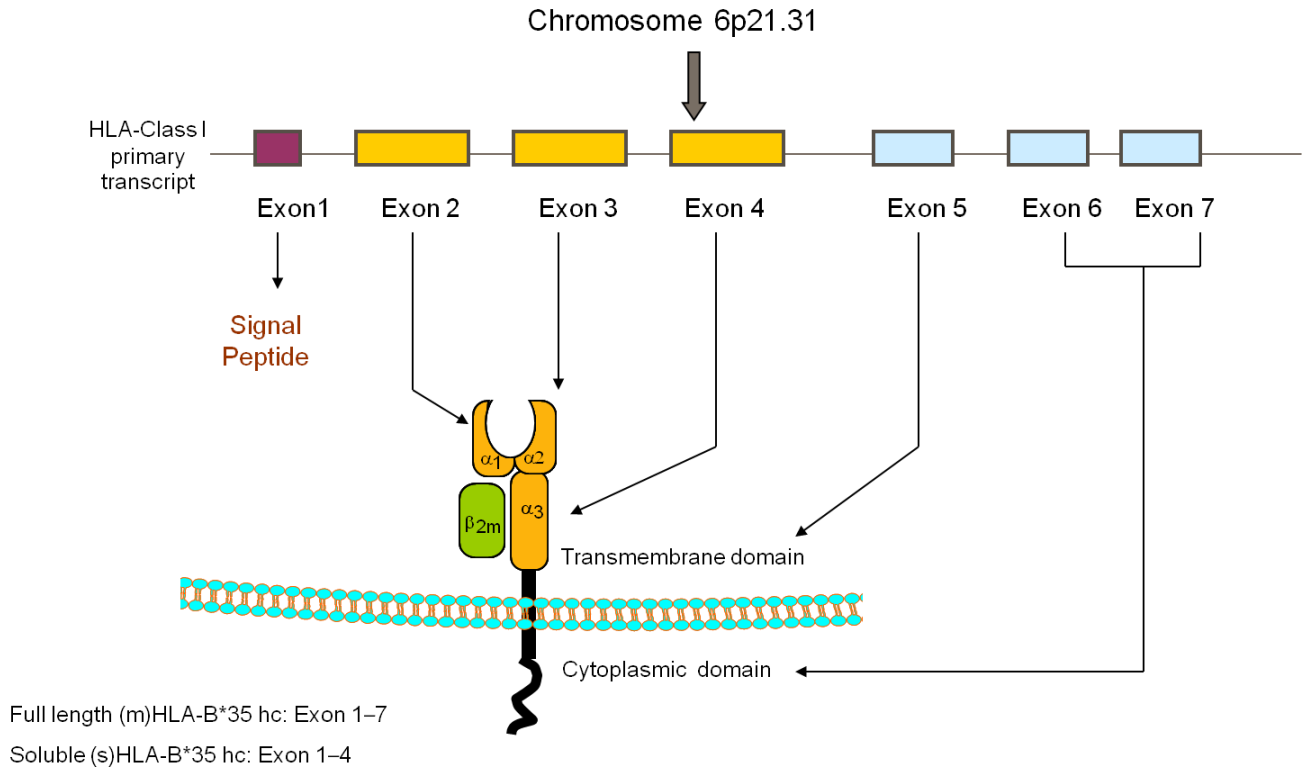
Full length (m)HLA-B*35:01 (Exon 1–7) (Figure 3.1) sequence was amplified from cDNA derived from an HLA-B*35:01 positive donor by PCR using the primers HLA-B1-TAS and HLA-B-TAAS-E7. The PCR products were ligated into the eukaryotic expression vector pcDNA3.1/V5-His using the pcDNA™3.1/V5-His TOPO® TA cloning kit. The mHLA-B*35:01 insert was then cut from the pcDNA.mHLA-B*3501 vector and cloned into the pRRL.PPT.SF.pre.V5-His vector. The pRRL.mHLA-B*3508 and pRRL.mHLA-B*3562 variants were generated by using site-directed mutagenesis (QuikChange® II XL site-directed mutagenesis kit) by mutating nucleotide(s) T to G using the primers Sdm_B3508_156_F and Sdm_B3508_156_R and CT to TG using the primers Sdm_B3562_156_F and Sdm_B3562_156_R, respectively, corresponding to AA position 156, applying the pRRL.mHLA-B*3501 plasmid as the template.

Vectors encoding for soluble (s)HLA-B*35/156 variants (Figure 3.1) were generated from pRRL.mHLA-B*35/156 vectors by introducing a stop codon (TGA) after Exon 4 utilizing site-directed mutagenesis with the primers Sdm_B35_sE4 and Sdm_B35_sE4_R.

Cloning of the HLA-B*35/156 inserts into pcDNA3.1/V5-His and pRRL.PPT.SF.pre.V5-His vectors was verified by restriction analysis and the sequences were verified by DNA-sequencing using an ABI Prism 3730 DNA analyzer. The vectors carrying the correct sequences were selected and endotoxin free plasmid DNA was extracted using the EndoFree® plasmid maxi kit for introducing the vectors into mammalian cells.

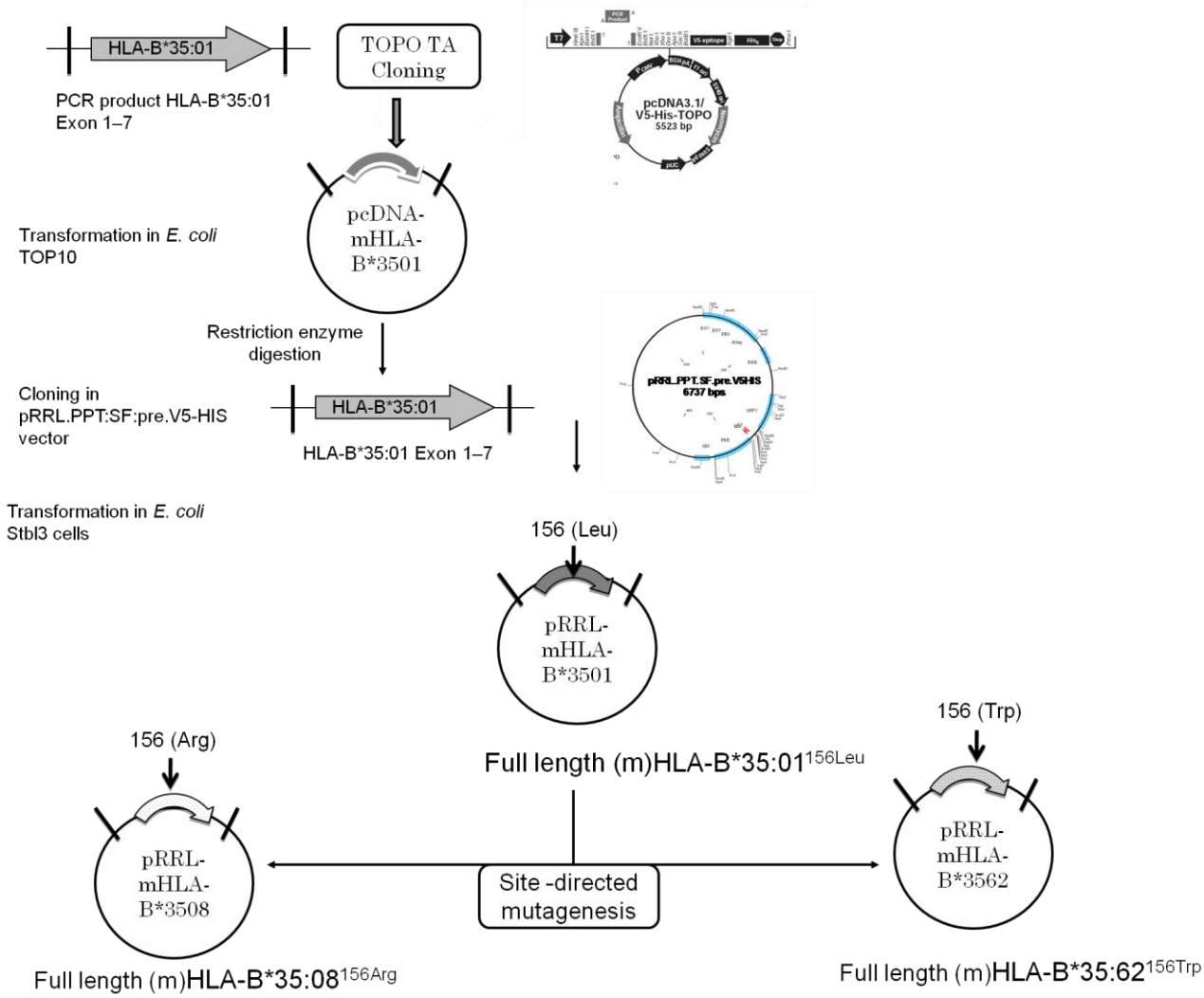
The experimental design for the construction of recombinant vectors with mHLA-B*35/156 or sHLA-B*35/156 is illustrated in Figure 3.2. The genomic map of HLA-B*35:01 with relevant primers and important residues is illustrated in Appendix I.

Figure 3.1 Strategy for expression of full length (m) or soluble (s)HLA-B molecules



HLA-B consists of Exon 1–7. To express membrane bound molecules (mHLA-B) Exon 1–7 was cloned in to an expression vector. To express soluble molecules (sHLA-B), truncated versions lacking the transmembrane and cytoplasmatic domain were cloned by introducing a stop codon after Exon 4.

Figure 3.2 Cloning strategy for construction of expression vectors



Full length HLA-B*35:01 cDNA (Exon 1–7) was cloned into pcDNA3.1/V5-His using the TOPO®-TA technology. HLA-B*3501 inserts were cut by restriction enzymes and the fragments ligated into the pRRL.PPT.SF.pre.V5-His vector. Site-directed mutagenesis primers were used to introduce the mutations, which could be finally verified by sequencing.

3.2.1.1 Amplification of DNA fragments

The polymerase chain reaction (PCR) is an enzymatic method, described by Mullis in 1987 [161] and 1990 [162], to amplify specific DNA fragments using template-homologues primers, dNTPs and a heat stable polymerase. The primer will hybridise with the denaturated starting DNA, this occurs anti-parallel in 3′–5′ direction on each DNA strand. The elongation of the primers that flank the target sequence is catalyzed through the polymerase.

The exponential amplification of the desired DNA fragment utilizing PCR requires the following three steps: (i) denaturation of double stranded DNA to single stranded DNA at 95°C, (ii) annealing of the primers, (iii) extension of the primers. At the end of a PCR reaction, the specific DNA target sequence will be exponentially accumulated in billions of copies called amplicons.

The BIO-X-ACT™ short mix was used for amplification of the template. The PCR setup is given below.

Reagents	**Volume**
2x PCR master mix (BIO-X-ACT™)	25.0 µl
Forward Primer (5 µM)	5.0 µl
Reverse primer (5 µM)	5.0 µl
Template (cDNA)	x µl
HPLC-H_2O	Final vol. 50.0 µl

Final concentration of cDNA in the PCR reaction was adjusted to 50 ng.

The thermal cycling conditions are given below.

	Temperature	**Time**
Step 1	94°C	4 min
Step 2 (35 cycles)		
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	45 sec
Step 3	72°C	7 min
Hold	4 °C	∞

3.2.1.2 Analysis of DNA by agarose gel electrophoresis

Agarose gel electrophoresis a method to separate DNA fragments according to their molecular weight. The method is based on the fact that DNA molecules migrate in an electric field to the anode due to their negative charge. In an electrical field the mobility of the DNA shows a linear dependence to their size [163]. For electrophoresis, the DNA is stained with aromatic cations, as for example Ethidiumbromide, that intercalates in the double helical structure of the DNA [164]. Ethidiumbromide enables recognition of DNA bands on a transilluminator

with a wavelength of 302 nm, an intensive purple florescence. Using an appropriate DNA length marker enables to determine the molecular weight of the DNA fragments.

3.2.1.3 Cloning of DNA into pcDNA™ 3.1/V5-His vector

The method to connect a PCR product with the pcDNA™ 3.1/V5-His vector is based on the principle of "TA-sticky-end" ligation. This method of ligation is enabled through the template-independent terminal transferase activity of Taq-DNA-Polymerase that adds to each generated PCR product a 3´-deoxyadenosine (A). These dATPs pair with the 3´-desoxythymidine (T) at the ligation side of the linearized vector. The covalent connection of both DNA molecules is catalyzed by the enzyme topoisomerase I [165]. The set up for TOPO®-TA cloning reaction is described below.

Reagents	**Volume**
Fresh PCR product	0.5 to 4.0 µl
Salt solution	1.0 µl
HPLC-H_2O	Final vol. 5.0 µl
pcDNA™3.1/V5-His TOPO® vector	1.0 µl

The reaction was mixed gently and incubated for 5 min at RT for ligation.

3.2.1.4 Ligation of DNA insert into a vector

Another method to ligate DNA fragments into an appropriate vector can be performed using T4 DNA ligase that facilitates the joining of two strands of DNA by a phosphodiester bond. The ligation process catalyzes the joining of the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive ended or blunt ended configuration. The set up for ligase reaction is given below.

Reagents	**Volume**
10x T4 DNA ligase buffer	1.0 µl
Vector DNA	x µl
Insert DNA	x µl
T4 DNA ligase	1.0 µl
HPLC-H_2O	Final vol. 10.0 µl

The ligation reaction was carried out with the optimum molar ratio of vector to insert (here 1:4). The ligation reaction was incubated at 16°C ON or at 4°C, depending on the size of the insert.

3.2.1.5 Transformation in chemically competent *E. coli*

Transformation is a process of introducing recombinant Plasmid DNA into host cells that generate together a replication-competent system. In the present work the method of choice was to transform DNA into competent bacterial cells [166]. The cell membranes of the competent cells allow them to uptake plasmid DNA. A certain volume of the ligation reaction was used to transform ligated DNA in One Shot® *E. coli* (TOP10 or Stbl3cells). Transformation of DNA in the chemically competent bacterial cells was achieved by heat shock method. This method promotes binding of plasmid DNA to the outer membrane of cells, renders the cell surface permeable to plasmid DNA and upon heat shock the DNA is uptaken by the *E. coli* cells.

Competent cells were thawed on ice for ~ 5−10 min. Around 1−5 µl of plasmid DNA (10 pg−100 ng) or 5−10 µl of ligation mixture was added to the thawed cells, mixed gently, and incubated on ice for at least 30 min. Thereafter the tubes incubated at 42°C for 30 seconds, immediately returned on ice and incubated for 2 min. Finally, 250 µl SOC medium was added to the cells to allow for developing the desired resistance introduced by the vector. The mixture was incubated at 37°C (shaking at ~450 rpm) for an hour.

3.2.1.6 Selection and expansion of the transformed bacteria

Since the used vectors carry a gene that mediates resistance against an antibiotic (for example Ampicillin), successfully transformed clones possess an advantage of selection. A volume of 100 - 200 µl from each transformation was spread on a pre-warmed selective plate (2x YT + Ampicillin) and incubated ON at 37°C.

Selected bacterial colonies were removed from the plate and resuspended in 5 ml of 2x YT media (supplemented with Ampicillin) and incubated (shaking at ~450 rpm) ON at 37°C.

3.2.1.7 Extraction of plasmids

The plasmid DNA was isolated using the modified alkaline lysis method [167]. Bacterial lysis was achieved by the treatment of Sodium dodecyl sulfate (SDS) and Sodium hydroxide (NaOH). The mixture was neutralized with Potassium acetate (CH_3COOK) causing the plasmid DNA to re-anneal rapidly. The recovery of plasmid DNA was performed by DNA adsorption on silica matrices [168].

For molecular biology grade plasmid DNA (QIAprep® spin miniprep kit) or endotoxin free transfection grade DNA (EndoFree® plasmid maxi kit) was extracted according to the manual, (QIAprep® miniprep handbook 05/2012 and EndoFree® plasmid purification handbook 05/2012). The concentration and quality of extracted plasmid DNA were estimated using agarose gel electrophoresis and NanoDrop.

3.2.1.8 Restriction endonuclease digestion of DNA

Restriction endonucleases recognize specific DNA sequences of 4–8 nucleotides in length and restrict DNA through hydrolyses of the phosphodiester bond. Plasmid DNA or PCR amplified DNA products were digested with one or more restriction enzymes at specific restriction sites for the analysis of DNA inserts and downstream purposes. Each restriction enzyme requires specific conditions for optimum activity. The DNA was treated with restriction enzyme(s) in appropriate buffer at correct temperature for the desired length of time, based on manufacturer's instructions. The reaction set up for a typical restriction endonuclease digestion is mentioned below.

Reagents	**Volume**
10x NEBuffer	1.0 µl
DNA	x µl
Restriction enzyme 1	1.0 µl
Restriction enzyme 2	1.0 µl
HPLC-H_2O	Final vol. 10.0 µl

Final concentration of DNA in the reaction was adjusted to 1 µg.

Finally the reaction was analyzed by agarose gel electrophoresis. For DNA samples that were supposed to be used for other manipulations, the reaction was purified using the QIAquick® PCR purification kit. The user's manual (QIAquick® Spin Handbook 05/2012) was followed.

3.2.1.9 Dephosphorylation of 5'-ends of plasmid DNA

Dephosphorylation of the 5'-ends of plasmid DNA is used to remove terminal 5'-phosphate groups. This process avoids self-ligation and re-circularization of plasmid DNA that has been linearized. The reaction set up for dephosphorylation process is given below.

Reagents	**Volume**
10x Antarctic phosphatase reaction buffer	1.0 µl
Plasmid DNA (cut)	x µl
Antarctic phosphatase (5 U/µl)	1.0 µl
HPLC-H_2O	Final vol. 10.0 µl

The reaction was incubated for 15 min at 37°C followed by heat inactivation for 5 min at 65°C. The linearized dephosphorylated plasmid DNA was used for ligation of desired DNA fragments.

3.2.1.10 Purification of DNA fragments from agarose gels

The QIAquick® gel extraction kit was used to isolate and purify a DNA fragment from an agarose gel following electrophoretic separation. This step is used to remove enzymes and buffers from the upstream processes. The user's manual (QIAquick® Spin Handbook 05/2012) was followed, the quality and quantity of purified DNA were accessed by agarose gel electrophoresis.

3.2.1.11 Site-directed mutagenesis

Site-directed mutagenesis (SDM) is an *in-vitro* technique that uses custom designed mutagenesis primers to introduce specific experimental mutations in a double-stranded DNA plasmid. SDM was first conceived by Kunkel in 1985 [169]. The QuikChange® II XL site-directed mutagenesis kit allows site-specific mutation in a double-stranded plasmid and

eliminates the need for subcloning and ssDNA rescue. SDM is carried out in three steps: (i) mutant strand synthesis by extension of mutagenesis primers with high fidelity *Pfu* Ultra DNA polymerase, (ii) *Dpn*I digestion of template and (iii) transformation. The reaction set up for mutant strand synthesis is mentioned below.

Reagents	**Volume**
10x Reaction buffer	5.0 µl
dNTP mix	1.0 µl
Primer 1 (25 ng/µl)	5.0 µl
Primer 2 (25 ng/µl)	5.0 µl
QuikSolution	3.0 µl
DNA template (final 10 ng)	0.5 µl
HPLC-H_2O	Final vol. 50.0 µl
Pfu Ultra DNA-Polymerase (2.5 U/µl)	1.0 µl

The thermal cycling conditions are given below.

	Temperature	**Time**
Step 1	95°C	1 min
Step 2 (18 cycles)		
Denaturation	95°C	50 sec
Annealing	60°C	50 sec
Extension	68°C	2 min/kb plasmid length
Step 3	68°C	7 min
Hold	4°C	∞

Following amplification of the mutation carrying DNA, the restriction enzyme *Dpn*I was used to digest the non-mutated parental methylated and hemimethylated DNA [170-171]. 1 µl of *Dpn*I (10 U/µl) was added to each amplification reaction and incubated at 37°C for an hour. Subsequently, the reaction was transformed into chemically competent *E. coli* cells.

3.2.1.12 Sequencing of DNA

DNA sequencing is a method to accurately define the nucleotide sequence of a given DNA section. The method used in this work is based on the chain termination-(dideoxy) method [172]. Here, primer elongations on a single strand DNA-matrice take place in a PCR-sequencing reaction with deoxynucleotides (dATP, dCTP, dGTP, dTTP). Fluorescence

labeled 2'-3'-dideoxynucleotide-5'-triphosphates (ddATP, ddCTP, ddGTP, ddTTP) are added to the DNA matrice, during the reaction their integration in the nucleotide chain leads to a base-specific termination of the reaction due to the lack of the 3´-OH-group for the 5´–3´-phosphodiester binding. The integration of dideoxynucleotides occurs stoachistic according to their concentration in the sequencing reaction that is significantly lower than that of the deoxynucleotides. Therefore, DNA fragments are generated, that are distinguished in their length each at one base pair. A laser detects the time delay of the fluorescence labeled products.The generated electropherograms are evaluated with appropriate software. The reaction setup for the sequencing reaction is mentioned below.

Reagents	**Volume**
BigDye®Terminator ready reaction mix	2.0 µl
DNA template	Variable
Sequencing primer (1 pmol)	1.5 µl
HPLC-H_2O	Final vol 10.0 µl

Final concentration of DNA in a sequencing reaction was adjusted to 150–300 ng.

The thermal cycling conditions are given below.

	Temperature	**Time**
Step 1	94°C	1 min
Step 2 (35 cycles)		
Denaturation	96°C	10 sec
Annealing	50°C	5 sec
Extension	60°C	4 min
Hold	4°C	∞

*Rapid thermal ramp is 1°C/sec.

Purification of sequencing reaction products

The sequencing reaction products were purified on Sephadex™. Approximately 1g Sephadex™ G-50 was dissolved in ~12 ml HPLC grade water and transferred to Micro-spin™ G-50 columns (about 3/4th full, ~1 ml per tube). The columns were centrifuged at 2500 rpm for 3 min at RT, the flowthrough discarded and the column was placed in a fresh microcentrifuge tube. The sequencing reaction sample was carefully added onto the centre of the gel material.

The columns were spun down at 2500 rpm for 2 min at RT to elute the sequencing reaction products. Appropriate volume of each purified sequencing reaction products were loaded into wells of MicroAmp® 96-well reaction plate and final volume was adjusted to 30 µl with water. The purified sequencing products were transferred to a capillary electrophoresis system (ABI Prism 3730 DNA analyser), the results were analysed utilizing the Data collection software v2.0.

3.2.2 Recombinant eukaryotic cell lines

LCL.TPN-, LCL.TPN+ or T2.TAP- cells were transduced with lentiviral particles encoding for recombinant mHLA-B*35/156 (Exon 1–7) or sHLA-B*35 (Exon 1–4) molecules. The mRNA levels of HLA-B*35/156 variants was quantified by real time PCR, surface expression of HLA-B*35/156 variants was assessed by flow cytometry, expression of sHLA-B*35/156 in the cell culture supernatant was quantified by ELISA.

3.2.2.1 Cultivation of human cell lines

The cell culture was performed under sterile conditions. The human lymphoblastoid cell lines (LCL.TPN-, LCL.TPN+ and T2.TAP-) grow in suspension cultures and were maintained in 10 % RPMI medium. The adherent HEK293T cells were cultured in 10 % DMEM medium.

All cells were cultivated in a CO_2 incubator at 37ºC, 5 % CO_2 and 99 % humidity. The cells were maintained in the logarithmic growth phase by routine subculture techniques and fed with fresh medium to replenish nutrients and keep a physiological pH (~7.4).

3.2.2.2 Determination of cell viability and density

The cell viability was determined by the Trypan blue exclusion process using the Neubauer counting chamber. It is based on the principle that live cells possess intact cell membranes that exclude Trypan blue dye, while non-viable cells with ruptured cell membrane will uptake the dye. A 1:1 dilution of the cell suspension and Trypan blue dye was prepared using a 0.4 % Trypan blue solution. The Neubauer counting chamber is a microscopic slide especially

designed to enable cell counting. The cover slip was placed on the central counting area of the Neubauer chamber. A volume of 10 µl sample was dispensed carefully on the loading groove, avoiding air bubbles. The central area of the Neubauer chamber consists of counting grids and each counting grid of the Neubauer chamber has 9 square subdivisions. Each of these big squares has the dimension of 0.1 mm depth and 1 x 1 mm^2 area. Each peripheral square is further subdivided into 16 small sub-squares (each small subsquare with the dimension of 0.1 x 0.25 x 0.25 mm^3). The cells were observed under the microscope and counted in all four peripheral squares. For an accurate determination, the total number of cells overlying one 1 mm^2 should be between 20–50 cells/square. The cell density was calculated using the following formula.

$$\text{Concentration (cells/ml)} = \frac{\text{Number of cells}}{\text{Number of squares x Volume of each square } (cm^3) \text{ x dilution factor}}$$

When the cells are counted on the peripheral squares, the formula can be derived as following.

$$\text{Concentration (cells/ml)} = \frac{\text{Number of cells x 10,000}}{\text{Number of squares x dilution factor}}$$

3.2.2.3 Cryopreservation of human cells

The human cells used in this study were cryopreserved using the freezing solution that contains the cryoprotective agent Dimethylsulfoxide (DMSO). The composition of the medium is given in chapter 3.1.12. All the procedures for cryopreservation were performed on ice. The number of cells and viability were determined using the Neubauer chamber and Trypan blue exclusion method. The cells were harvested and resuspended in cold freezing medium, aliquots of 1 ml cell suspension were dispensed into cryogenic storage vials and placed in Nalgene® Cryo 1°C isopropanol freezing container ON at -80°C and later stored at -150°C.

For revival of the frozen cells, the cryogenic vials were taken out of -150°C freezer and quickly thawed at 37°C. The cells were resuspended in PBS and spun down to remove DMSO. The cells were resuspended in fresh media.

3.2.2.4 Transfection of HEK293T cells for lentivirus production

Human embryonic kidney (HEK) cells are the most widely used mammalian expression system for production of recombinant proteins. The biochemical machinery of HEK293T cells is capable of carrying out most of the post-translational folding and processing required to generate functional, mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acids [173]. HEK293T cells are easy to transfect and efficient in production of lentiviral particles.

Lipofectamine® 2000 is a cationic liposome formulation used for the transfection of nucleic acids into eukaryotic cells. Lipofectamine's cationic lipid molecules are provided with a neutral co-lipid (helper lipid) that helps in formation of liposomal vesicles. These vesicles aid in the transfer of nucleic acid in the cytoplasm and make them available for replication or expression. A second generation packaging system (packaging plasmid psPAX2 and VSV-G envelope expressing plasmid pMD2G) was used for the production of lentiviral particles in HEK293T cells.

Approximately 5 x 10^6 HEK293T cells were plated on each 10 cm petri dish in 5 ml complete media. It is important to make sure that the cells have a low passage number and are healthy with greater than 90 % viability. For transfection, endotoxin free plasmid DNA was used (see chapter 3.2.1). 10 µg transfer vector along with 5 µg packaging plasmid (psPAX2) and 5 µg envelope plasmid (pMD2G) were incubated with Lipofectamine® 2000 reagent (Invitrogen) for 20 min at RT, finally the mixture was added to plates coated with 5 X 10^6 HEK293T cells. The medium was exchanged after 8 hours to avoid toxic side effects of Lipofectamine® 2000 reagent. The plates were incubated further at 37°C for 24 hours. Finally, supernatants containing lentiviral particles were filtered through a 0.45 µm Millex® syringe filter units to remove HEK293T cells and cellular debris, the virus was concentrated by centrifuging overnight at 4°C, 15,000 rpm. The supernatants were carefully removed and the virus pellet resuspended in 1 ml complete media.

3.2.2.5 Lentiviral transduction of target cells

1 x 10^6 target cells were resuspended in the virus suspension and plated in 24 well tissue culture plates. 8 μg/ml protamine sulfate was added to each well and the cells were incubated with the virus for 8 hours at 37°C, finally the virus was removed and the media was exchanged. Transduced cells were maintained in complete RPMI medium supplemented with 10% FCS, as described in chapter 3.2.2.1.

3.2.2.6 Analysis of HLA-B*35/156 surface expression

Flow cytometry is a laser based technology that measures multiple biophysical properties of cells including particle's relative size, relative granularity or internal complexity and relative fluorescence intensity. A flow cytometer comprises of three main systems: (i) the fluidics system that transports particles or cells in a stream to the laser beam, (ii) the optics system that consists of lasers to illuminate the particles in the sample stream and optical filters to direct the light signals to the detectors and (iii) the electronics system that converts the detected light signals into electronic signals that can be processed by the computer [174].

Surface expression of HLA-B*35/156 molecules on transduced cells was analyzed by flow cytometry using the antibodies anti-bw6-FITC and W6/32-PE. Anti-bw6 recognizes the bw6 epitope on HLA-B*35 molecules while W6/32 recognizes trimeric HLA-A/B/C-β2m-peptide complexes. A total of 0.5 x 10^6 cells were washed twice with PBS containing 0.5 % BSA and then incubated with 5 μl of each antibody for 30 min at 4°C. Following incubation, the cells were washed twice with PBS and data was acquired using FACS Canto A (BD Biosciences). Flow cytometry analysis was performed after proper instrument setting and compensation. The data were analyzed using BD FACSDIVA™ software and FlowJo software (version 7.6.5). The results were interpreted in terms of Median florescence intensity (MFI). The MFI of the sample was then normalized to the MFI of the negative control (untransduced cells) to obtain the normalized MFI (nMFI). The nMFI was calculated using the following formula [175].

$$nMFI = \frac{MFI_{sample}}{MFI_{negative\ control}}$$

Statistical analyses were performed using GraphPad Prism software (version 5.0) [176]. The One way ANOVA was used to test the significant differences on the surface expression between the HLA-B*35/156 groups. The Bonferroni and Tukey's multiple comparison tests were used to compare the surface expression between the HLA-B*35/156 groups. The *p values* <0.05 were considered significant.

3.2.2.7 Quantitative detection of sHLA-B*35/156 molecules

An enzyme linked immune sorbent assay (ELISA) is a method to detect antibodies as well as antigens [177-178]. On a solid phase, in most cases a polystyrene micro-titer plate, one of the reaction-partners, either antibody or antigen, is bound. In the following a probe containing the other reaction-partner that is coupled to an enzyme will be added and the binding between antibody and antigen can be detected through an enzymatic catalysation, leading to a color change of the transparent substrate added to the reaction. The color change can be measured at a given wavelength in a photometer.

For quantitative verification of sHLA molecules in the supernatant a DAS-ELISA (Double antibody sandwich ELISA) was applied. An antibody is bound to a solid phase, to that antibody the searched antigen was added. Finally a detection antibody that is specific for another epitope of the antigen was added. The detection antibody is bound to an enzyme, that catalyzes the color change of the transparent substrate.

To detect sHLA molecules in the supernatant of recombinant cells, a mouse anti-human HLA-A/B/C mAb (W6/32) was used as a capture antibody. W6/32 is an anti-HLA-A/B/C antibody [179-180], recognizing all HLA class I molecules that are associated with ß2m. W6/32 binds to the AA Lys at position 121 of the hc and residues 45 and 89 of ß2m. The rabbit anti-human β2m pab was used as the detection antibody. A horseradish peroxide (HRP) conjugated goat anti-rabbit IgG pab was used as secondary detection antibody. The spectrophotometric analysis of ELISA plates was carried out by using the Gen5™ data analysis software. The

clones with the highest expression of sHLA-B*35/156 molecules were used for large scale production.

3.2.3 Biophysical analysis of HLA-B*35/156 interactions with PLC

Immunoprecipitation experiments were performed in order to analyze the differential interaction of the HLA-B*35/156 hc and distinct PLC components. An anti-TAP1 pab was used to target protein complexes, followed by western blot analysis using antibodies against HLA-B hc, TPN, CRT or ERp57.

3.2.3.1 Immunoprecipitation of HLA-B*35 and PLC complexes

Immunoprecipitations (IPs) were utilized to study the interactions of HLA-B*35/156 hc with certain components of the PLC. IP is one of the widely applied immunochemical technique that allows precipitation of protein complexes by targeting one of the reaction partners. A selected antibody recognizing one of proteins is immobilized on a solid phase as sepharose beads. Cell lysates are added to capture and immobilize the immune complex on the beads. Any proteins not precipitated on the beads are washed away. The protein complex is then eluted from the beads and dissociated by using NuPAGE® LDS sample buffer. Following SDS-PAGE samples were analyzed utilizing western blot.

A total of 1 x 10^7 mHLA-B*35/156 expressing LCL.TPN- or LCL.TPN+ cells were lysed for 30 min on ice in Digitonin lysis buffer. The lysates were collected by centrifugation at 13,000 rpm for 15 min at 4ºC and pre-cleared with Protein A-sepharose beads CL-4B for an hour. The immunoprecipitation was performed with Protein A-sepharose beads CL-4B covalently coupled to rabbit anti-TAP1 pab for an hour at 4ºC. After series of washing, the supernatants from wash buffers were removed and 25–50 µl of 2x NuPAGE® LDS sample buffer was added. The mixture was boiled at 95°C for 20 min to recover the immunoprecipitates bound to the sepharose beads.

Sodium dodecylsulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The principle of Sodium dodecylsulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) is based on the method described by Laemmli in 1970 [181]. During heat denaturation of proteins in the presence of an SDS and DTT excess, a complex of SDS and protein is formed, which negative charge is proportional to the proteins mass. In an electric field, the molecular mesh effect of a polyacrylamide matrix separates SDS-protein complexes according to their stokes-radius and their velocity of migration is logarithmic to the molecules size. Immunoprecipitates were applied on a NuPAGE Novex® 4–12 % Bis-Tris protein gel. The separation of proteins was performed at 125 V and stopped when the bromphenolblue front reached the lower border of the gel.

Western Blot

The principle of western blot is based on the method described by Towbin *et al* in 1979 [182]. Western blotting is a technology that enables the transfer of electrophoretical fractionated proteins on a PVDF membrane. The product is an exact replica of the separated molecules by SDS-PAGE. Under certain conditions, the immunreactivity of proteins might be preserved. Using specific antibodies, the proteins are detectable directly on the membrane [183].

Following SDS-PAGE transferred to PVDF membranes (iBlot® transfer stack). Western blot analysis was performed using the iBlot®Gel Transfer Device 7 minute iBlot™ blotting system according to user manual for iBlot® 2 Dry B lotting System version 15 December 2013. The blots were incubated in blocking solution (3 % skimmed milk in PBS) for an hour at RT to avoid any non-specific binding and then with a specific HRP-conjugated (rabbit anti-CRT pab) or unconjugated (mouse anti-Erp57 mab, goat anti-HLA-B pab or goat anti-TPN pab) primary antibodies for an hour at room temperature. Following primary incubation, the blots were washed three times with PBST and then incubated for an hour with respective HRP-conjugated secondary antibodies. The blots were developed using Roti®-Lumin substrate and digital images were obtained by exposing the membranes to a chemiluminescent FlorChem™ imaging system with automatic image capture. Alternatively, the blots can be developed using TMB substrate for quick visualization.

3.2.3.2 Coupling of antibody with horseradish peroxidase (HRP)

Anti-TAP1 pab that was used for immunoprecipitation of the PLC was raised in rabbit. Detection of CRT component using rabbit anti-CRT pab (primary) and anti-rabbit IgG-HRP (secondary) would lead to nonspecific and false positive reaction related to rabbit anti-TAP1 pab. Due to this reason, rabbit anti-CRT pab was directly coupled with HRP using EZ-Link™ plus activated peroxidase kit, as described in manufacturer's instruction leaflet. In order to remove free amino groups, 20 µl of anti-CRT antibody was dialysed and desalted in 0.2 M Carbonate bicarbonate buffer (pH 9.4) at 4ºC for an hour. Peroxidase solution was prepared by dissolving 1mg of lyophilized EZ-Link plus activated peroxidase powder in 100 µl of ultrapure water. Then 1.5 µl of this peroxidase was mixed with the desalted antibody and incubated at RT in dark for an hour. After incubation, 0.5 µl of Sodium cyanoborohydride was added and incubated at RT for 15 min. Finally the reaction was stopped by adding 1 µl of quenching buffer. The reaction was left to stand at RT for 15 min. The antibody HRP conjugate can be stored at 4°C for up to 4 weeks.

3.2.4 Large scale production of sHLA-B*35/156 molecules

The controlled mass production of secretory proteins by eukaryotic cells is very sensitive due to the high requirements that those cells have towards nutrients and cultivation conditions as pH, temperature and oxygen maintenance. Furthermore, those cells are sensitive towards shearing forces and toxic products of metabolism. The large scale production of sHLA-B35/156 molecules was performed in the bioreactor CELLine. In this bioreactor system the protein concentration increased from 10 µg/ml up to 80 µg/ml.

sHLA-B35*/156 molecules producing B-LCLs cells were expanded in two compartment bioreactors CELLine classic 1000 and supernatants were harvested weekly. Supernatants were tested for production of sHLA-B*35 molecules by sandwich ELISA as described in chapter 3.2.2.7. The supernatants, containing the sHLA-peptide complexes were affinity purified using N-hydroxysuccinimide (NHS)-activated HiTrap columns.

3.2.4.1 Cultivation of B-LCLs in a two compartment bioreactor

The bioreactor CELLine comprises of two compartments, cell or intracellular compartment and medium or extracellular compartment. The two compartments are separated by 10 kDa semi-permeable, cellulose acetate membrane that allows the diffusion of secreted molecules among these compartments. Optimal cell vitality was achieved by supplementation of cell culture medium in the extracellular space with 5 % RPMI and in the intracellular space with 10 % RPMI.

After the inoculation of the cells into the bioreactor, the first harvest was done after 10–14 days depending on the cell density in the cell compartment. After the first harvest, the supernatants were collected weekly. In order to harvest the cells, the medium from the extracellular compartment was aspirated using a VACUSAFE vacuum pump and discarded. The supernatant from the intracellular compartment containing sHLA molecules was harvested using a 25 ml serological pipette. After harvesting 20 ml of 10 % RPMI was transferred into the intracellular compartment, 1000 ml of 5 % RPMI was transferred to the extracellular compartment. Supernatants were frozen at -20°C until further processing.

3.2.4.2 Purification of sHLA molecules on an immobilized antibody

The use of NHS-activated sepharose as coupling reagents for affinity-chromatographic applications was first described by Cuatrecasas *et al.* in 1972 [184]. NHS-activated HiTrap columns are ready to use columns that are used to couple an appropriate ligand via Epichlorhydrin (3-Chlor-propylenoxide) spacer of 6-atoms bound to the sepharose matrix. This spacer is activated through N-hydroxysuccinimide. Primary amino groups of the antibody will be covalently bound to the activated spacer of the sepharose. To the column used in this work, the anti HLA-A/B/C mab (W6/32) was coupled.

The affinity purification was performed on the BioLogic DuoFlow system. Before starting the purification procedure, the BioLogic DuoFlow system was cleaned with 20 % ethanol followed by HPLC grade water. The 5 ml NHS-activated HiTrap column coupled to the W6/32 mab was assembled to the BioLogic DuoFlow System.

sHLA molecules bound to the column were eluted with 3 column volumes elution buffer. Five fractions with each fraction containing 3ml eluate were collected. The column was washed with 25 ml start buffer and 20 ml storage buffer. The column was stored in storage buffer at 4°C until further use. Aliquots of the elution fractions were tested for the presence of trimeric sHLA complexes by ELISA.

3.2.5 Mass spectrometric analysis of peptides

Mass spectrometry is an analytical technique to determine the mass of a molecule by measuring the mass-to-charge ratio (m/z) of its ion. A typical mass spectrometer consists of three components: an ion source, a mass analyzer, and a detector. The basic principle of a mass spectrometer revolves around the generation of multiple ions from the sample under investigation, followed by their separation according to the specific m/z [185]. The sHLA-B*35-bound high binding (HB) peptides as well as low binding (LB) peptides were recovered as described and subjected to Matrix assisted laser desorption/ionization-Time of flight (MALDI-TOF) mass spectrometry (MS) for quantitative estimation of peptides. Finally the peptides were sequenced using Eksigent nano-LC Ultra 2D HPLC coupled to LTQ Orbitrap ion trap mass spectrometry (Thermo Scientific) (Figure 3.3). MALDI-TOF-MS and nano-LC ultra 2D HPLC-MS/MS were performed by TOPLAB GmbH, Germany.

3.2.5.1 Isolation of sHLA-B*35/156-bound peptides

The eluate after affinity purification contains sHLA-B*35/156 trimeric complexes. This eluate was filtered through an Amicon ultra-15 filter unit with a molecular weight cut off (MWCO) of 10 kDa and the peptides detected in the flowthrough were considered to be of low binding (LB) affinity. The retentate was further acidified by treatment with 0.1 % trifluoroacetic acid (TFA) and filtered through a MWCO 10 kDa filter to elute the peptide of high affinity (HB).

3.2.5.2 Quantitative analysis of peptide pools

The general principle of MALDI-TOF-MS involves the volatilization of an analyte embedded in an UV-absorbing matrix followed by time-of-flight (TOF) mass spectrum analysis [186]. Upon the laser irradiation of the mixture of sample and matrix, the matrix absorbs the ultraviolet light (nitrogen laser light, wavelength 337 nm) and converts it to heat energy. This causes the heating and vaporisation of the sample and subsequent generation of charged ions of various sizes on the sample slide. A potential difference V_0 between the sample slide and ground induces the transfer of these ions, consequently the time of ion flight to reach the detector depends on their m/z values. Since its initial application by Karas *et al.* in 1987 [187] and Tanaka *et al.* in 1988 [188], MALDI-TOF-MS is currently being used for analysis of wide variety of biomolecules. MALDI-TOF-MS can be used for the identification of proteins by the so-called peptide mass mapping or peptide mass fingerprinting technique. In this study, MALDI-TOF-MS can be used for quantitative analysis of peptide pools. A saturated solution of peptide sample and matrix was prepared. The peptide composition of HPLC fractions was analyzed by MALDI-TOF-MS in the linear, positive ion and reflector mode.

3.2.5.3 Sequencing of peptides

The peptides were analyzed by a capillary Eksigent HPLC system equipped with reverse phase PepMap™ C18 column (75 µm internal diameter and 3 µm beads) and C18 trap column (0.3 x 10 mm). HPLC grade degassed solvents (solvent A: 98% deionized water with 2% acetonitrile and 0.1% acetic acid, solvent B: 98% acetonitrile with 2% water and 0.1% acetic acid) were used as mobile phase solvents. The solution containing peptides was transferred to a sample vial and placed into the loading tray at 4°C. Autosampler device settings were regulated to adjust the loop volume. Volumes of 10 µl sample were loaded onto the C18 trap column. If the samples were much diluted, the peptides were enriched by using ZipTip® SCX tip device. After back flushing from the trapping column, the sample was loaded on a reverse-phase C18 column. The peptides on the capillary reverse phase C18 column were separated with increasing acetonitrile concentration.

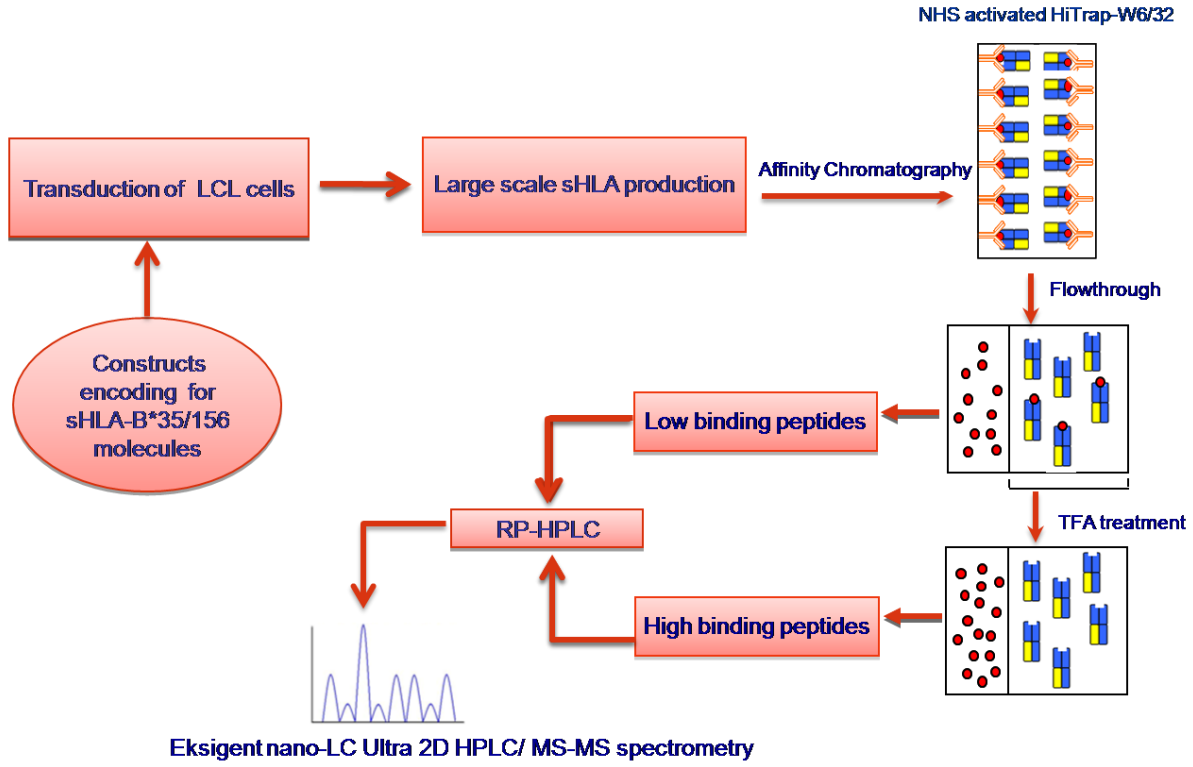
The Eksigent HPLC system was on-line connected to the electrospray LTQ-Orbitrap XL mass spectrometer. The ion source emitter was positioned to initiate and maintain a stable

electrospray. The spray tip was inspected properly as any form of tip blockade may result in an unstable electron spray and negative impact on the detection of peptides in the mass spectrometer. The LTQ Orbitrap mass spectrometer was operated in a data dependent mode, automatically switching between MS and MS/MS acquisition. Full-scan mass spectra were acquired with the LTQ Orbitrap XL mass spectrometer and the three most abundant precursor ions were selected for fragmentation by CID.

3.2.5.4 Analysis of mass spectrometric data

From the MS/MS data in each LC run, Mascot generic files were created using Mascot software (http://www.matrixscience.com). The mass spectrometry data were then served on Mascot server via a Mascot Daemon interface (www.matrixscience.com). Database queries for peptide sequences and peptide source were carried out by Mascot software [189] using the SwissProt 2012_11 human and the respective decoy databases. UniProtKB/Swiss-Prot 2012_11 release of 28-Nov-12 contains 538585 sequence entries, comprising 191240774 AAs abstracted from 215068 references (http://www.uniprot.org).

Figure 3.3 Isolation of sHLA-B*35/156-bound peptides and mass spectrometric analysis



sHLA-B*35 molecules were generated by soluble HLA technology [190]. Constructs encoding for sHLA-B*35/156 molecules were transduced into LCL.TPN- or LCL.TPN+ cells. Supernatant containing sHLA molecules was purified by affinity chromatography on immobilized antibody (mab W6/32). Trimeric complexes were eluted using 0.1 M Glycine/HCl buffer (pH 2.7). Elution fractions were filtered through a 10 kDa MWCO membrane to obtain low binding (LB) peptides. The retentate containing trimeric complexes was then treated with 0.1 % Trifluoroacetic acid (TFA) to elute high binding (HB) peptides. Peptides were purified by RP-HPLC and subjected to mass spectrometry using an Eksigent nano-LC Ultra® 2D HPLC coupled to an Orbitrap ion trap for peptide sequencing.

3.2.6 Structural analysis of mismatches

The molecular visualization software program (PyMOL) (http://www.pymol.org) was used to generate the 3D computational graphics of HLA molecules. The list of crystallographic structures used for the computer simulation is listed in Table 3.17. The crystallographic structures were retrieved from the Research collaboratory for structural bioinformatics (RCSB) Protein data bank (PDB) (www.rcsb.org/pdb). There is currently no structure of HLA-B*35:62 available, in order to understand the structural implication of the micropolymorphism at position 156 on HLA-B*35 molecules, a computational simulation was performed to exchange AAs at position 156. The structures of HLA-B*35:01 (2AXG) [37]

was overlaid with that of B*35:08 (2AXF) [37], a model of B*35:62 was generated by mutation of Leu at 156 (B*35:01, 2AXG) to Trp using YASARA/FoldX software.

Table 3.18 List of crystallographic structures used for computer simulation

PDB	HLA-peptide	Peptide source	Reference
2AXG	HLA-B*35:01^{APQPAPENAY}	EBV BZLF1	Tynan *et al*., 2005 [37]
2AXF	HLA-B*35:08^{APQPAPENAY}	EBV BZLF1	Tynan *et al*., 2005 [37]

3.2.7 Bioinformatics tools for prediction of viral epitopes

HLA-B*35/156 variants are clinically relevant as they have been shown to bind the various viral epitopes, implicating that these alleles are able to overcome viral immune evasion strategies. Moreover, the results of this study show that these alleles were able to present peptides independent of TPN and TAP, indicating that they would be able to present peptides while these PLC components are down regulated during viral infections. The knowledge about the peptide repertoire of HLA-B*35/156 variants enables the prediction of viral derived epitopes. Those predicted viral epitopes would help to shed light on the structural impact of position 156 on viral epitope binding as well as would provide a prerequisite for T cell based anti viral therapies [191-193].

Table 3.19 List of prediction tools

Name	Source/Reference	Utility
SYFPEITHI	(http://www.syfpeithi.de) [140]	Prediction of probable epitopes likely to be presented by a defined HLA type for a sequence of AAs
Immune epitope database (IEDB)	(http://www.iedb.org) [146]	Prediction of set of epitopes and their ability to bind to a specific HLA molecule
MHC-I antigen presentation prediction (MAPPP/FragPredict)	(http://www.mpiib-berlin.mpg.de) [155]	Prediction of epitopes likely to be cleaved by the proteasomal enzymes

3.2.7.1 SYFPEITHI and IEDB prediction tools

The anchor motif data from HLA-B*35/156 derived self peptides were used to predict the probable epitopes. Certain prediction tools are based on the databases for peptide motifs from self peptides. SYFPEITHI (http://www.syfpeithi.de) and IEDB (http://www.iedb.org) epitope prediction tools were used to determine the possible immune epitopes of viral immune evasion proteins likely to be presented by HLA-B*35:01.

3.2.7.2 Proteasomal cleavage prediction tools

However, there are no such databases available for epitope prediction of HLA-B*35:08 or HLA-B*35:62 restricted peptides. A different approach was used to predict the likely viral epitopes presented by these alleles upon infection. The proteasomal cleavage prediction tools together with the knowledge of anchor motif from the experimental data in this study were employed to predict the probable viral epitopes likely to be presented by these alleles. Proteasomal cleavage prediction tools estimate the efficiency of a peptide or its N-terminally prolonged precursors to be liberated from its source protein. In this study, MAPPP (FragPredict) [155] was used to generate assortment of peptides that are most likely to be produced by proteasomal cleavage. Based on the anchor motif data obtained identified in this study, the potential viral epitopes that are likely to be naturally presented by HLA-B*35:08 and B*35:62 were predicted.

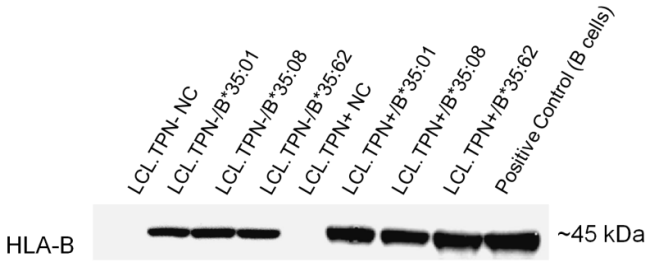
4 RESULTS

4.1 Transduction of the lentiviral constructs in the target cells

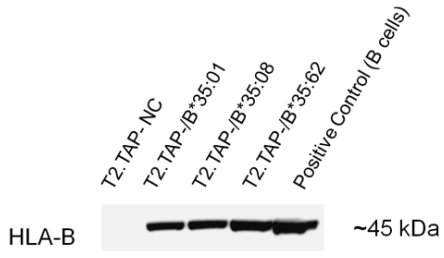
pRRL.mHLA-B*35/156 and pRRL.sHLA-B*35/156 vectors were constructed successfully and transduced in the target cells: LCL.TPN- (LCL 721.220, HLA-/TPN-/TAP+), LCL.TPN+ (LCL 721.221, HLA-/TPN+/TAP+) or T2.TAP- (HLA-/TPN+/TAP-). The transfection grade endofree plasmids encoding for mHLA-B*35/156 and sHLA-B*35/156 were used to transfect HEK293T cells for production of lentiviral particles. Following transfection, the targets cells were transduced with lentivirus encoding for mHLA-B*35/156 (Exon 1–7) or sHLA-B*35 (Exon 1–4) molecules. The protein expression of mHLA-B*35/156 hc in the transduced cells was determined by western blot using HLA-B specific antibody (Figure 4.1). The expression of sHLA-B*35/156 molecules was verified by sandwich ELISA. Surface expression of pHLA complexes, in the cells transduced with constructs encoding for mHLA-B*35/156 hc, were assessed by flow cytometry.

Figure 4.1 mHLA-B*35/156 hc expression in the target cells

A



B



Western blot analysis of HLA-B in the target cells. Cell lysates were extracted from target cells trasduced with vectors encoding for mHLA-B*35/156 hc and analysed by western blot technique using anti-HLA-B (Article N-20) antibody. A) Protein expression of mHLA-B*35/156 variants in LCL.TPN- and LCL.TPN+ cells. B) Protein expression of mHLA-B*35/156 variants in T2.TAP- cells. B cells from HLA-B*35 positive donor were used as positive controls. Annotations: NC - negative control (untransduced cells).

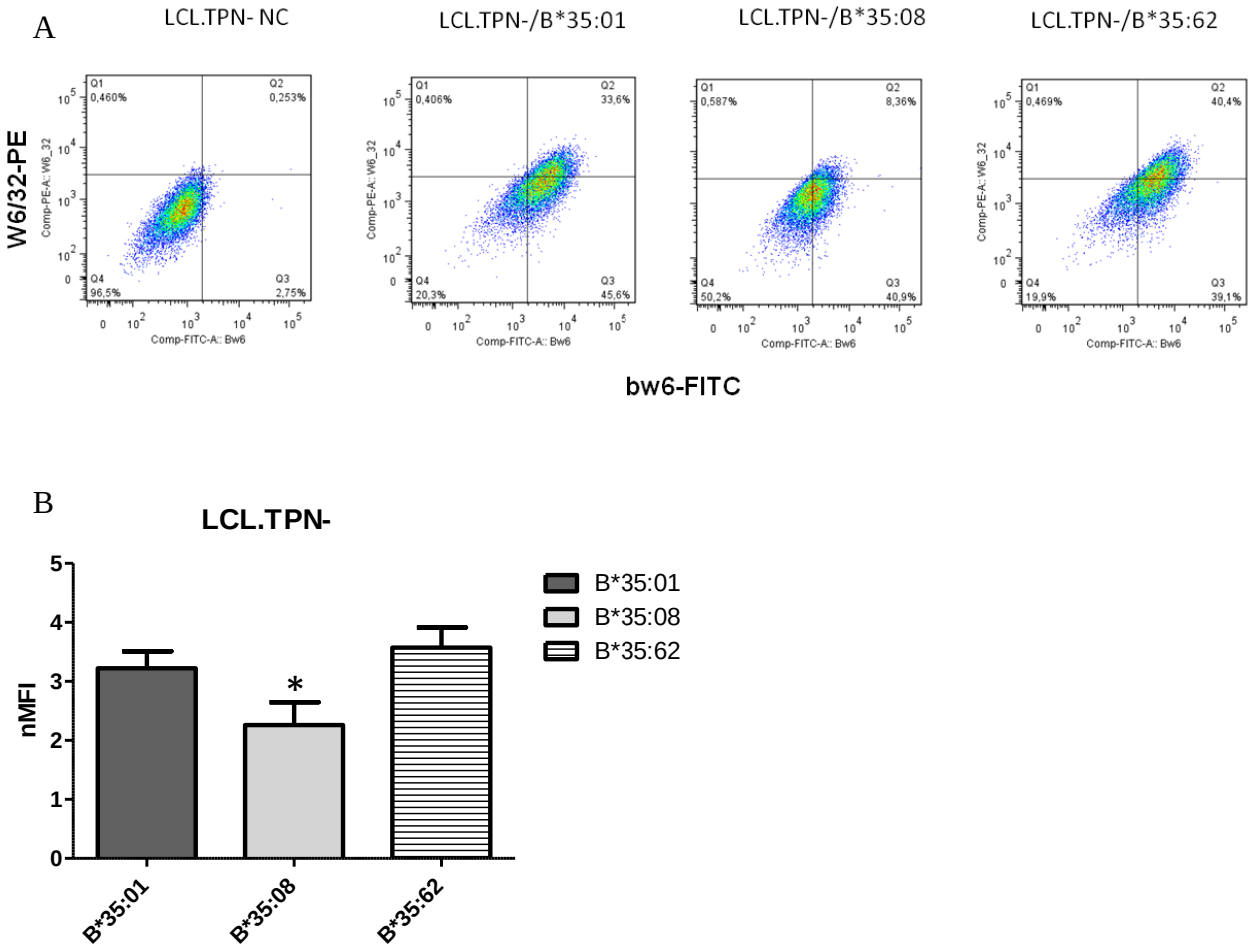
Analysis of surface expression of HLA-B*35/156

To understand the impact of micropolymorphism at position 156 in HLA-B*35/156 variants on TPN or TAP dependence, LCL.TPN-, LCL.TPN+ or T2.TAP- cells were transduced with mHLA-B*35/156 variants. Surface expression of mHLA molecules on the transduced cells was analyzed by flow cytometry using anti-bw6-FITC and anti-HLA-A/B/C-PE (W6/32-PE) antibodies. Trimeric complexes of HLA hc, β2m and peptide were recognized by W6/32 antibody, while the anti-bw6 antibody recognizes the bw6 epitope on HLA-B*35 molecules.

4.1.1 Surface expression on LCL cells

Flow cytometric analysis showed differential surface expression of mHLA-B*35/156 molecules on LCL.TPN- cells. All allelic B*35/156 variants were able to load peptides independent of TPN. However, the surface expression of mHLA molecules loaded with a peptide was found to be influenced by the type of polymorphism at position 156. The expression of mHLA-B*35:08 was found to be comparatively low in the absence of TPN compared to HLA-B*35:01 and B*35:62. This data illustrates that the surface expression of HLA-B*35:08 is relatively more TPN-dependent than the surface expression of the other allelic variants investigated. Data from a representative experiment, of at least three independent ones is shown in Figure 4.2A. Data represented in terms mean $\pm$ SD of nMFI values are shown in Figure 4.2B.

Figure 4.2 Surface expression of mHLA-B*35/156 on LCL.TPN- cells

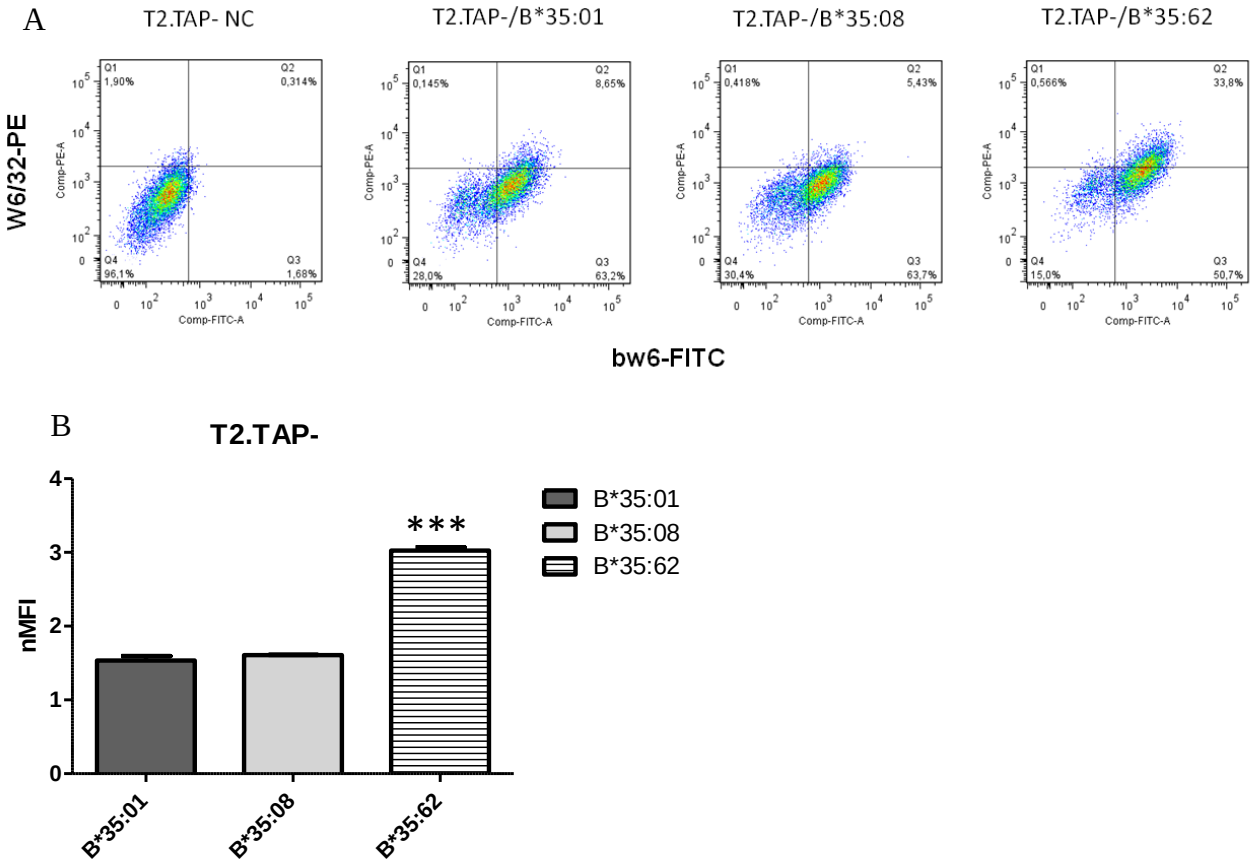


FACS analysis of mHLA-B*35/156 variants on LCL.TPN- (LCL 721.220, HLA-/TPN-/TAP+). A) Cells were analyzed for surface expression of mHLA complexes using anti-bw6-FITC and anti-HLA-A/B/C-PE (W6/32-PE) labeled monoclonal antibodies. A higher amount of molecules could be detected for mHLA-B*35:01 and B*35:62 on LCL.TPN- cells in comparison to the amount of B*35:08 molecules. B) Bar diagram showing mean ± SD of normalized Median florescence intensity (nMFI) values measured in terms of anti-HLA-A/B/C-PE (W6/32-PE) antibodies from three independent experiments. Significant difference was observed with the surface expression of HLA-B*35:08 compared to B*35:01 and B*35:62. Annotations: NC - negative control (untransduced cells). Stars indicate the level of significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.1.2 Surface expression on T2.TAP- cells

Flow cytometric analysis showed differential surface expression of mHLA-B*35/156 molecules on T2 cells. Results demonstrated that the surface expression of mHLA-B*35:62 was relatively more independent of TAP compared to those of mHLA-B*35:01 and B*35:08. Significant difference was observed with the surface expression of HLA-B*35:62 compared to B*35:01 and B*35:08. Figure 4.3A illustrates the FACS data from a representative experiment, carried out at least three times and Figure 4.3B represents the statistical significance of the surface expression in terms of nMFI.

Figure 4.3 Surface expression of mHLA-B*35/156 on T2.TAP- cells



FACS analysis of mHLA-B*35/156 variants on T2.TAP- (HLA-/TPN+/TAP-)cells.A) Cells were analyzed for surface expression of mHLA complexes using anti-bw6-FITC and anti-HLA-A/B/C-PE (W6/32-PE) labeled monoclonal antibodies. Flow cytometric analysis show relatively higher surface expression of HLA-B*35:62 compared to B*35:01 and B*35:08, thereby indicating that the surface expression of B*35:62 is comparatively more independent of TAP. B) Bar diagram showing mean ± SD of normalized Median florescence intensity (nMFI) values measured in terms of anti-HLA-A/B/C-PE (W6/32-PE) antibodies from three independent experiments. Significant difference was observed with the surface expression of HLA-B*35:62 compared to B*35:01 and B*35:08. Annotations: NC - negative control (untransduced cells). Stars indicate the level of significance * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

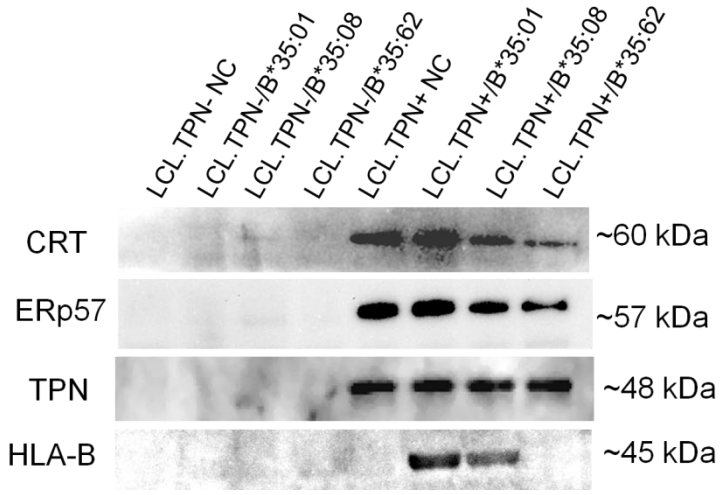
4.2 Analysis of biophysical interaction of HLA-B*35 /156

HLA class I hc assemble in the ER with β_2m and peptide to form pHLA heterotrimeric complexes. The assembly of stable pHLA complex and its surface expression requires its biophysical association with components of PLC, in particular with TPN and TAP for acquiring high affinity peptides.

To determine the impact of mismatch at position 156 on the association of HLA-B*35/156 variants with TAP in the presence or absence of TPN, LCL.TPN- or LCL.TPN+ cells expressing mHLA-B*35/156 variants were immunoprecipitated with an anti-TAP1 antibody. The immunoprecipitates were analyzed by western blotting using antibodies against the HLA-B hc and selected PLC components. The association of mHLA-B*35/156 molecules and TAP could not be detected in TPN-deficient cells, indicating the significance of TPN in bridging the TAP and HLA molecule. However, a strong association of mHLA-B*35:01 and B*35:08 with TAP was demonstrated in TPN-competent cells. Surprisingly no such interaction could be detected for mHLA-B*35:62, indicating its complete PLC-independent mode of peptide loading. All the experiments were performed at least three times. Figure 4.4 shows a representative result for one of the experiments.

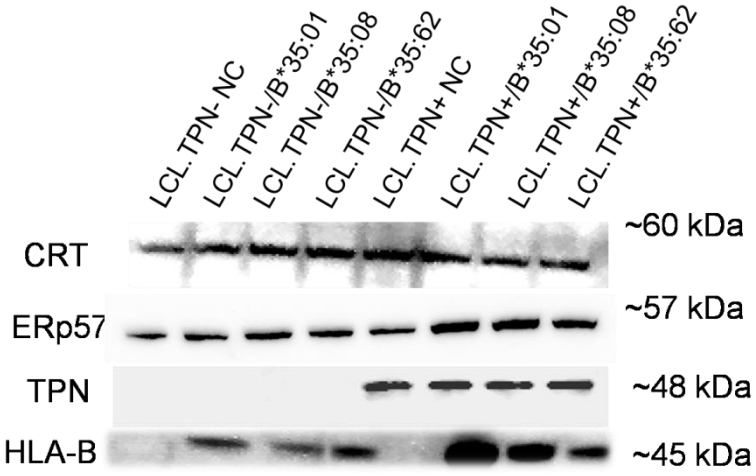
Figure 4.4 Biophysical interaction of mHLA-B*35/156 molecules with PLC components

A



Immunoprecipitation with anti-TAP1 antibody

B



Lysate controls

Immunoprecipitations with lysates from LCL.TPN- and LCL.TPN+ cells transduced with full length (m)HLA-B*35/156 constructs. The lysates of the cells were immunoprecipitated with anti-TAP1 antibody. The immunoprecipitates were then immunoblotted with antibodies against HLA-B hc, CRT, ERp57 and TPN. A) IP using anti-TAP1 antibody and immunoblotting against CRT, ERp57, TPN and HLA-B. B) Lysate controls showing levels of CRT, ERp57, TPN and HLA-B in LCL.TPN- and LCL.TPN+ cells. In cells lacking TPN, the interaction of HLA-B*35/156 and TAP was not detected. In TPN-competent cells, HLA-B*35:01 and B*35:08 was found to be strongly associated with TAP while no such association could be demonstrated with B*35:62. Lysate controls showed presence of CRT, ERp57 and HLA-B in all the LCL.TPN- and LCL.TPN+ transduced cells. Untransduced LCL.TPN- and LCL.TPN+ cells show no detectable HLA-B hc in the lysate controls.

4.3 Large scale production of sHLA-B*35 molecules

To investigate the repertoire of presented peptides by HLA-B*35/156 variants, LCL.TPN- and LCL.TPN+ cells were transduced with constructs encoding for sHLA-B*35/156 molecules. sHLA production was tested by ELISA as described and selected clones were cultured in the bioreactors. sHLA containing supernatants were harvested weekly and purified by affinity chromatography using NHS-activated HiTrap columns. Low binding (LB) peptides were recovered from elution fractions by size exclusion filtration through a 10 kDa MWCO membrane. High binding (HB) peptides were obtained by treating the retentate with 0.1 % TFA following size exclusion filtration. Peptides were quantitatively analyzed by MALDI-TOF-MS and subjected to mass spectrometry using an Eksigent nano-LC Ultra 2D HPLC coupled to an Orbitrap ion trap for subsequent peptide sequencing.

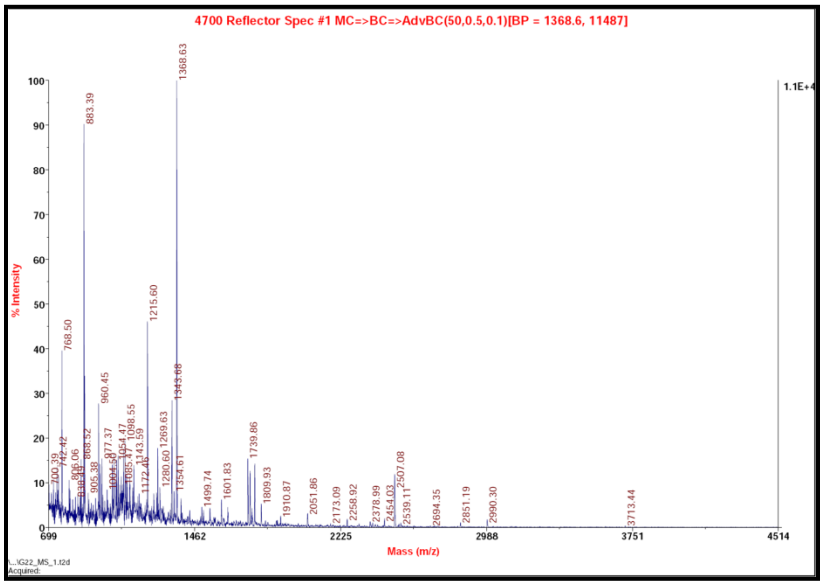
4.4 Mass spectrometric analysis of peptides

Both LB and HB Peptides recovered from affinity purification were subjected to MALDI-TOF-MS for quantitative estimation of peptides. Subsequently, the peptides were sequenced using Eksigent nano-LC Ultra 2D HPLC coupled to LTQ Orbitrap ion trap mass spectrometry.

4.4.1 MALDI-TOF-MS analysis of peptide pools

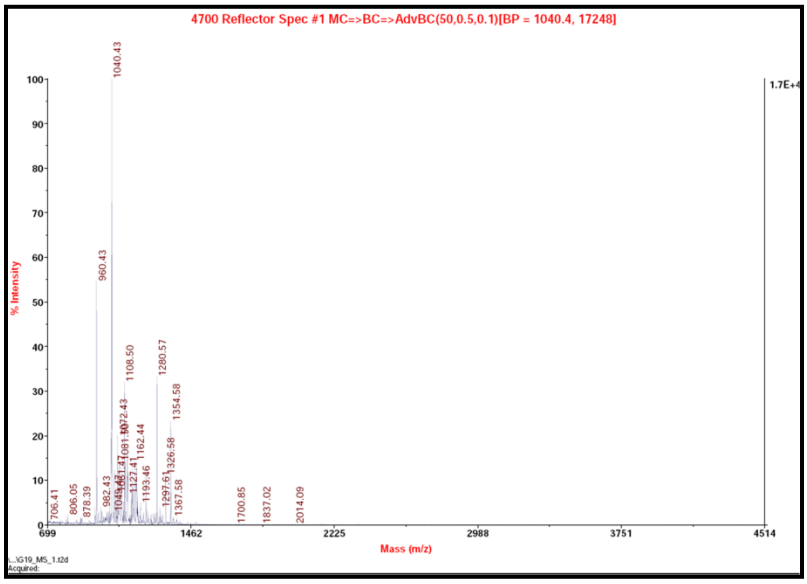
For quantitative analysis of peptide pools, LB as well as HB peptides were subjected to MALDI-TOF-MS. A representative example of a MALDI-TOF spectrum (Figure 4.5 and 4.6) from this study is illustrated below.

Figure 4.5 MALDI-TOF spectrum of a sHLA-B*35:08 derived LB peptide pool



The peaks represent to the peptide ion fragments, corresponding to Appendix V. The x axis represents the m/z value and the y axis represents the percentage intensity of the signal.

Figure 4.6 MALDI-TOF spectrum of a sHLA-B*35:08 derived HB peptide pool



The peaks represent to the peptide ion fragments, corresponding to Appendix V. The x axis represents the m/z value. nd the y axis represents the percentage intensity of the signal.

4.4.2 Peptide profiling

4.4.2.1 Summary of peptides

A total of 233, 274 and 509 peptides restricted by sHLA-B*35:01, B*35:08 and B*35:62, respectively, are given in Table 4.1. There was apparently a shift in the number of single peptides obtained from sHLA-B*35/156 variants, although the sHLA-B*35/156 molecule concentration post peptide elution was equal.

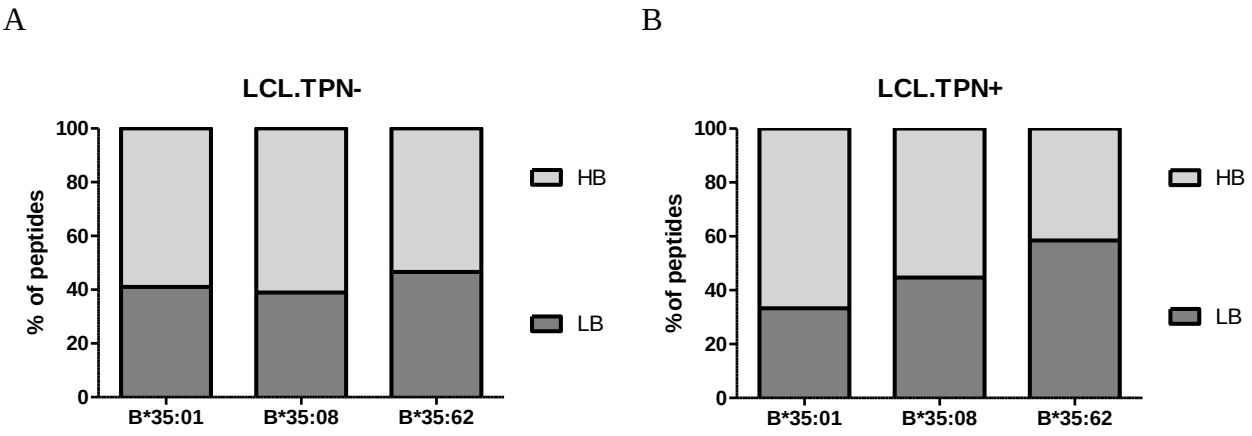
Comparative analysis of HB and LB peptides facilitates the association of the strength of peptide binding in the PBR and distinct AA exchanges in the HLA hc. The peptide profile of peptides acquired in the presence or in the absence of TPN showed relatively higher numbers of HB peptides in HLA-B*35:01 and B*35:08. In contrast more LB peptides could be recovered from sHLA-B*35:62 molecules in the presence or absence of TPN.

Table 4.1 Profile of peptides

HLA-B*35 allele	HB/LB peptides	Peptides (N)
B*35:01 (TPN-)	LB	39
	HB	56
B*35:01 (TPN+)	LB	46
	HB	92
B*35:08 (TPN-)	LB	37
	HB	58
B*35:08 (TPN+)	LB	80
	HB	99
B*35:62 (TPN-)	LB	124
	HB	142
B*35:62 (TPN+)	LB	142
	HB	101

This table shows the number of LB or HB peptides. Annotations: LB - low binding peptides; HB - high binding peptides, TPN+ - acquired in the presence of TPN, TPN- - acquired in the absence of TPN.

Figure 4.7 Differential stability of sHLA-B*35/156 molecules



Bar diagram indicating recovery of LB and HB peptides. The x axis represents the peptide recovery from HLA-B*35 subtypes while the y axis represents percentage prevalence of LB and HB peptides. Dark grey represents the LB peptide, light grey the HB peptides. A) Analysis of the total LB and HB peptides acquired in LCL.TPN- cells. B) Analysis of the total LB and HB peptides acquired in LCL.TPN+ cells. sHLA-B*35:62 molecules derived from LCL.TPN+ cells showed remarkably higher appearance (58.44 %) of LB than HB peptides. sHLA-B*35:01/35:08 molecules in contrast revealed the presentation of comparatively higher percentages of HB peptides than LB peptides.

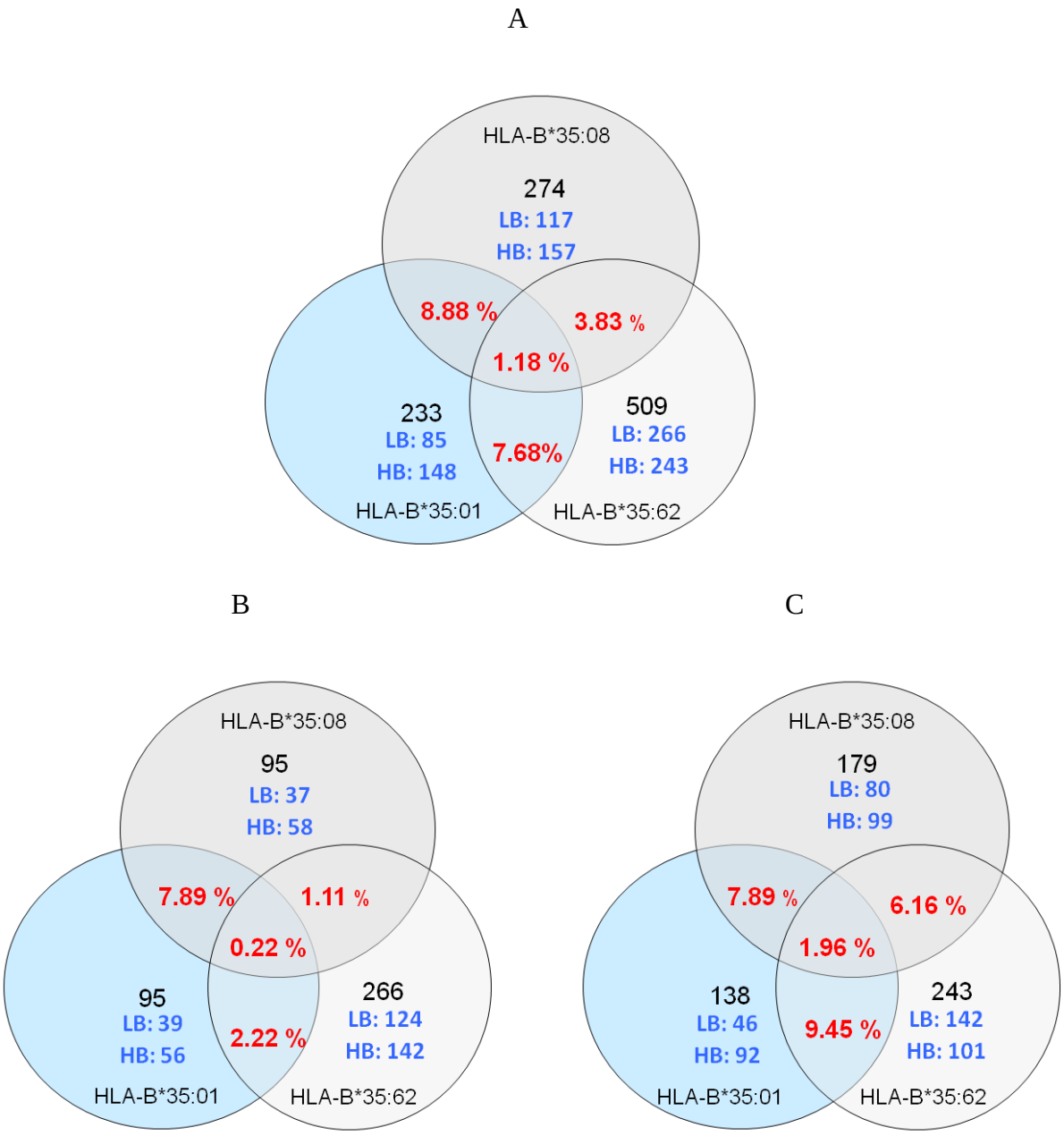
4.4.2.2 Shared peptide analysis

sHLA-B*35/156 variants were all expressed in LCL cells to ensure that the source of the proteomic content is the same. However, the HLA-B*35/156 allotypes were found to share a very small subset of their overall peptide repertoire, both in the presence and absence of TPN. Shared peptide analysis shows that 12 peptides (1.18 %) of the overall peptides were shared among three HLA-B*35/156 variants (Table 4.2). Among these shared peptides only a 12-mer peptide (0.22 %), ALSTGEKGFGYK (Peptidyl-prolyl cis-trans isomerase A) was acquired in the absence of TPN while 11 peptides (1.96 %) were acquired in the presence of TPN (Figure 4.8).

Only 3.83 % of the peptides were shared between HLA-B*35:08 and HLA-B*35:62, while 7.68 % of peptides were shared between HLA-B*35:01 and B*35:62. HLA-B*35:01 and B*35:08 share an overall peptide repertoire of 8.88 %. Between the three allelic variants a highly variable peptide repertoire could be observed it becomes obvious how the differential peptide loading pathways lead to a divergent selection of the proteomic content. The differential association of

the HLA-B*35 allelic variants with the loading complex reflects on the peptide selection. This was highlighted by the numbers of shared peptides given in Table 4.2 and Figure 4.8.

Figure 4.8 Shared peptides of sHLA-B*35/156 variants



Shared peptide analysis. A) Overall shared peptide repertoire of HLA-B*35:01, B*35:08 or B*35:62 restricted peptides. B) Shared peptide analysis of peptides acquired in the absence of TPN. C) Shared peptide analysis of peptide acquired in the presence of TPN. A small percentage (1.18 %) of the overall peptide repertoire was found to be shared between the three different allotypes. Among the pool of peptides, 0.22 % and 1.96 % of eluted peptides were shared both in absence and presence of TPN, respectively.

Table 4.2 Sequence and origin of shared peptides

Peptides sequence	Origin	Source
		TPN
A L S T G E K G F G Y K	Pept.-prolyl cis-trans isomer. A	-
I G P L G L S P K	60S ribosomal prot. L12	+
L P F D K E T G F	SRA stem-loop-interacting RNA-bind. prot.	+
F P N A I E H T L	Ubiquitin-like modifier-activating enzyme 1	+
L P Q E A F E K Y	Struct. mainten. of chrom. prot. 3	+
F P D E T H E R Y	Glycosylphosphatidylinositol anchor attachment 1 prot.	+
L P N D G D E K Y	Cell death prot. 2-like	+
Y P N G V V V H Y	Vam6/Vps39-like prot.	+
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.	+
V P E E G G A T H V Y	A-kinase-interacting prot. 1	+
H A V S E G T K A V T K Y T S A	Histone H2B type 1-J	+
K L E K E E E E G I S Q E S S E E E Q	prot. HMG-I/HMG-Y	+

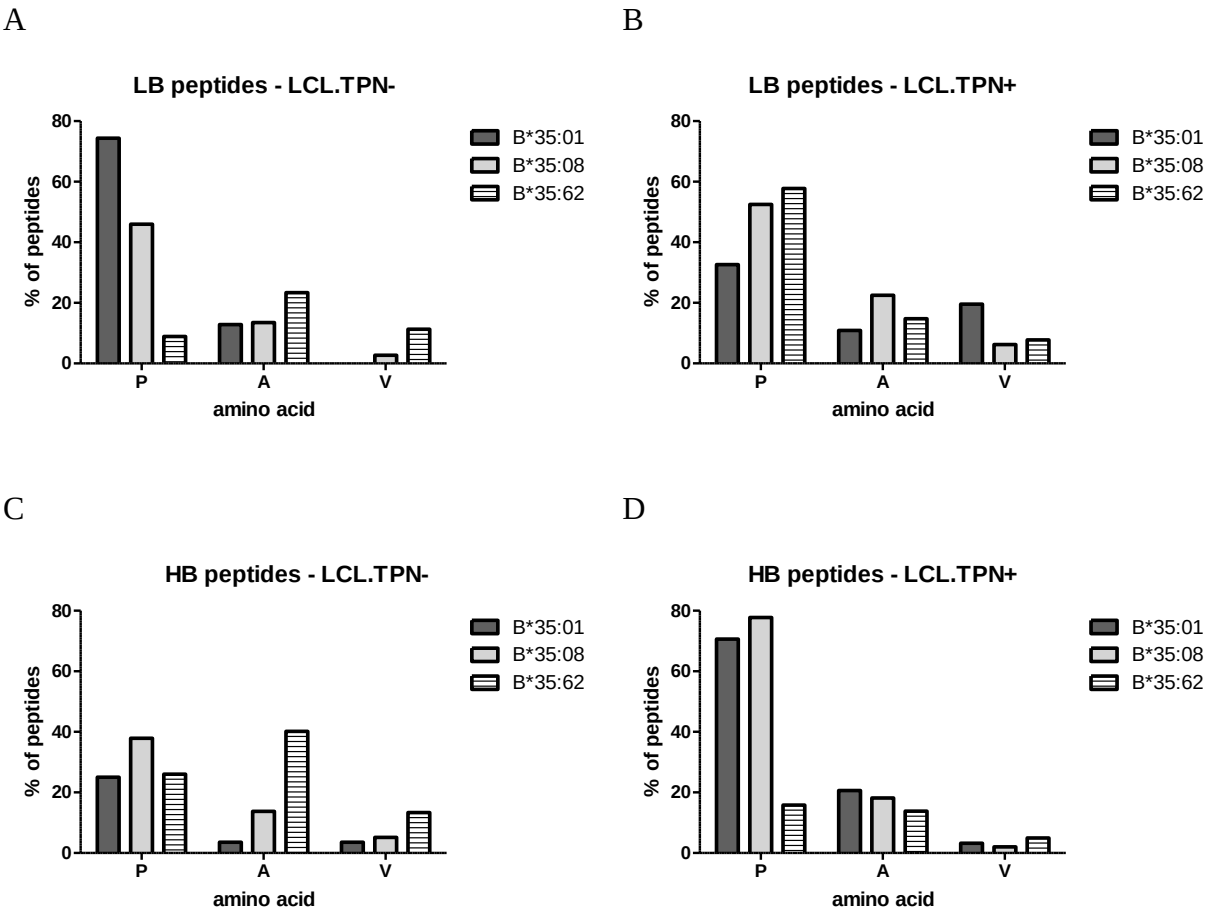
Peptides sequences are depicted in N-terminal to C-terminal orientation. Anchor positions p2 and pΩ are underlined.

4.4.2.3 Peptide anchor motifs

Frequencies of amino acids at peptide position p2

Analysis of the peptides presented by sHLA-B*35/156 molecules demonstrated Pro as p2 anchor (Figure 4.9). The preference for Pro at p2 was decreased for HLA-B*35:01 restricted LB peptides acquired in the presence of TPN. HLA-B*35:08 and B*35:62 were additionally anchored at p2 by Ala and Val. HLA-B*35:62 restricted peptides acquired in the absence of TPN are exclusively anchored by Ala at p2 (Figure 4.9).

Figure 4.9 p2 anchor positions of sHLA-B*35/156 restricted peptides

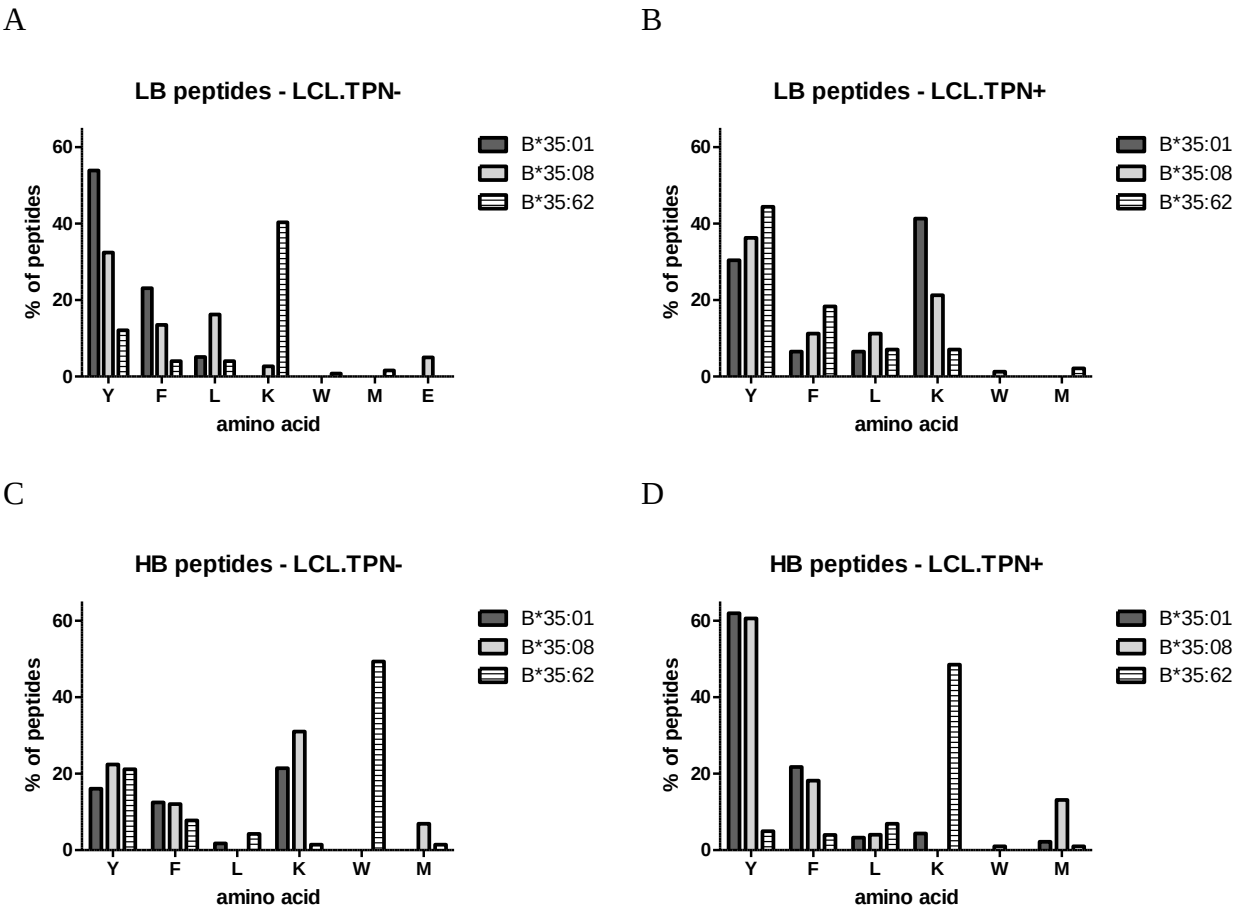


Frequencies of the AAs occurring at peptide position p2 among LB and HB sHLA-B*35/156 restricted peptides. The x axis represents the AA residues at p2. The y axis represents the percentage prevalence of individual AAs at p2. Black, grey or crossed bars represent the alleles HLA-B*35:01, B*35:08 or B*35:62. A) and B) Frequencies of AAs occurring at p2 in LB peptides. C) and D) Frequencies of AAs occurring at p2 in HB peptides. Pro was the most frequently occurring AA at p2 position among the HB and LB peptides. However, HLA-B*35:62 preferred peptides with Ala at p2 in the absence of TPN.

Frequencies of amino acids at peptide position pΩ

Comparison of AA frequencies from sHLA-B*35/156 restricted peptides showed a preference for Tyr, Phe, Leu or Lys at pΩ (Figure 4.10). Unlike HLA-B*35:01 and B*35:08, B*35:62 preferentially demonstrated Trp at pΩ when TPN is absent. Approximately 50 % of sHLA-B*35:62 restricted HB peptides acquired in the absence of TPN exhibited a preference for Trp at pΩ. Furthermore, 13.13 % of sHLA-B*35:08 restricted HB peptides acquired the presence of TPN were preferentially anchored by Met at the C-terminal position.

Figure 4.10 pΩ anchor positions of sHLA-B*35/156 restricted peptides



Frequencies of the AAs occurring at peptide position pΩ among LB and HB sHLA-B*35/156 restricted peptides. The x axis represents the AA residues at pΩ. The y axis represents the percentage prevalence of individual AAs at pΩ. Black, grey or crossed bars represent the alleles HLA-B*35:01, B*35:08 or B*35:62. A) and B) Frequencies of AAs occurring at pΩ in LB peptides. C) and D) Frequencies of AAs occurring at pΩ in HB peptides. HLA-B*35/156 variants preferentially presented the peptides with Tyr, Phe, Leu and Lys at C-terminal.

4.4.2.4 Length distribution of peptides

The majority of sHLA-B*35/156 restricted peptides was found to be of canonical length (8–10 AAs). However peptides of non-canonical lengths (>10 AAs) could also be recovered by sHLA-B*35/156 variants. sHLA-B*35:62, in particular, was found to preferentially present peptides of non-canonical length (>50 %) both in the presence and absence of TPN (Figure 4.11). Among the peptides presented, 33.68 % and 36.84 % non-canonical peptides were presented by sHLA-B*35:01 and B*35:08, respectively, in the absence of TPN. In the presence of TPN, sHLA-

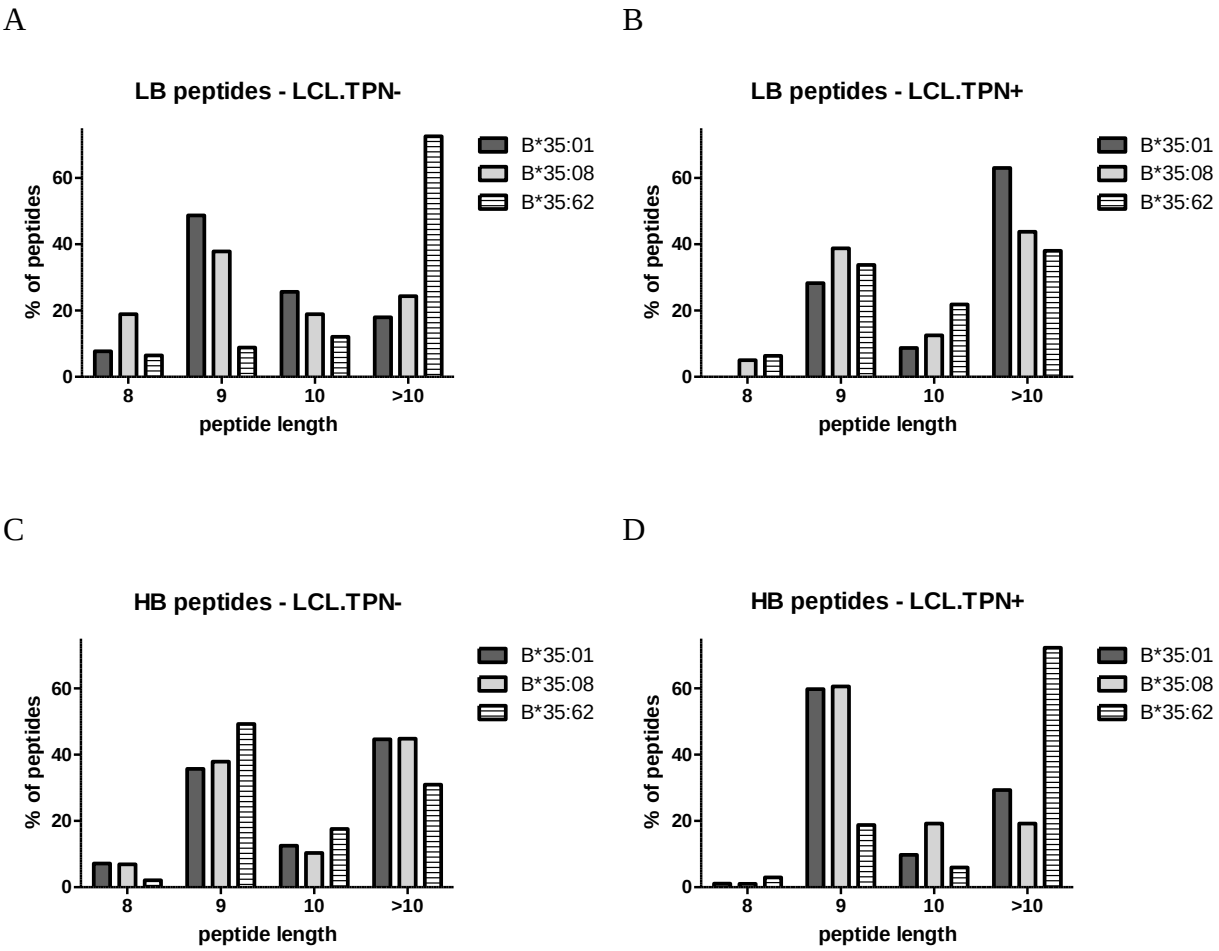
B*35:01 was shown to present 40.58 % non-canonical peptides, while sHLA-B*35:08 was shown to present the lesser percentage of longer peptides.

A relatively higher percentage of HB peptides recovered from sHLA-B*35:01 or B*35:08, in the presence of TPN, was found to be of canonical lengths (70.65 % for sHLA-B*35:01 and 80.81 % for sHLA-B*35:08), this is in contrast to this set of peptides recovered from sHLA-B*35:62 (27.72 %). In the absence of TPN, both canonical and non-canonical sHLA-B*35/156 restricted HB peptides were observed. The length analysis of the recovered peptides showed that the majority of sHLA-B*35:01 and B*35:08 restricted LB peptides were of canonical lengths. Contrary to that observation, most of the sHLA-B*35:62 restricted LB peptides acquired in the absence of TPN were of non-canonical length (72.58 %) (Figure 4.11).

HLA-B*35/156 variants were found to present peptides of non-canonical length and are anchored by Pro at p2. The analysis of longer peptides showed a preference for Pro at p2 for HLA-B*35:01 and HLA-B*35:08 restricted peptides (Appendix VI). However, HLA-B*35:62 restricted peptides of extraordinary length were preferentially anchored by Ala at p2 position (31.01 %).

The results highlight that a single mismatch at position 156 in HLA-B*35 molecules could alter the anchor motif and repertoire of presented peptides. Striking differences in peptide repertoire was observed with HLA-B*35:62 molecules.

Figure 4.11 Length distribution of sHLA-B*35/ 156 restricted peptides

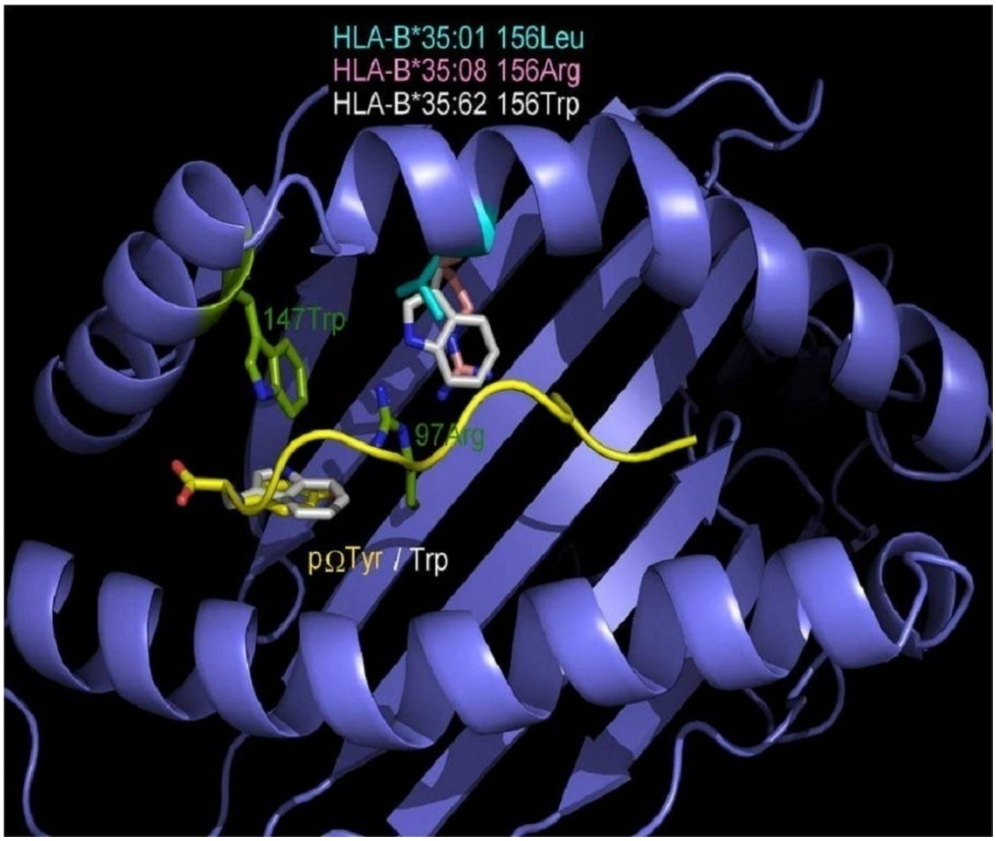


Length distribution of HB and LB peptides. Peptide length (AA) is given on the x axis and the percentage prevalence of peptides is given on the y axis. Black, grey or crossed bars represent the alleles HLA-B*35:01, B*35:08 or B*35:62. A) and B) Length distribution of LB peptides. C) and D) Length distribution of HB peptides. In the presence of TPN, the majority of HLA-B*35:01 and B*35:08 restricted HB peptides were of canonical length. HLA-B*35:62 restricted LB peptides that were acquired in the absence of TPN, were of non-canonical length.

4.5 Molecular modelling

sHLA-B*35:62 restricted peptides that were acquired in the absence of TPN are predominantly anchored by a C-terminal Trp. Residue 156 that distinguishes the three allelic variants is not part of pocket F and thus has no direct influence on the peptides C-terminus. To understand the unexpected alteration of the C-terminal peptide anchor, YASARA/FoldX software was utilized for generating a model of HLA-B*35:62. Crystal structures of HLA-B*35:01 (2AXG) [37] and B*35:08 (2AXF) [37] are available and were overlayed. Since no structure of HLA-B*35:62 is available, a model of HLA-B*35:62 was generated by mutating 156Leu in HLA-B*35:01 to 156Trp. In the HLA-B*35:62 model, the stacking arrangement of 147Trp and 156 Trp against 97Arg alters the F pocket indirectly and allows for a C-terminal Trp of the bound peptides. (Figure 4.12).

Figure 4.12 Model of HLA-B*35:62



Molecular modelling of position 156 in HLA-B*35 utilizing YASARA/FoldX software. Structural overlay of HLA-B*35:01 and B*35:08 both bound to a decamer peptide (APQPAPENAY) and the modeled structure of HLA-B*35:62. PDB: 2AXG [37] and 2AXF [37].

4.6 Prediction of viral epitopes

Several viral derived immune evasion proteins target host antigen presentation pathway at various periods of time with the ultimate aim to prevent the presentation of viral epitopes. For example, HCMV US6 targets TAP, HCMV US3 down-regulates TPN, HCMV US2 induces degradation of HLA class I molecules, US10 retains HLA class I molecules in ER and HCMV IE1 transactivates the viral gene expression. However, HLA-B*35/156 alleles studied here can present peptides independent of TPN and/or TAP, indicating the important role of these alleles in identification of viral epitopes. In this context it would be interesting to understand whether these alleles could present the peptides derived from these immune evasion proteins. This study soughts to understand the probable immune evasion derived viral epitopes likely to be presented by HLA-B*35/156 alleles.

The knowledge of peptide anchor motifs enables to predict viral peptides that are likely to be presented by distinct HLA alleles. To date prediction tool databases incorporate data for HLA-B*35:01 restricted viral or pathogenic peptides. As an example, from the HCMV US6 protein the nonamers H**P**SHRLLT**L** **(**SYFPEITHI**)** (Table 4.3) or L**A**LLCSIT**Y** (IEDB) (Table 4.4) would be predicted to bind to HLA-B*35:01. The anchor motifs of the predicted viral peptides correspond to the HLA-B*35:01 specific anchor motifs described in this thesis.

Peptide motifs are not available for all allelic variants. For HLA-B*35:62 peptide binding data were unknown. To enable the prediction of peptide ligands for such an unknown allele, the first step is to determine the individual peptide binding profile. The next step for peptide prediction in this study was the implementation of MAPPP (FragPredict), a proteasomal cleavage prediction tool [194] that virtually performs proteasomal cleavage of selected proteins. Proteasomal cleavage tools generate peptides trimmed correctly at the C-terminus [62, 148], the N-terminus of peptides to be bound to HLA class I molecules is always produced with an extension and later trimmed by aminopeptidases of the ERAAP complex [149]. From a list of this predicted peptides, the best fitting ones were selected based on the anchor motif data from this thesis.

HLA-B*35:08 specific peptide anchor motifs show a preference for Tyr, Phe, Leu and Lys at the C-terminal position and Pro, Ala and Val at N-terminus. HLA-B*35:62 prefers Trp, Tyr, Phe, Leu or Lys at the C-terminus and Ala at the N-terminus. Based on the data for HLA-B*35:62 peptide anchor motifs, the list of predicted peptides from the HCMV US6 protein was applied to

search peptides that would fit in the B*35:08 or B*35:62 PBR. Two peptides with the highest proteasomal score and anchor motif for HLA-B*35:08 and HLA-B*35:62 were D**A**DDSWKQ**L** (0.8023) and DG**A**VWNAFR**L** (0.8943). Peptides with a C-terminal Trp and an N-terminal Ala could not be detected. Peptide SHR**P**ICYNDTGDCDADDS**W** (0.4999), with Pro at N terminal position (Table 4.5) was predicted by this approach, although unlikely to be presented in real scenario.

Few peptides with an N-terminal Ala and a C-terminal Trp could be predicted from the HCMV US3 protein. These peptides that fits the anchor motif of HLA-B*35:62 include FR**V**EENQC**W** (1), IKS**A**HFRVEENQC**W** (0.6763), PQ**V**RMDYSSQTIN**W** (0.4999), C**V**PQVRMDYSSQTIN**W** (0.4999). Peptide epitopes derived from US10 that would be likely to be presented include P**V**YDSGTP**M** (0.8605), PS**P**KTLRASA**W** (0.9999), QF**P**SPKTLRASA**W** (1), L**A**ICLLWW**L** (0.8124), RN**P**LAICLLW**W** (1). The probable epitopes predicted for HCMV US2 include T**V**DCNLSMM**W** (0.9999), L**A**LRLVLQGDVI**W** (0.9999), K**A**DYGGVGEN**L** (0.5572), R**P**WKSTAKHP**W** (0.8595) and K**A**WVGLWTS (0.9999). Similarly, the peptide epitopes derived from HCMV IE1 that are likely to be presented by HLA-B*35:62 were V**P**EDKREM**W** (0.8843) and I**V**PEDKREM**W** (1).

Table 4.3 Prediction of HLA-B*35:01 restricted HCMV US6 peptides using SYFPEITHI

HLA-B*35:01 nonamers

Pos	1 2 3 4 5 6 7 8 9	score
114	H P S H R L L T L	23
157	L L V V I L A L L	16
107	L P C D L D I H P	15
3	L L I R L G F L L	14
149	L Y L C C G I T L	14
162	L A L L C S I T Y	14
7	L G F L L M C A L	13
21	R S S R D P K T L	13
25	D P K T L L S L S	13
141	R H G F F A V T L	13
156	T L L V V I L A L	13
1	M D L L I R L G F	12
2	D L L I R L G F L	12

HLA-B*35:01 decamers

114	H P S H R L L T L M	19
149	L Y L C C G I T L L	14
1	M D L L I R L G F L	13
66	L G E D F A H Q C L	13
85	H K S R P N D R N L	13
107	L P C D L D I H P S	13
140	E R H G F F A V T L	13
155	I T L L V V I L A L	13
156	T L L V V I L A L L	13
2	D L L I R L G F L L	12
21	R S S R D P K T L L	12
23	S R D P K T L L S L	12
46	R P V C Y N D T G D	12
88	R P N D R N L E G R	12

SYFPEITHI prediction tool for estimation of HCMV US6 derived probable peptides likely to be presented by HLA-B*35:01. Based on the guidelines of this web prediction tool, high scores indicate higher binding efficiency.

Table 4.4 Prediction of HLA-B*35:01 restricted HCMV US6 peptides using IEDB

Allele	#	Start	End	Length	Peptide	Method used	Percentile_rank
HLA-B*35:01	1	162	170	9	LALLCSITY	Consensus (ann/comblib_sidney2008/smm)	0.2
HLA-B*35:01	1	145	157	13	FAVTLYLCCGITL	ann	0.7
HLA-B*35:01	1	158	170	13	LVVILALLCSITY	ann	0.7
HLA-B*35:01	1	159	170	12	VVILALLCSITY	ann	1
HLA-B*35:01	1	160	170	11	VILALLCSITY	Consensus (ann/smm)	1.2
HLA-B*35:01	1	145	158	14	FAVTLYLCCGITLL	ann	1.3
HLA-B*35:01	1	114	123	10	HPSHRLLTLM	Consensus (ann/smm)	1.35
HLA-B*35:01	1	142	150	9	HGFFAVTLY	Consensus (ann/comblib_sidney2008/smm)	1.8
HLA-B*35:01	1	39	50	12	VPRTKSHRPVCY	ann	1.8
HLA-B*35:01	1	114	122	9	HPSHRLLTL	Consensus (ann/comblib_sidney2008/smm)	1.9
HLA-B*35:01	1	70	78	9	FAHQCLQAA	Consensus (ann/comblib_sidney2008/smm)	2
HLA-B*35:01	1	157	170	14	LLVVILALLCSITY	ann	2
HLA-B*35:01	1	70	77	8	FAHQCLQA	Consensus (ann/smm)	2.2
HLA-B*35:01	1	107	119	13	LPCDLDIHPSHRL	ann	2.2
HLA-B*35:01	1	123	136	14	MNNCVCDGAVWNAF	ann	2.3
HLA-B*35:01	1	107	120	14	LPCDLDIHPSHRLL	ann	2.5
HLA-B*35:01	1	125	136	12	NCVCDGAVWNAF	ann	2.7
HLA-B*35:01	1	70	79	10	FAHQCLQAAK	Consensus (ann/smm)	2.85
HLA-B*35:01	1	161	170	10	ILALLCSITY	Consensus (ann/smm)	2.9
HLA-B*35:01	1	107	116	10	LPCDLDIHPS	Consensus (ann/smm)	3.0
HLA-B*35:01	1	134	145	12	NAFRLIERHGFF	ann	3.2
HLA-B*35:01	1	58	70	13	DADDSWKQLGEDF	ann	3.5
HLA-B*35:01	1	114	127	14	HPSHRLLTLMNNCV	ann	4.1

IEDB prediction tool for estimation of HCMV US6 derived probable peptides likely to be presented by HLA-B*35:01. Based on the guidelines of this web prediction tool, low percentile ranks indicate good binders.

Table 4.5 Prediction of HLA-B*35:08/B*35:62 restricted HCMV peptides

HCMV US6
C A L P T P G E R S S R D P K (0.4999)
C A L P T P G E R S S R D P K T L (0.499999)
L P T P G E R S S R D P K (0.4999)
S P R Q Q A C L P R T K S H R P I C Y (0.4999)
D A D D S W K Q L (0.8023)
E D F A H Q C L L A A K (0.5023)
E G R L T C Q R V S R L L P C D L (0.4999)
L L P C D L D I H P S H R L (0.4999)
L L P C D L D I H P S H R L L (0.4999)
D G A V W N A F R L (0.8943)
V W N A F R L I E R H G F F (0.4999)
HCMV US3
H F R V E E N Q C W (1)
I K S A H F R V E E N Q C W (0.6763)
P Q V R M D Y S S Q T I N W (0.4999)
C V P Q V R M D Y S S Q T I N W (0.4999)
C V P Q V R M D Y (0.8485)
A A C V P Q V R M (0.6116)
K Y A A C V P Q V R M D Y (0.4999)
M V D I T L S T R W (1)
G H P V T H T V D M (0.9999)
G H P V T H T V D M V D I T L (0.4999)
T V D M V D I T L (0.4999)
G G H P V T H T V D M (0.4999)
G I V S Q S Y M D R L (0.5002)
L A D S V P R P L (0.9698)
L A I L A V L F L (1)
L V L A I L A V L (1)
L V L A I L A V L F (0.9850)
L V L A I L A V L F L (0.9999)
M K P V L V L A I L (1)
M K P V L V L A I L A V L (1)
M K P V L V L A I L A V L F (1)
M K P V L V L A I L A V L F L (1)

HCMV US2
T V D C N L S M M W (0.9999)
V T V D C N L S M M W (0.9879)
I V F Y S I N I T L L (1)
I V F Y S I N I T L L V L (0.6745)
R V D Y T S S A Y (0.6488)
R V D Y T S S A Y M (0.6147)
A L R L V L Q G D V I W (0.9999)
L A L R L V L Q G D V I W (0.9999)
K A D Y G G V G E N L (0.5572)
F A R G S I V G N M (0.5648)
R P W K S T A K H P W (0.8595)
K A W V G L W T S M (0.9999
HCMV US10
R N P L A I C L L W (1)
R N P L A I C L L W W (1)
L A I C L L W W L (0.8124)
Q F P S P K T L R A S A W (1)
P S P K T L R A S A W (0.9999)
G T A T A T E A L (0.7844)
G T A T A T E A L Y (0.6252)
G T A T A T E A L Y I L (0.8106)
T E A L Y I L L P T E L (0.5022)
L S S P E G N R P R N Y (0.5116)
S S P E G N R P R N Y (0.5001)
S S P E G N R P R N Y S A T L (0.4999)
P V Y D S G T P M (0.8605)
G V L M N L T Y L W (0.7340)
HCMV IE1
T P V T K A T T F L (0.9999)
T P V T K A T T F L Q T M (0.7534)
L A E E S L K T F (0.5164)
L A E L V K Q I K (0.5000)
I V P E D K R E M W (1)
V P E D K R E M W (0.8843)
V P E D K R E M W M (0.5002)

A A N K L G G A L (0.9676)
Q A K A R A K K D E L (0.7561)
Q A M A A L Q N L (0.5321)
M A Y A Q K I F K I L (0.5010)
Y A Q K I F K I L (1)
K V L T H I D H I F (0.7363)
K V L T H I D H I F M (1)
K V T S D A C M M T M (0.5414)
I T K P E V I S V M K (0.5000)
F A Q Y I L G A D P L (0.5587)
E P T A S G G K S T H P M (0.5005)

List of probable predicted peptides derived from viral immune evasion proteins for HLA-B*35:08 and HLA-B*35:62 (based on anchor motifs deducted from self peptide analysis in this study). The peptides are generated using FragPredict (MAPPP) software. The sequences displayed in the table are the peptides with most accurate motifs and highest proteasomal score.

5 DISCUSSION

Human leukocyte antigen (HLA) matching is the main barrier to haematopoietic stem cell transplantation (HSCT). Clinical data demonstrated that precise HLA matching of potential donors is associated with higher success rates compared to those that are mismatched at one or more loci [195-196], yet the availability of a perfectly matched donor:recipient pair is only about 30 % for Caucasians [52]. Besides the number of mismatches, the nature of exchanged AAs and their location also influences post transplantation outcome. It becomes evident that the understanding of how to accept the mismatched donors and recipients and still have successful clinical outcomes determines the future of bone-marrow transplantation. For this reason it is necessary to study systematically the effect of polymorphism on the immune function of HLA molecules. Considerable efforts have been made over the last years to distinguish non-permissive HLA mismatches that dramatically increase the risk of post-transplantation outcomes from permissive HLA mismatches [197-198]. A measurement of histocompatibility is to determine the impact of a particular mismatch on the mode of peptide loading and repertoire of selected and presented peptides. This understanding would be helpful in risk estimation of mismatches when no clinical information is available.

HLA class I molecules play a major role in adaptive immunity by binding cytosol derived peptides and presenting them on the surface of antigen presenting cells for surveillance by CD8+ T cells. These T cells have the properties to discriminate self from non-self, thus leading to the detection of pathogen derived peptides and hence those infected cells could be targeted for the cellular immune response. Furthermore, self peptides are prevented from immune destruction via the immune self-tolerance. HLA class I molecules, especially HLA-B, are the most polymorphic proteins in the human proteome [199]. HLA polymorphisms are mostly located within the PBR, thereby specifying and diversifying the nature and repertoire of the bound peptides. Differential peptide binding specificities of HLA class I allotypes have significant consequences on immune recognition of pathogens [14], disease progression [200-201], drug induced toxicity [202], cancer [203-204] and transplantation outcomes [205]. Every single pHLA complex constitutes a unique landscape that is altered through the most variable part of the trimeric molecule, the bound peptide.

The loading of peptides onto HLA class I molecules is a complex procedure and facilitated by the PLC, where TAP and TPN are essential components. TPN supports peptide loading, stabilizes the TAP complex and thus indirectly promotes the accessibility of peptides in the ER [206]. Furthermore, TPN is known to function in peptide editing and loading of high affinity peptides [91, 93]. In the absence of TPN, interaction of class I molecules to TAP is disrupted, resulting in unstable pHLA complexes [89] since TAP plays an important role in the translocation of peptides from the cytosol to the ER, making them available for presentation by HLA class I molecules [70, 207]. Most of the HLA class I molecules rely strictly on the functions of TPN and TAP for efficient peptide loading. Certain viral proteins and cancer proteins target these PLC components to prevent the presentation of immunogenic peptides and recognition by cytotoxic CD8+ T cells. Nevertheless, recent studies document host strategies to circumvent these immune evasions by choosing pathways [97, 208-209] that could operate independent of these PLC components. Certain allelic HLA class I variants that load peptides independent from TPN and/ or TAP are therefore still able to present a fraction of the intracellular peptide repertoire to the immune system. However, TPN or TAP-independent peptide presentation might lead to a differential peptide repertoire that would be selected and subsequently presented, since those peptides are not optimized for stabilizing the respective HLA allele. HLA class I variants that are TPN and/or TAP-independent, differ from the PLC-dependent alleles often exclusively by one AA difference [91, 115, 210]. Understanding the effect of polymorphism on the immune function of a given HLA molecule can only take the form of a measure of histocompatibility. The similarity of allele peptide-binding profiles and repertoires is such a measure.

This study was focused on determining the mode of antigen presentation utilized by HLA-B*35 allotypes that differ at one single AA at position 156, HLA-B*35:01^{156Leu}, B*35:08^{156Arg} and B*35:62^{156Trp}. HLA-B*35:01 and B*35:08 are described to present viral peptides [130-133], making them presumably PLC-independent. The remaining questions was what type of peptides (features, binding motif, length) they would present in the presence or absence of TPN and if the Leu/Arg156 vs Trp156 exchange influences the HLA/PLC interaction.

All three HLA-B*35/156 variants studied were expressed on the cell surface of TPN-deficient cell lines (LCL.TPN-), suggesting a TPN-independent mode of peptide acquisition and presentation for these molecules. However, the spectrum of TPN dependence among these

allotypes was found to vary with the nature of AA at position 156. The data revealed the influence of microploymorphism at position 156 on the surface expression and stability of pHLA complexes on the cell surface.

A recent study by Rizvi *et al* (2014) [211] also demonstrated a TPN-independent mode of peptide loading for HLA-B*35:01. The 156 micropolymorphism distinguishing the HLA-B*35/156 allotypes causes a functional disparity for their TPN interaction. Unlike HLA-B*35:01^{156Leu} and B*35:62^{156Trp}, surface expression of B*35:08^{156Arg} was decreased by more than 2 folds in the absence of TPN, indicating partial dependence of B*35:08 on TPN for peptide loading and presentation.

In addition to TPN independence, certain class I molecules have been shown to bypass the classical antigen presentation pathway that stringently requires TAP for efficient peptide loading. TAP-independent presentation of viral derived peptides on TAP-deficient RMA-S cells was reported for vesicular stomatitis virus, rauscher murine leukemia virus, sendai virus, and influenza viral infections [212-215]. To investigate if the mismatch at position 156 influence the dependence of HLA-B*35 variants on TAP, we analyzed the surface expression of HLA-B*35/156 allotypes in a TAP-deficient cell line (T2.TAP-). Our results showed that the surface expression of HLA-B*35:62 was relatively independent of TAP compared to that of HLA-B*35:01 and B*35:08. The mechanism behind the TAP-independent mode of peptide presentation still remains unknown. Shi *et al*. (1998) [216] hypothesized the role of signal sequences that can direct the peptide translocation and help in cell surface presentation in TAP-deficient cells. Another plausible explanation could be that the peptides were possibly translocated into the ER in a TAP-independent manner with the help of some unknown proteins.

Loading of peptides on HLA class I molecules and the formation of stable pHLA complexes requires proper biophysical interaction of the HLA molecules with components of the PLC. Immunoprecipitation experiments were carried out in order to understand the functional impact of 156 polymorphism on the association of the HLA-B*35/156 hc with components of the PLC. Pull down experiments were performed by immunoprecipitation of the PLC with an anti-TAP1 antibody followed by targeting the HLA-B hc or other components of PLC by western blot technique. The results demonstrated differential association of TAP with the HLA-B*35:01^{156Leu} hc, B*35:08^{156Arg} hc or the B*35:62^{156Trp} hc. The HLA-B*35/156 hc could not be detected in any

of the TAP complexes from cells lacking TPN (LCL.TPN- cells), since the association between TAP and the HLA hc is mediated through TPN. TPN plays an important role in steady state expression of TAP [85], and also helps in the stabilization of the heterodimeric TAP1/TAP2 complex. In contrast, in TPN positive cells (LCL.TPN+), a strong association of both HLA-B*35:01 hc and HLA-B*35:08 hc with TAP could be detected. The observations validates the accepted role of TPN in bridging a HLA class I molecule and the TAP complex [217-218]. However, unlike for HLA-B*35:01 and B*35:08, the association of the B*35:62 hc with TAP could not be confirmed. This result could mean either HLA-B*35:62 does not incorporate into the PLC and does not utilize the TAP complex for peptide loading, or the transit period of HLA-B*35:62 into the PLC is so concise that the interaction could not be detected using this experimental approach.

The results from the immunoprecipitation experiments were in synchrony with the flow cytometric studies where the surface expression of HLA-B*35:62 was found to be comparatively independent of TAP. The observed results could be suggestive that HLA-B*35:62 might be very weakly or not at all associated with TAP. The findings postulate the involvement of an unknown alternate pathways for peptide selection and presentation by HLA-B*35:62. Given that all the three HLA-B*35/156 variants share the same AA sequence except for the single AA polymorphism at position 156, Trp156 in HLA-B*35:62 would be the most likely factor regulating the association of this allele with TAP and modulating a differential mode of peptide loading. Similar observations were made in the HLA-B*44 group, where a single polymorphism at position 116 or 156, was found to effect the association of the HLA hc with TAP. HLA-B*44:02$^{116Asp/156Asp}$ which differ from HLA-B*44:03$^{116Asp/156Leu}$ by a single AA at position 156 and from HLA-B*44:05$^{116Tyr/156Asp}$ at position 116, bound strongly to TAP, while HLA-B*44:03$^{116Asp/156Leu}$ [210] and HLA-B*44:05$^{116Tyr/156Asp}$ [219] did not.

Several studies revealed how certain allelic variants that differ at one or more AAs might vary in their TAP association. For instance, it was found that HLA-A*68:07$^{116His/70Gln}$ is associated much stronger with TAP than HLA-A*68:03$^{116Asp/70His}$ [220]. In 1996, Neisig *et al* [210] demonstrated that HLA-B alleles with aromatic AAs at position 116 could be a better TAP binder compared to the others. It was observed that among the HLA-B*15 allotypes, HLA-B*15:10^{116Tyr} showed a stronger association with TAP compared to HLA-B*15:18^{116Ser} [221]. In addition to these studies, Magnacca *et al* (2012) [222], demonstrated the functional role of Cys at position 67, in

HLA-B*27:05^{116Asp} and HLA-B*27:09^{116His} alleles, both of which can acquire peptides independent of TAP. They used TAT-driven chimeric carrier protein, carrying HLA-B*27 restricted epitopes, for the delivery of peptides and to ensure the TAP-independent peptide supply. They demonstrated that the mutation of 67Cys in HLA-B*27:05^{116Asp} would affect the TAP-independent peptide presentation, while this mutation did not show any effect in HLA-B*27:09^{116His}. This finding provided the evidence that interaction of neighbouring AA residues with certain polymorphic residues can alter the mode of peptide loading. In case of HLA-B*35:62^{156Trp}, it is possible that Trp at position 156 alters the overall conformation of the PBR sufficiently to affect its interaction with neighbouring AA residues, thereby rendering a TAP-independent mode of peptide loading.

After demonstrating the relationship between the mode of peptide loading and nature of AAs at position 156, the aim of this work was to validate if the polymorphic differences at this position could alter the peptide binding specificities of HLA-B*35 molecules. Recently, Badrinath *et al* (2012) [115] demonstrated the impact of micropolymorphism at position 156 on the repertoire of presented peptides and mode of peptide loading by HLA-B*44 alleles. Knowledge on variations in the peptide repertoire would support the prediction of immunological outcomes following transplantation. It is most likely that the post-transplantation outcome could be associated to recipient specific peptide repertoire and its set of shared peptides by the donor HLA molecule [223].

Soluble HLA technology [190, 224] was utilized to characterise the repertoire of peptides presented by HLA-B*35/156 variants. The peptide binding profiles of three naturally occurring molecules, HLA-B*35:01^{156Leu}, B*35:08^{156Arg} and B*35:62^{156Trp}, were analyzed. In the present study it could be demonstrated how a single mismatch at residue 156 in HLA-B*35/156 allotypes changes the peptide binding groove sufficiently to alter the features of the selected peptide repertoire.

Position p2 and the C-terminal position pΩ of a peptide are significant for an effective binding in the PBR of most allotypes [35, 225] and thus determine allelic specificity. Hence, the HLA-B*35/156 bound peptides were investigated for the peptide binding anchor motifs at p2 and pΩ. The results demonstrated similar peptide binding preferences for HLA-B*35:01 and B*35:08, however HLA-B*35:62 showed striking differences for the anchor motif at p2 and pΩ,

especially in TPN-deficient cells. The mass spectrometric analysis revealed that HLA-B*35:01 as well as B*35:08 restricted peptides acquired in the presence or absence of TPN are preferentially N-terminal anchored by Pro at p2. The preference for Pro at p2 for HLA-B*35:01 and B*35:08 is consistent with previous studies [226-229]. In contrast, surprisingly the binding motif of HLA-B*35:62 showed an unusual preference for Ala at p2, in the absence of TPN. Comparison of AA frequencies in peptides derived from sHLA-B*35/156 showed a preference of Tyr, Phe, Leu or Lys at pΩ. Unlike HLA-B*35:01 and B*35:08, B*35:62 preferentially bind peptides with Trp at pΩ in the absence of TPN. These results implies the influence of Trp at position 156 in HLA-B*35:62 on alteration of peptide selectivity by TPN. To validate the possible structural implication of position 156 on HLA-B*35:62 molecule, a structural model of HLA-B*35:62 was generated by mutating Leu at position 156 in the HLA-B*35:01 structure (2AXG) [37] to Trp. This mutational model of HLA-B*35.62 revealed a stacking arrangement of 147Trp and 156Trp against 97Arg. The residue triad, 147Trp/156Trp/97Arg, was found to be highly selective for a C-terminal Trp of the bound peptide.

Moreover, a subtle difference was observed regarding the sources and biological functions of the HLA-B*35:62 derived peptides compared to those from B*35:01 or B*35:08, thus adding evidences for the potential role of Trp at position 156 in HLA-B*35 allele, in altering the repertoire of presented peptides (Appendix VII).

It was also demonstrated that HLA-B*35/156 variants were able to present peptides of non-canonical lengths (>10 AAs). Generally, it was considered that HLA class I molecules present peptides of canonical length (8–10 AAs). The limit for peptide length restriction depends, in part, by structure and conformation of PBR. It was reported that peptides of non-canonical length could be bound by HLA-B*35:01 and B*35:08 molecules and such peptides can be highly immunogenic [131, 134, 230]. HLA-B*35/156 alleles were found to present longer peptides with Pro at p2 (Appendix VI). Generally, peptides with an N-terminal Pro are less efficiently translocated by TAP [231-232]. Moreover, since ERAAP is unable to cleave “X-Pro” bonds [233], it is most likely that the X-Pro-Xn peptides would be accumulated within ER. Because of these reasons, it is unlikely that long peptides with Pro at p2 would be efficiently translocated and presented. The property of HLA-B*35 alleles to present long peptides with an N-terminal Pro can be attributed to the ability of Pro to introduce a kink in the peptide [234-235], thereby allowing the accommodation of looping conformation of long peptides.

Furthermore, the aim was to understand if the micropolymorphism at position 156 would impact the stability of pHLA complexes. To achieve this objective, analysis of peptide binding affinities, as reflected by profile of LB and HB peptides, were performed and correlated with the results of flow cytometry and immunoprecipitation experiments.

Analysis of peptides presented by HLA-B*35:01 and B*35:08 molecules in the presence of TPN (LCL.TPN+ cells) displayed the majority of them being HB peptides. It was expected that the absence of TPN would lead to the presentation of a higher quantity of LB peptides. However, no remarkable difference between the percentages of HB and LB peptides were observed from peptides presented by these alleles in the absence of TPN. Especially, it was expected that HLA-B*35:08, which was found to be relatively more dependent on TPN, for surface expression, would present peptides of low affinity in the absence of TPN. This result suggests that the probable dependence of HLA-B*35:08 on TPN is on egress of pHLA complex from ER to cell surface and their stabilization on cell surface rather than inside the cell. The results could be comparable to the findings observed in peptides associated with HLA-B*08:01 and HLA-A*02:01, where the peptides acquired in absence of TPN were found to be of unexpected higher affinity than those acquired in its presence [236]. Moreover, Raghuraman *et al* (2002) [96], using an insect cell-reconstituted system and peptide translocation assay, observed that TPN does not alter the peptide translocation efficiency and presence of TPN unexpectedly slightly reduced the affinity of TAP complexes for peptides, suggesting that TPN is less likely to alter the peptide selectivity by TAP and hence the features of TAP-translocatable peptides. These results indicated the role of TPN in stabilizing a peptide receptive conformation of the PBR, but not to function as a peptide editor to discriminate between low and high binding peptides. Furthermore, a peptide receptive conformation of the PBR may not always mean the conformation of HLA molecule with HB affinity or tightly bound peptides.

Besides this, HLA-B*35:62 was shown to present a higher percentage of LB peptides in the presence of TPN, that could be indirectly attributed to its TAP-independence character. The selectivity of TAP is important because it helps in peptide selection by translocation of peptides of optimal length and sequence to their corresponding HLA class I molecules [237-238]. Our experimental data revealed that HLA-B*35:62 could acquire peptides independent of TAP. It most likely that TAP is not being utilized for selection of HLA-B*35:62 specific peptides, therefore poorly selected peptides are presented by B*35:62, even in the presence of TPN. All

these observations imply that the AA mismatch at position 156 in HLA-B*35 variants have the potential to alter the stability of pHLA complexes and influence the mode by which TPN or TAP functions.

HLA-B*35/156 variants studied here are found to present peptides independent of TPN and/or TAP, indicating that these alleles could have probable role in the presentation of viral epitopes. The peptide-binding characteristics of individual HLA class I proteins are shown to be a major factor determining the immunorecognition of pathogens [239]. Moreover, refolding assays on conformational stabilities have shown that TPN-independent allotypes were found to be more assembly competent and are in a more stable peptide-receptive conformation compared with TPN-dependent allotypes [211, 240]. This result highlighted the added advantage of these TPN-independent alleles for pathogen recognition. Paradoxically, dependence of individual HLA class I molecule on TPN can influence the assembly and stability of individual HLA class I molecules and have a subsequent impact on disease progression. Rizvi *et al* (2014) [211] demonstrated the association of greater TPN-independent HLA-B assembly with higher hazard ratios for AIDS. TPN facilitates the occupancy of HLA class I molecule with the optimal peptides. HLA class I molecules loaded with slow-dissociating peptides are probably more stable at the cell surface and thus expected to present peptides to the CD8+ T cells over longer time. On the other hand, loading of fast-dissociating and suboptimal peptides on the TPN-independent HLA molecules may result in a more transient peptide presentation to CD8+ T cells.

Moreover, the presence of Trp at position 156 in HLA-B*35:62 was shown to confer a TAP-independent mode of peptide loading that could be suggestive of conferring the ability of peptide presentation via non-classical pathways and its potential role in immune response against viral infections. However, such differences on the mode of peptide loading can have interference on the alliance with ER quality control factors, stabilities of antigenic peptide associations with HLA-B molecules and hence the abilities of HLA-B molecules to mediate immune responses during infections. HLA-B*35:62 is a rare allele occurring sparsely in Hispanic population (http://www.allelefrequencies.net; http://www.ebi.ac.uk) and further studies are needed to better understand the reason for its failure to be selected in the course of host-pathogen co-evolution.

Knowledge of the peptides likely to be presented on HLA-B*35/156 molecules upon the viral infections would shed light on virus induced modulation of the immune system and further

applied for peptide based-immunotherapies. Mass spectrometric techniques are efficiently used for the characterization of endogenous self peptides presented in healthy cells. It has to be taken into account, that in the context of a viral infection, viral peptides are present in smaller proportion compared to self peptides, and hence the fishing of the virally derived peptides among the large pool of self peptides would not challenging. Herr *et al* (1999) [241], for the first time, demonstrated the natural presentation of EBV LMP 2A peptide by EBV transformed B-LCL cells derived from a donor expressing HLA-A*02:01 allele. Following this study, few other naturally processed HLA restricted T cell epitopes derived from hepatitis B virus [242], Epstein-Barr virus [243], borna disease virus [244] and vaccinia virus [245] were described. Although recent advances in MS derived computational analysis have eased the characterization of virally derived peptides, the output of detection of the naturally presented viral epitopes is very low.

With the current innovations made in the field of bioinformatics, various prediction tools are developed to predict non-self peptides that could bind to a defined HLA molecule. Prediction tools were used to determine probable peptides, derived from some potential viral immune evasion proteins. As for example, this study focus on some of the extensively studied immune evasion proteins derived from HCMV: HCMV US6, HCMV US3, HCMV US2, HCMV US10 and HCMV IE1, that target various components of PLC at different points of viral infection. Based on the peptide sequence data and the anchor motif knowledge collected from published reports, the databases SYFPEITHI and IEDB were used to predict various viral epitopes likely to be presented by HLA-B*35:01. As an example, H**P**SHRLLT**L** (SYFPEITHI) or L**A**LLCSIT**Y** (IEDB) were predicted to be the most probable viral epitopes derived from the HCMV US6 protein, a potent inhibitor of TAP. These prediction tools are based on the reliable databases of self peptide sequences known to bind the HLA molecules. Since to date no peptide anchor motifs were available for HLA-B*35:62, a different approach was followed to predict the viral epitopes likely to be presented by this unique allele. The proteasomal cleavage prediction tool MAPPP was used to predict a list of peptide sequences that would be the potential result of proteasomal cleavage. From those lists of peptides, the best fitting peptides, based on the anchor motifs determined in this thesis, were identified as the candidate epitopes likely to be presented by this allele.

One important limitation of this study is related to the *in-vitro* experiments. The artificial experimental setup can never fully mimic the complexity of normal human system where several background phenomenon may come into play. For instance, the HLA-immunopeptidome studies were carried out in the lentiviral transduction based over-expression system. The results of the surface expression of the particular HLA-B*35/156 allele may not be same in a real intact human body. The exact outcomes in a natural human system depend on many factors including the haplotype and biology of each individual. Another limitation is related to the methodological constraints of peptide purification procedure used in the study, as this approach cannot address the post-translational modifications. It has been reported in various studies that the immunological responses can be influenced by several post-translational modifications of peptides including glycosylation [246] and phosphorylation [247-248]. However, majority of peptides presented by HLA class I molecules are unmodified. Moreover a potential bias in result interpretation can be introduced due to database related limitation since the identification of peptide sequences and sources is dependent on the database and allows detection of peptides that are included in the database. In spite of exponentially growing sequence databases, the drawback of this approach to identify entirely new peptide which has not been included in the database could be a potential challenge to protein identification by current database dependent tandem MS (MS-MS) method. With regards to HLA binding prediction tools, a marked limitation is that predictions can only be applied for those alleles for that peptide binding data are documented. And the precision of methods for HLA peptide binding prediction depends essentially on the available anchor motif data [249]. In addition to this, most of the prediction tools are limited to the peptides of canonical lengths. In this context, prediction of peptides of non-canonical lengths could be an obvious limitation, since it was found in this study that HLA-B*35/156 alleles are able to present peptides of unusual lengths. Moreover, immunogenicity of the predicted peptides cannot be defined, whether these predicted peptides would potently stimulate T cells and elicit immunological memory or commence the T cell unresponsiveness leading to an anergic state. Overall, this approach is a representative of the basic HLA-I antigen presentation pathway and provides a basic foundation for better understanding of the peptide binding specificities of HLA class I molecules.

Our study highlights how a single AA mismatch at position 156 orchestrates the mode of peptide loading and repertoire of presented peptides. This study as a whole is a part of our continual

effort to provide better explanation and estimation of outcomes of HLA mismatches through biochemical and structural analysis of key polymorphic position in HLA alleles. The results of this thesis will provide future directions to HSCT research for improving the therapeutic outcomes of stem cell transplantation. In addition to this, the observations of our study results would guide towards designing novel strategies for the treatment of viral infections.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The main function of HLA class I molecules is to present intracellular digested peptides of self or non-self origin for surveillance by cytotoxic T cells. Immune epitopes presented on class I molecules are scanned by T cell receptors on CD8+ T cells. Even a single AA mismatch at key residues of the HLA class I molecule could sufficiently alter the conformation of the PBR, thereby altering the mode of peptide loading and the whole spectrum of presented peptides. This project was aimed to understand the impact of single AA substitution at position 156 on peptide binding specificities by HLA-B*35 molecules and their biophysical interaction with dedicated parts of the PLC.

Key findings of this thesis:

- All three HLA-B*35/156 variants studied (HLA-B*35:01, B*35:08 and B*35:62) recruit peptides independently of TPN.
- Micropolymorphism at residue 156 alters the degree of TPN dependence. Surface expression of HLA-B*35:08 was found to be relatively more dependent on TPN compared to that of B*35:01 and B*35:62.
- Micropolymorphism at residue 156 in HLA-B*35 molecules have a remarkable impact on their biophysical interaction with components of the PLC. Immunoprecipitation experiments illustrate an association of the HLA-B*35:01 and B*35:08 hc with TAP while the B*35:62 hc does not.
- Comparative analysis of presented peptides by HLA-B*35/156 variants revealed that micropolymorphism at residue 156 alters the peptide repertoire and stability of pHLA complexes.
- HLA-B*35:01 and B*35:08 restricted peptides were found to preferentially anchor with Pro at the p2 position.
- In TPN deficient cells, HLA-B*35:62 restricted peptides were preferentially anchored by Ala at p2.
- HLA-B*35:01 and B*35:08 restricted peptides were found to preferentially anchor with Tyr, Phe, Leu or Lys at the pΩ position.

- In TPN deficient cells, HLA-B*35:62 restricted peptides were preferentially anchored by Trp at pΩ.
- All three HLA-B*35/156 variants were shown to present peptides of non-canonical lengths. HLA-B*35:62 was shown to present comparatively higher percentage of non-canonical peptides both in presence and absence of TPN.

The knowledge of the comparative allele specific peptide binding spectra and biophysical interactions with components of the PLC among HLA-B*35/156 variants identified the importance of micropolymorphism at residue 156 on the PBR of HLA class I molecule. More specifically Trp at position 156 was identified to render a TAP independent mode of peptide loading and unique peptide binding specificities for HLA-B*35:62.

Polymorphism at residue 156 within the PBR of HLA class I molecules are associated with severe acute GvHD and transplant rejection. Therefore, donor:recipient pairs with such AA mismatches should be avoided. This thesis clearly showed the effect of 156 mismatches on functional differences between subtypic variants.

Furthermore, the findings of this thesis including the definition of a PLC independent mode of peptide loading conferred by micropolymorphism will guide towards personalized vaccination therapies.

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Appendix I

Peptide sequences of HLA-B*35/156 hc (Exon 1–7)

HLA-B*35:01 362 aa
MRVTAPRTVLLLLWGAVALTETWAGSHSMRYFYTAMSRPGRGEPRFIAVGYVDDTQFVRF
DSDAASPRTEPRAPWIEQEGPEYWDRNTQIFKTNTQTYRESLRNLRGYYNQSEAGSHIIQ
RMYGCDLGPDGRLLRGHDQSAYDGKDYIALNEDLSSWTAADTAAQITQRKWEAARVAEQ**L**
RAYLEGLCVEWLRRYLENGKETLQRADPPKTHVTHHPVSDHEATLRCWALGFYPAEITLT
WQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEP
SSQSTIPIVGIVAGLAVLAVVVIGAVVATVMCRRKSSGGKGGSYSQAASSDSAQGSDVSL
TA

HLA-B*35:08 362 aa
MRVTAPRTVLLLLWGAVALTETWAGSHSMRYFYTAMSRPGRGEPRFIAVGYVDDTQFVRF
DSDAASPRTEPRAPWIEQEGPEYWDRNTQIFKTNTQTYRESLRNLRGYYNQSEAGSHIIQ
RMYGCDLGPDGRLLRGHDQSAYDGKDYIALNEDLSSWTAADTAAQITQRKWEAARVAEQ**R**
RAYLEGLCVEWLRRYLENGKETLQRADPPKTHVTHHPVSDHEATLRCWALGFYPAEITLT
WQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEP
SSQSTIPIVGIVAGLAVLAVVVIGAVVATVMCRRKSSGGKGGSYSQAASSDSAQGSDVSL
TA

HLA-B*35:62 362 aa
MRVTAPRTVLLLLWGAVALTETWAGSHSMRYFYTAMSRPGRGEPRFIAVGYVDDTQFVRF
DSDAASPRTEPRAPWIEQEGPEYWDRNTQIFKTNTQTYRESLRNLRGYYNQSEAGSHIIQ
RMYGCDLGPDGRLLRGHDQSAYDGKDYIALNEDLSSWTAADTAAQITQRKWEAARVAEQ**W**
RAYLEGLCVEWLRRYLENGKETLQRADPPKTHVTHHPVSDHEATLRCWALGFYPAEITLT
WQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEP
SSQSTIPIVGIVAGLAVLAVVVIGAVVATVMCRRKSSGGKGGSYSQAASSDSAQGSDVSL
TA

Residues in red indicates different AA residue occurring at position 156 on HLA-B*35 hc.

Appendix II

Nucleotide sequences of HLA-B*35/156 hc (Exon 1–7)

HLA-B*35:01 1089 bp
ATGCGGGTCACGGCGCCCCGAACCGTCCTCCTGCTGCTCTGGGGGGCAGTGGCCCTGACC
GAGACCTGGGCCGGCTCCCACTCCATGAGGTATTTCTACACCGCCATGTCCCGGCCCGGC
CGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACCCAGTTCGTGAGGTTC
GACAGCGACGCCGCGAGTCCGAGGACGGAGCCCCGGGCGCCATGGATAGAGCAGGAGGGG
CCGGAGTATTGGGACCGGAACACACAGATCTTCAAGACCAACACACAGACTTACCGAGAG
AGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACATCATCCAG
AGGATGTATGGCTGCGACCTGGGGCCCGACGGGCGCCTCCTCCGCGGGCATGACCAGTCC
GCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGAGCTCCTGGACCGCGGCG
GACACCGCGGCTCAGATCACCCAGCGCAAGTGGGAGGCGGCCCGTGTGGCGGAGCAGCTG
AGAGCCTACCTGGAGGGCCTGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAG
GAGACGCTGCAGCGCGCGGACCCCCCAAAGACACACGTGACCCACCACCCCGTCTCTGAC
CATGAGGCCACCCTGAGGTGCTGGGCCCTGGGCTTCTACCCTGCGGAGATCACACTGACC
TGGCAGCGGGATGGCGAGGACCAAACTCAGGACACTGAGCTTGTGGAGACCAGACCAGCA
GGAGATAGAACCTTCCAGAAGTGGGCAGCTGTGGTGGTGCCTTCTGGAGAAGAGCAGAGA
TACACATGCCATGTACAGCATGAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGAGCCA
TCTTCCCAGTCCACCATCCCCATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTT
GTGGTCATCGGAGCTGTGGTCGCTACTGTGATGTGTAGGAGGAAGAGCTCAGGTGGAAAA
GGAGGGAGCTACTCTCAGGCTGCGTCCAGCGACAGTGCCCAGGGCTCTGATGTGTCTCTC
ACAGCTTGA

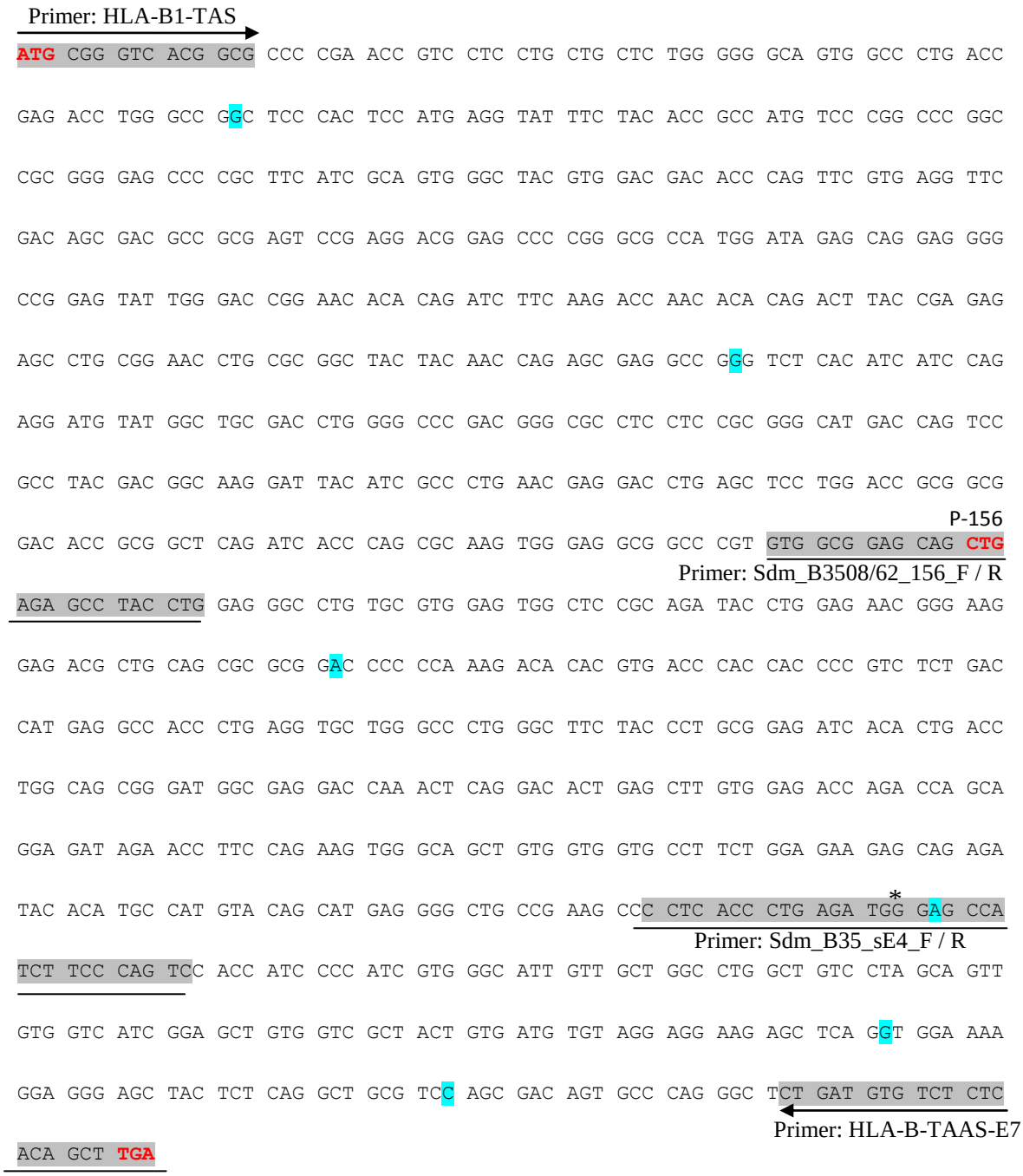
HLA-B*35:08 1089 bp
ATGCGGGTCACGGCGCCCCGAACCGTCCTCCTGCTGCTCTGGGGGGCAGTGGCCCTGACC
GAGACCTGGGCCGGCTCCCACTCCATGAGGTATTTCTACACCGCCATGTCCCGGCCCGGC
CGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACCCAGTTCGTGAGGTTC
GACAGCGACGCCGCGAGTCCGAGGACGGAGCCCCGGGCGCCATGGATAGAGCAGGAGGGG
CCGGAGTATTGGGACCGGAACACACAGATCTTCAAGACCAACACACAGACTTACCGAGAG
AGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACATCATCCAG
AGGATGTATGGCTGCGACCTGGGGCCCGACGGGCGCCTCCTCCGCGGGCATGACCAGTCC
GCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGAGCTCCTGGACCGCGGCG
GACACCGCGGCTCAGATCACCCAGCGCAAGTGGGAGGCGGCCCGTGTGGCGGAGCAGCGG
AGAGCCTACCTGGAGGGCCTGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAG
GAGACGCTGCAGCGCGCGGACCCCCCAAAGACACACGTGACCCACCACCCCGTCTCTGAC
CATGAGGCCACCCTGAGGTGCTGGGCCCTGGGCTTCTACCCTGCGGAGATCACACTGACC
TGGCAGCGGGATGGCGAGGACCAAACTCAGGACACTGAGCTTGTGGAGACCAGACCAGCA
GGAGATAGAACCTTCCAGAAGTGGGCAGCTGTGGTGGTGCCTTCTGGAGAAGAGCAGAGA
TACACATGCCATGTACAGCATGAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGAGCCA
TCTTCCCAGTCCACCATCCCCATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTT
GTGGTCATCGGAGCTGTGGTCGCTACTGTGATGTGTAGGAGGAAGAGCTCAGGTGGAAAA
GGAGGGAGCTACTCTCAGGCTGCGTCCAGCGACAGTGCCCAGGGCTCTGATGTGTCTCTC
ACAGCTTGA

HLA-B*35:62 1089 bp
ATGCGGGTCACGGCGCCCCGAACCGTCCTCCTGCTGCTCTGGGGGGCAGTGGCCCTGACC
GAGACCTGGGCCGGCTCCCACTCCATGAGGTATTTCTACACCGCCATGTCCCGGCCCGGC
CGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACCCAGTTCGTGAGGTTC
GACAGCGACGCCGCGAGTCCGAGGACGGAGCCCCGGGCGCCATGGATAGAGCAGGAGGGG
CCGGAGTATTGGGACCGGAACACACAGATCTTCAAGACCAACACACAGACTTACCGAGAG
AGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACATCATCCAG
AGGATGTATGGCTGCGACCTGGGGCCCGACGGGCGCCTCCTCCGCGGGCATGACCAGTCC
GCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGAGCTCCTGGACCGCGGCG
GACACCGCGGCTCAGATCACCCAGCGCAAGTGGGAGGCGGCCCGTGTGGCGGAGCAGTGG
AGAGCCTACCTGGAGGGCCTGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAG
GAGACGCTGCAGCGCGCGGACCCCCCAAAGACACACGTGACCCACCACCCCGTCTCTGAC
CATGAGGCCACCCTGAGGTGCTGGGCCCTGGGCTTCTACCCTGCGGAGATCACACTGACC
TGGCAGCGGGATGGCGAGGACCAAACTCAGGACACTGAGCTTGTGGAGACCAGACCAGCA
GGAGATAGAACCTTCCAGAAGTGGGCAGCTGTGGTGGTGCCTTCTGGAGAAGAGCAGAGA
TACACATGCCATGTACAGCATGAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGAGCCA
TCTTCCCAGTCCACCATCCCCATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTT
GTGGTCATCGGAGCTGTGGTCGCTACTGTGATGTGTAGGAGGAAGAGCTCAGGTGGAAAA
GGAGGGAGCTACTCTCAGGCTGCGTCCAGCGACAGTGCCCAGGGCTCTGATGTGTCTCTC
ACAGCTTGA

Nucleotides in red indicates different bases corresponding to position 156 on HLA-B*35 hc.

Appendix III

Genomic map of HLA-B*35:01 (1089 bp) with relevant primers and important residues

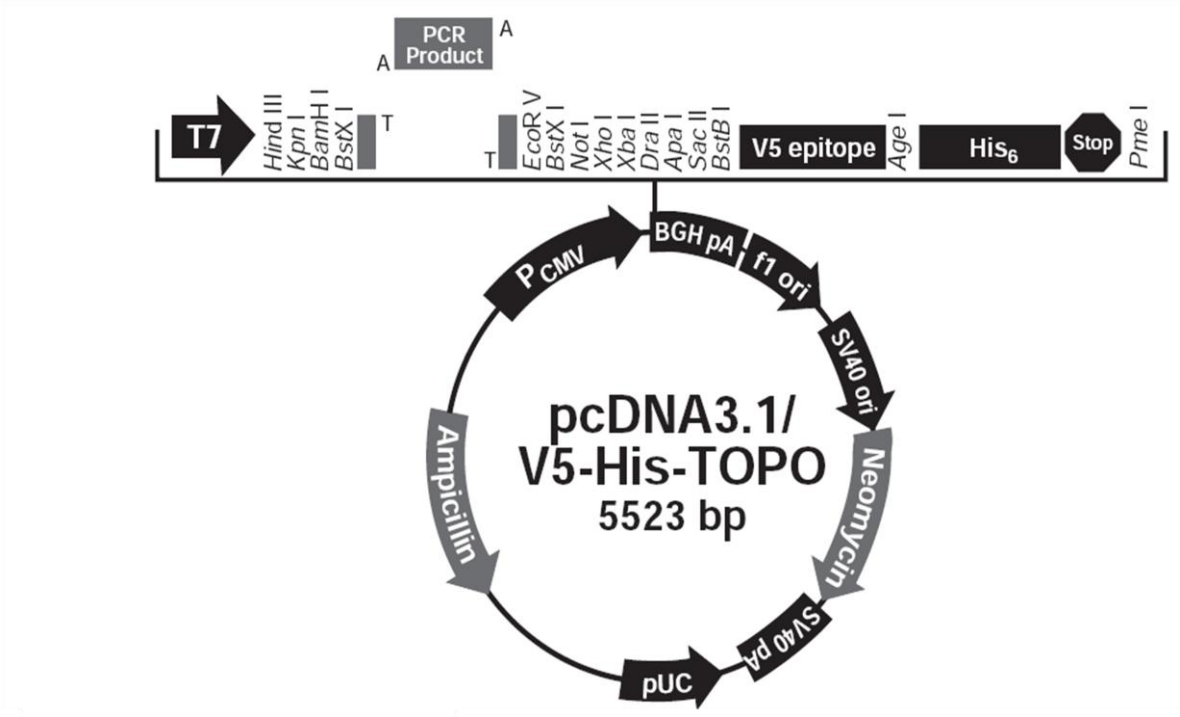


Nucleotides highlighted in grey indicate the location of respective primers for cloning or site directed mutagenesis. The arrows indicate the direction of primers. Nucleotides highlighted in light blue indicate exon boundaries (start of following exon) of HLA-B*35:01 hc (http://hla.alleles.org/data/txt/b_nuc.txt). P-156 denotes position 156 on HLA-B*35 hc. Symbol (†) indicate site of nucleotide exchanged in site-directed mutagenesis primers.

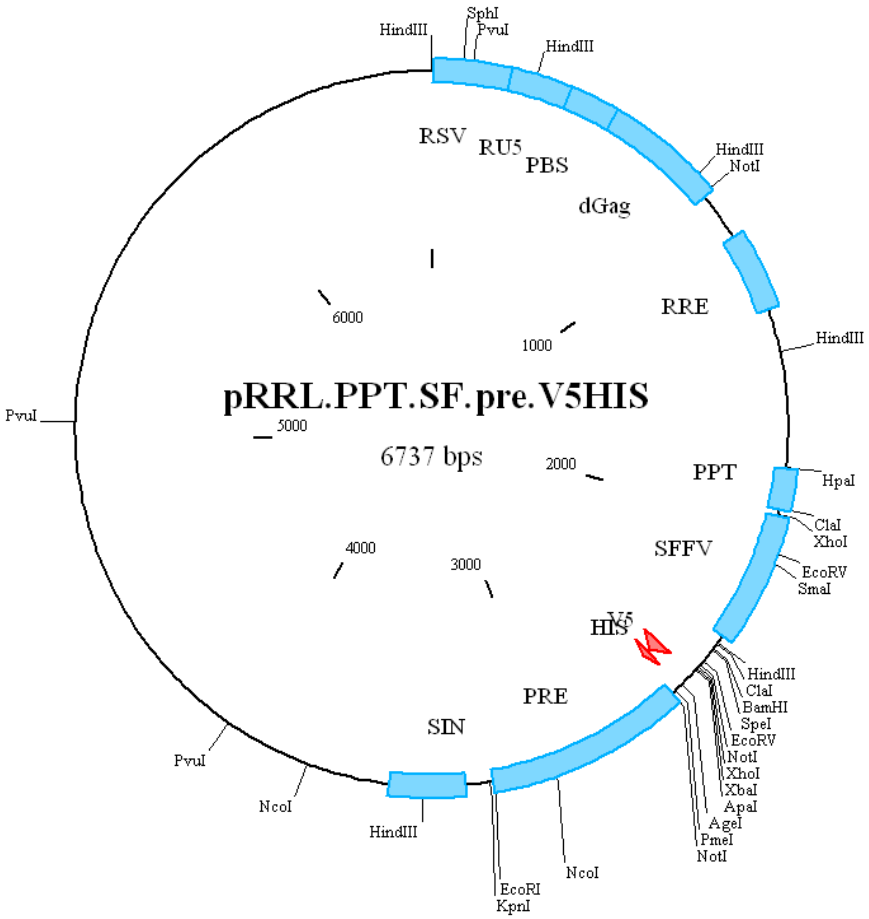
Appendix IV

Plasmid maps

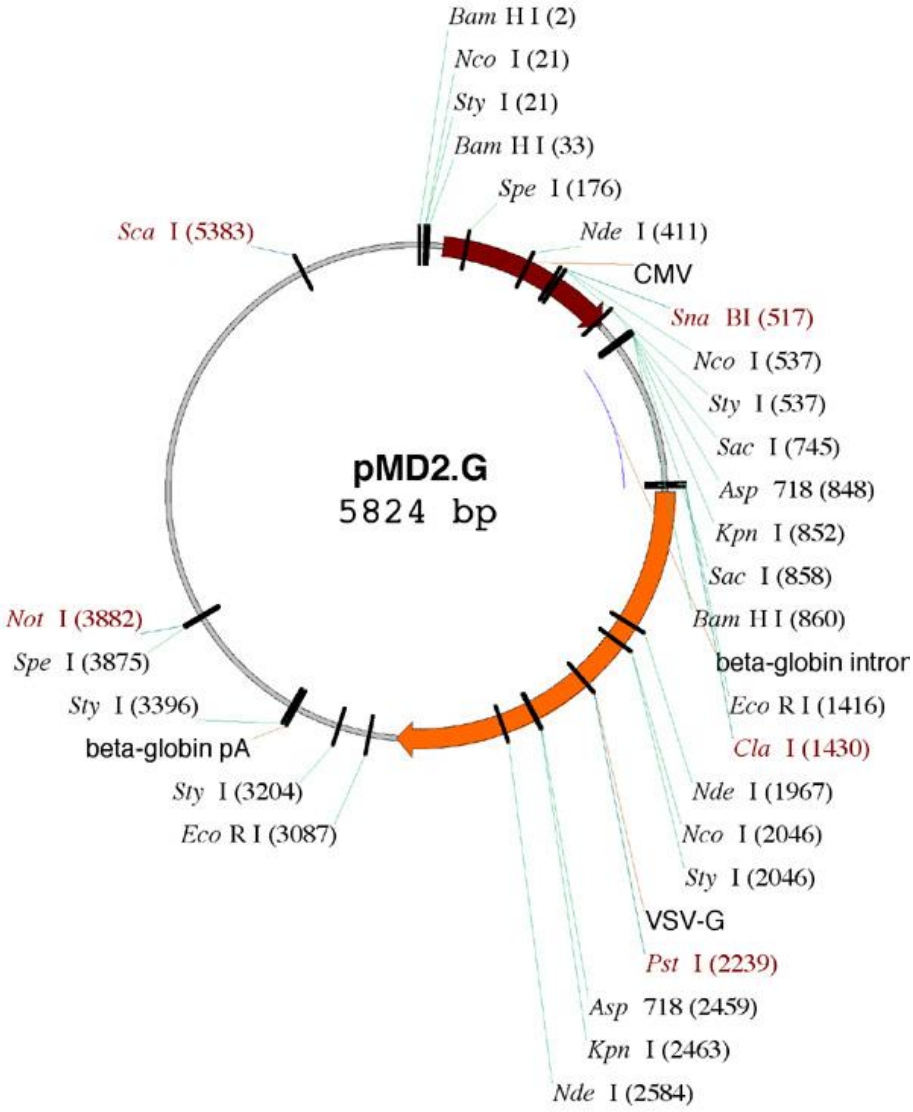
Map of pcDNA™ 3.1/V5-His TOPO®



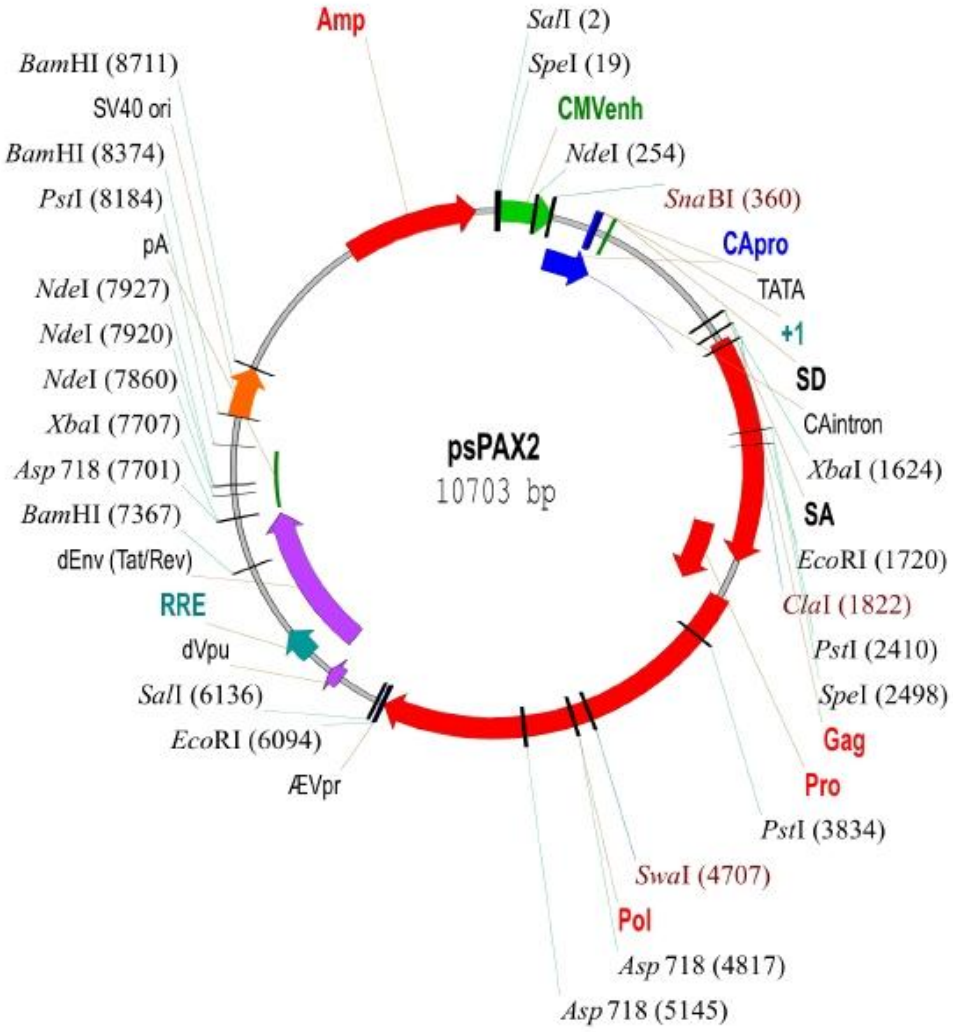
Map of pRRL.PPT.pre.V5HIS



Map of pMD2.G


pMD2.G
5824 bp
Bam H I (2)
Nco I (21)
Sty I (21)
Bam H I (33)
Spe I (176)
Nde I (411)
CMV
Sna BI (517)
Nco I (537)
Sty I (537)
Sac I (745)
Asp 718 (848)
Kpn I (852)
Sac I (858)
Bam H I (860)
beta-globin intror
Eco R I (1416)
Cla I (1430)
Nde I (1967)
Nco I (2046)
Sty I (2046)
VSV-G
Pst I (2239)
Asp 718 (2459)
Kpn I (2463)
Nde I (2584)
Sca I (5383)
Not I (3882)
Spe I (3875)
Sty I (3396)
beta-globin pA
Sty I (3204)
Eco R I (3087)

Map of psPAX2



Appendix V

List of peptides eluted from cells transduced with constructs encoding for sHLA-B*35/156 molecules

Low binding peptides associated with HLA-B*35:01 (origin LCL.TPN- cells)

Peptide position																				Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				
E	A	F	L	N	N	Q	Y													Spectrin beta chaon
P	A	G	V	V	N	K	Y													7-dehyd. Cholesterol reductase
M	A	L	A	D	S	A	I													Insulin receptor substrate 4
M	P	E	D	V	K	N	F	Y												Tubulin polyglutamylase complex subunit 2
Q	P	M	E	V	Q	E	G	Y												Transcription intermediary factor 1-beta
L	P	D	T	R	S	E	A	Y												Cyclin-T1
M	P	T	T	G	I	N	E	Y												Guanine nucleotide-binding prot. subunit alpha-15
L	A	L	V	T	L	L	S	F												Prot. O-mannosyl-transferase 2
V	P	V	G	E	K	T	T	Y												Melanotransferrin
Y	P	T	Q	P	G	Q	G	Y												RNA-binding prot. FUS
I	P	V	T	I	I	T	G	Y												COBW domain-containing prot. 1
L	P	P	P	P	P	G	S	F												Mitogen-activated prot. kinase kinase kinase 5
L	P	Q	D	V	I	L	K	F												Probable ATP-dependent RNA helicase DDX27
L	P	D	E	A	S	E	A	F												Huntingtin
I	A	L	K	A	V	T	N	F												Bromodomain-containing prot. 7
E	A	A	V	A	I	K	A	M												Eukaryotic translation initiation factor 5A-1-like
M	P	Q	G	A	P	R	L	Y												Phosphatidylinositol 3,4,5-trispho-dep Rac prot.

F P D K P I T Q Y	Dolichyl-diphosphooligosaccharide—prot.
Y P V D L G D K F	DNA-directed RNA polymerases I, II, and III subunit RPABC3
M P A D T N K A F	ER membrane prot. complex subunit 3
L P N D G D E K Y	Programmed cell death prot. 2-like
T P A G V V N K Y	7-dehydrocholesterol reductase
Y P A P E R L Q E Y	Putative WAS prot. family homolog
H P I I P E Q S T F	Suppressor of G2 allele of SKP1 homolog
F P E T T S P H E Y	Neuroblastoma-amplified sequence
L P I D P N E P T Y	Inhibitor of growth prot. 1
H P L S L T S D Q Y	Interferon regulatory factor 3
I P I A G R D I T Y	Actin-related prot. 3B
S P A Q E D G K V Y	Hematopoietic cell signal transducer
Y K C V S C T K T F	Zinc finger prot. 668
L P S P V T A Q K Y	Elongation factor 2
F P M T H G N T G F	Poly(rC)-binding prot.
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.
M L N I V Q D S A L L	Ral GTPase-activating prot. subunit beta
M P V A A R E A S I Y	V-type proton ATPase catalytic subunit A
S P V N S S K Q P S Y	ATP synthase lipid-binding prot., mitochondrial
I P Y H S E V P V S L	Spectrin beta chain, non-erythrocytic 1
D P A P L G A G N L G P	Prot. DPCD
P A N G A V T L P A P P P	Rho guanine nucleotide exchange factor 26

High binding peptides associated with HLA-B*35:01 (origin LCL.TPN- cells)

Peptide position 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 Ligands	Source
T G E G F Y K Y	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
I Q K F P V G R	Transmembrane protease serine 9
P A Q Y P P P P	RNA binding prot. fox-1 homolog 3
N E E N I F I I	Protocadherin-18
H P T I I S E S F	T-complex prot. 1 subunit delta
H P T S V I S G Y	T-complex prot. 1 subunit alpha
A P E E H P V L L	Actin, cytoplasmic 1
S G V S L A A L K	Histone H1.2
P V P V L V E D T	Mitogen-activated prot. kinase kinase kinase 5
M D N Y S T G Y D	Nuclear receptor subfamily 4 group A member 2
I D C L S Q K Q F	Activating signal cointegrator 1
T P D E I D H V F	Poly [ADP-ribose] polymerase 14
A Y D A T H L V K	S-formylglutathione hydrolase
I P L P L I K S Y	Cyclin-dependent kinase 2
L P D E I Y H V Y	N-terminal Xaa-Pro-Lys N-methyltransferase 1
S N L E N I D F K	Adenylosuccinate lyase
I P N E I I H A L	Heterogeneous nuclear ribonucleoprot. M
N V I R D A V T Y	Histone H4
L P Q E A F E K Y	Structural maintenance of chromosomes prot. 3
L K D D E V A Q L	L-lactate dehydrogenase B chain
A G L Q F P V G R	Histone H2A.Z
Y P V E H P D K F	Transitional endoplasmic reticulum ATPase

I D C L S Q K Q F	Activating signal cointegrator 1
V E N Q I E K V F	Translation factor GUF1, mitochondrial
K L E D G P K F L K	Elongation factor 1-alpha 1
G G V V G I K V D K	Fructose-bisphosphate aldolase A
G S G T A E V E L K	Pyruvate kinase isozymes M1/M2
Q E K I V Q C Q K A	Inactive phospholipase C-like prot. 1
M F C Q A A R V D L	E3 ubiquitin-prot. ligase TRIM41
H P L S L T S D Q Y	Interferon regulatory factor 3
L P S P V T A Q K Y	Elongation factor 2
P E L A K S A P A P K	Histone H2B type 1-L
P D P A K S A P A P K	Histone H2B type
E A K V K F E E R Y K	60S ribosomal prot. L27
P E P A K S A P A P K	Histone H2B type 2-E
V L P G V D A L S N I	Phosphoglycerate kinase 1
L L K V L S F T H P T	Germinal center-associated signaling and motility prot.
N F G I G Q D I Q P K	60S ribosomal prot. L7a
S P V N S S K Q P S Y	ATP synthase lipid-binding prot., mitochondrial
A S T S S N S A S S F	Zinc finger homeobox prot. 3
V G G T S D V E V N E K	60 kDa heat shock prot., mitochondrial
C D T R P Q L L M R G C	Integrin beta-2
A R V I T E E E K N F K	60S ribosomal prot. L13
A L S T G E K G F G Y K	Peptidyl-prolyl cis-trans isomerase A
H P I H L G D E Q H S Q Y	RING finger prot. 10
K M K I H G V V A F K C E	Neurexin-1-alpha
N R Q D P S Q E E E G A A	RUN and SH3 domain-containing prot. 1
A G N L G G G V V T I E R	60S ribosomal prot.
F S I V R D P A A L A R S A	Galactose-3-O-sulfotransferase 4
S N T A G S Q S Q V E T E A	Peptidyl-prolyl cis-trans isomerase FKBP4
N T K G G D A P A A G E D A	40S ribosomal prot. S25

E V S T N T A M I Q T S K T E	Keratin, type I cytoskeletal 13
P Y G S R S P F E H S V E H K	Chromodomain-helicase-DNA-binding prot. 1
A S G N Y A T V I S H N P E T K	60S ribosomal prot. L8
A S S E G G T A A G A G L D S L H K	Actin-related prot. 2/3 complex subunit 1B

Low binding peptides associated with HLA-B*35:01 (origin LCL.TPN+ cells)

Peptide position																				Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				
N	V	A	D	L	H	E	K	Y												Proteasome subunit beta type-5
T	V	F	D	L	V	E	E	Y												Nuclear pore complex prot. Nup107
L	P	Q	E	A	F	E	K	Y												Structural maintenance of chromosomes prot. 3
T	A	T	Q	L	A	V	N	K												T-complex prot. 1 subunit eta
H	P	I	R	I	A	D	G	Y												T-complex prot. 1 subunit epsilon
N	P	I	S	T	V	T	E	L												T-complex prot. 1 subunit delta
N	A	V	N	L	A	I	K	Y												WD repeat and HMG-box DNA-binding prot. 1
H	A	V	S	P	I	A	K	Y												PAP-associated domain-containing prot. 5
I	G	P	L	G	L	S	P	K												60S ribosomal prot. L12
E	A	I	G	A	V	I	H	Y												Neurochondrin
S	P	I	D	V	V	E	K	Y												Kelch-like prot. 12
T	P	A	G	V	V	N	K	Y												7-dehydrocholesterol reductase
A	P	E	E	H	P	V	L	L												Actin, cytoplasmic 1
L	P	S	P	V	T	A	Q	K	Y											Elongation factor 2
I	A	R	D	E	G	G	K	A	F											ADP/ATP translocase 2 OS
T	P	A	P	V	E	K	S	P	A											Histone H1.5
Y	V	H	D	D	G	R	V	S	Y											Glia maturation factor gamma
T	V	I	D	E	V	R	T	G	T	Y										Tubulin alpha-1A chain
S	P	V	N	S	S	K	Q	P	S	Y										ATP synthase lipid-binding prot., mitochondrial
P	E	L	A	K	S	A	P	A	P	K										Histone H2B type 1-L
P	D	P	A	K	S	A	P	A	P	K										Histone H2B type 1-H
P	D	P	S	K	S	A	P	A	P	K										Histone H2B type 3-B

K Q T Y S T E P N N L K	60S ribosomal prot. L28
R Q Q P G P S E H I E R	Vasodilator-stimulated phosphoprot.
L T S D D V K E Q I Y K	40S ribosomal prot. S13
Q S L P P G L A V K E L	60S ribosomal prot. L38
I A V D G E P L G R V S F	Peptidyl-prolyl cis-trans isomerase A
L K A N P F G G A S H A K	40S ribosomal prot. S23
F G F G D S R G G G G N F	Heterogeneous nuclear ribonucleoprot.s A2/B1
N A G A V I G K G G K N I K	Heterogeneous nuclear ribonucleoprot. K
K I K E K Y I D Q E E L N K	Putative heat shock prot. HSP 90-beta 2
A S I K K G E D F V K T L K	Malate dehydrogenase, mitochondrial
V A K V T G G A A S K L S K	60S ribosomal prot. L35
D T G K T P V E P E V A I H R	40S ribosomal prot. S20
H G S Y E D A V H S G A L N D	T-complex prot. 1 subunit alpha
S Q V I S N A K N T V Q G F K	Heat shock 70 kDa prot. 4
N Q Q I T H A N N T V S N F K	Heat shock prot. 105 kDa
P E P V K S A P V P K K G S K	Histone H2B type 1-M
K L T G K D V N F E F P E F Q L	40S ribosomal prot. S7
H A V S E G T K A V T K Y T S A	Histone H2B type 1-K
A S I P F S V V G S N Q L I E A K	Septin-2
S A I N E V V T R E Y T I N I H K	60S ribosomal prot. L31
H S G P G P A G F P V P N Q P V Y	Phospholipid scramblase 1
S V P T S T V F Y P S D G V A T E K	Transketolase
T I L S N Q T V D I P E N V D I T L K	60S ribosomal prot. L9
T A E A G G V T G K G Q D G I G S K A E K	Poly [ADP-ribose] polymerase 1

High binding peptides associated with HLA-B*35:01 (origin LCL.TPN+ cells)

Peptide position																				Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				
F	P	G	P	S	K	P	F													T-cell-specific surface glycoprot. CD28
Y	P	V	E	I	H	E	Y	L												Nuclear nucleic acid-binding prot. C1D
N	P	D	D	V	F	R	E	F												DnaJ homolog subfamily B member 6
N	P	V	N	Y	G	R	P	Y												Ribosome biogenesis prot. TSR3 homolog
Y	P	V	D	L	G	D	K	F												DNA-directed RNA polymerases I, II, and III subunit RPABC3
S	A	A	S	E	Q	H	V	F												Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16
Q	A	V	A	D	A	V	T	Y												Proteasome subunit beta type-6
H	H	P	A	A	A	A	A	Y												Transcription factor MafA
N	V	A	D	L	H	E	K	Y												Proteasome subunit beta type-5
M	P	Q	E	K	S	P	G	Y												G patch domain-containing prot. 2-like
L	P	D	E	I	Y	H	V	Y												N-terminal Xaa-Pro-Lys N-methyltransferase 1
D	P	F	V	D	R	I	G	Y												Nucleoporin NUP188 homolog
Q	P	M	E	V	Q	E	G	Y												Transcription intermediary factor 1-beta
F	P	A	G	K	V	P	A	F												Elongation factor 1-gamma
M	P	Q	G	A	P	R	L	Y												Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac prot.
T	A	C	A	P	V	S	H	Y												Phosphorylated adapter RNA export prot.
Y	P	N	G	V	V	V	H	Y												Heat shock-related 70 kDa prot. 2
T	P	I	Q	S	K	E	A	Y												Adenylate kinase isoenzyme 4, mitochondrial
M	P	N	G	T	V	Q	R	F												Nucleosome-remodeling factor subunit BPTF
M	P	E	D	V	K	N	F	Y												Tubulin polyglutamylase complex subunit 2
L	P	D	T	R	S	E	A	Y												Cyclin-T1
Y	P	L	D	V	Q	K	E	F												Probable RNA polymerase II nuclear localization prot.

I P L P L I K S Y	Cyclin-dependent kinase 2
T P G G T R I I Y	Eukaryotic translation initiation factor 4E-binding prot.
Y P D E Y H G E Y	Calponin-3
H P T D P L T S F	Prot. THEMIS2
N A I S V T T S Y	RNA polymerase-associated prot. CTR9 homolog
N A A Q T S V A Y	Arginine--tRNA ligase, cytoplasmic
A G L Q F P V G R	Histone H2A type 1-A
T A A E T H Y T Y	Upstream stimulatory factor 1
L P F D K E T G F	SRA stem-loop-interacting RNA-binding prot., mitochondrial
N P L Q K D P Q Y	Prot. phosphatase 1 regulatory subunit 7
M P Q G G G Q H Y	Prot. SSXT
H P I Q T Q A Q Y	La-related prot. 4
F P V I Y D V K Y	CCR4-NOT transcription complex subunit 7
Y P N S H T H Y F	CCR4-NOT transcription complex subunit 1
F P I P G E P G F	Actin-related prot. 2/3 complex subunit 3
F P A K V T A H W	Trafficking prot. particle complex subunit 5
T A A G L M H T F	Selenide, water dikinase 2
L P Q E A F E K Y	Structural maintenance of chromosomes prot. 3
F P E E D K K T Y	NADH dehydrogenase [ubiquinone] 1 subunit C2
I P N E I I H A L	Heterogeneous nuclear ribonucleoprot. M
L P N D G D E K Y	Programmed cell death prot. 2-like
I P A A V K L T Y	E3 ubiquitin-prot. ligase UBR5
T P A G V V N K Y	7-dehydrocholesterol reductase
D A G P P T H A F	E3 ubiquitin-prot. ligase pellino homolog 1
M P A E I V E L H	Transcriptional repressor prot. YY1
E A A V A I K A M	Eukaryotic translation initiation factor 5A-1
S P I D V V E K Y	Kelch-like prot. 12
T A S A V V Q H M	Splicing factor 3B subunit 1
T P M F V V K A Y	Elongation factor 2

F P N A I E H T L	Ubiquitin-like modifier-activating enzyme 1
H A D G T I V R Y	Intraflagellar transport prot. 172 homolog
T P A E I R E E F	DnaJ homolog subfamily C member 11
A P V E V T H N F	Regulator of nonsense transcripts 1
L P P G V H I S Y	Integrin beta-7
N A F Y E H A Q T Y	Trafficking prot. particle complex subunit 11
Q P W E E I K T S Y	Ectonucleoside triphosphate diphosphohydrolase 1
S P A Q E D G K V Y	Hematopoietic cell signal transducer
F P A G I Y D T K Y	Target of EGR1 prot. 1
F A V D L E H H S Y	Exosome component 10
F P V K G L K T G Y	1-phosphatidylinositol bisphos phosphodiest.gamma
N V V K L L G E Q Y	Zinc finger prot. ubi-d4
Q A F Q E R L N S Y	Nuclear pore complex prot. Nup155
F P M T H G N T G F	Poly(rC)-binding prot. 2
M P S Q V V K G G A F	EH domain-containing prot.
M P E P Q A P G R Y F	Ig mu chain C region, Ig mu heavy chain disease prot.
V P C D S N E A N E M	Sperm prot. associated with the nucleus on the X chrom A
H P T D I T S L D Q Y	Endoplasmin
L P F P D E T H E R Y	Trafficking prot. particle complex subunit 2-like prot.
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.
E A F D E L L A S K Y	Regulator of G-prot. signaling 2
D A I R S L A S V S Y	ATP-dependent RNA helicase DDX50
R P F E E N G A C K Y	Zinc finger prot. 36, C3H1 type-like 1
V P E E G G A T H V Y	A-kinase-interacting prot. 1
L P F D G S P K I T Y	HERV-V_19q13.41 provirus ancestral Env polyprot. 1
N P E N L A T L E R Y	Eukaryotic translation initiation factor 3 subunit K
M P V G P D A I L R Y	Large proline-rich prot. BAG6
Q V H P D T G I S S K	Histone H2B type 1-A
L P Y K A T E N D I Y	Heterogeneous nuclear ribonucleoprot. F

Q P L L I I G K G A A Y	2-hydroxyacyl-CoA lyase 1
S L V S K G T L V Q T K	Histone H1.1
F P A V G E P N I Q Q Y	UDP-N-acetylglu. N-Acetylglu.tranf. prot.
V P F E S E D N Q G I V Y	Prot. flightless-1 homolog
M P W G D P N Y R S A N F	RING finger prot. 114
H P I S S E E L L S L K Y	Prot. C-ets-1
H A F Q P Q S G T V E A M	Cirhin
T P W E E S T N D I S H Y	Phosphoinositide 3-kinase regulatory subunit 5
T L H P D L G T D K D K E Q W K	L-lactate dehydrogenase A chain
L I A P V A E E E A T V P N N K	L-lactate dehydrogenase B chain
H A V S E G T K A V T K Y T S A	Histone H2B type 1-J
K L E K E E E E G I S Q E S S E E E Q	High mobility group prot. HMG-I/HMG-Y

Low binding peptides associated with HLA-B*35:08 (origin LCL.TPN- cells)

Peptide position																				Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				
R	Y	R	T	N	T	G	F													Intelectin-2
I	D	S	K	C	L	H	F													Histone acetyltransferase KAT5
I	D	D	Y	E	L	P	L													Inactive dipeptidyl peptidase 10
M	A	D	S	S	P	A	L													LysM and peptidoglycan-binding domain-containing prot. 2
S	P	S	E	K	E	D	E													Biorientation of chromosomes in cell division prot. 1-like
M	A	D	E	A	A	L	A													NAD-dependent prot. deacetylase sirtuin-1
M	A	L	A	D	S	A	I													Insulin receptor substrate 4
V	Y	S	H	D	G	V	S	L												Frataxin, mitochondrial
F	P	E	G	S	V	E	L	Y												40S ribosomal prot. S3
M	S	V	Q	A	L	Q	S	L												RAD9, HUS1, RAD1-interacting nuclear orphan prot. 1
V	P	L	A	F	G	P	P	P												Structure-specific endonuclease subunit SLX1
A	A	T	L	E	E	G	N	P												Trinucleotide repeat-containing gene 18 prot.
F	P	D	K	P	I	T	Q	Y												Dolichyl-diphosphooligosaccharide--prot. glycosyltransferase
L	P	A	V	G	E	H	V	F												E3 SUMO-prot. ligase CBX4
I	P	Y	Q	D	R	E	S	Y												Zinc finger prot. 106 homolog
F	P	S	S	N	V	H	V	Y												DnaJ homolog subfamily B member 12
M	P	D	E	A	T	P	H	Y												Alpha-mannosidase 2
F	P	I	P	G	E	P	G	F												Actin-related prot. 2/3 complex subunit 3
L	P	S	E	I	E	V	K	Y												Anaphase-promoting complex subunit 7
L	P	N	D	G	D	E	K	Y												Programmed cell death prot. 2-like
P	G	H	P	G	P	S	E	P												Microtubule-associated serine/threonine-prot. kinase 4
H	P	D	A	P	M	S	Q	V	Y											Mortality factor 4-like prot. 1

G Q K L E G L L R Q	Leucine-rich repeat-containing prot. 16C
L K S T V S S L L Q	BPI fold-containing family A member 2
Y K C V S C T K T F	Zinc finger prot. 668
F P D V P D K E N F	Prot. CASC5
F P D T G S D H S Y	THAP domain-containing prot. 11
A F G G G E R V S L	Putative uncharacterized prot. SPANXA2-OT1
I P A S N T A D E E Y	Inositol 1,4,5-trisphosphate receptor type 2
F P Q E E A I I D K Y	All-trans-retinol 13,14-reductase
Y M I F D P N N P L M	Zinc finger homeobox prot. 4
S E P A A A A A M A L A L	Paraspeckle component 1
V S E T A S G S V T Q P K	CD2-associated prot.
V G N G S A L P T N D N S Y	Corneodesmosin
Y Q E A A A A G T F L A V E F T	Phosphopantothenate--cysteine ligase
F P P G A Q A D D G R D P E S R R E	RING finger prot. 126
D V A W S P K N I D R R C Y N Y T G I A D A C	Di-N-acetylchitobiase

High binding peptides associated with HLA-B*35:08 (origin LCL.TPN- cells)

Peptide position																				Ligands	Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
P	A	Q	G	A	K	Y	R													P A Q G A K Y R	Annexin A6
Q	L	Y	G	G	R	A	A													Q L Y G G R A A	Lipocalin-like 1 prot.
S	K	Q	A	E	E	E	F													S K Q A E E E F	Threonine--tRNA ligase, cytoplasmic
A	L	D	R	I	V	E	Y													A L D R I V E Y	Prot. lunapark
P	A	K	S	A	P	A	P	K												P A K S A P A P K	Histone H2B type 1-H
K	P	P	P	P	P	P	P	P												K P P P P P P P P	Mediator of RNA polymerase II transcription subunit 19
L	P	Q	E	A	F	E	K	Y												L P Q E A F E K Y	Structural maintenance of chromosomes prot. 3
F	P	D	K	P	I	T	Q	Y												F P D K P I T Q Y	Dolichyl-diphospholip--prot.glycosyltransferase
V	Y	P	A	D	V	V	L	F												V Y P A D V V L F	Uridine-cytidine kinase 1
H	P	D	K	K	I	V	A	Y												H P D K K I V A Y	Ataxin-10
T	P	D	E	I	D	H	V	F												T P D E I D H V F	Poly [ADP-ribose] polymerase 1
H	P	N	D	D	D	V	H	F												H P N D D D V H F	5'-3' exoribonuclease 1
P	G	P	P	P	P	G	P	H												P G P P P P G P H	Prot. transport prot. Sec24D
L	P	D	T	R	S	E	A	Y												L P D T R S E A Y	Cyclin-T1
L	V	P	A	T	A	E	P	P												L V P A T A E P P	Telomere length regulation prot. TEL2 homolog
P	H	L	G	P	P	V	P	P												P H L G P P V P P	POU domain, class 3, transcription factor 3
P	P	G	P	P	P	P	Q	P												P P G P P P P Q P	Prot. phosphatase 1 regulatory subunit 28
L	P	D	T	L	K	V	T	Y												L P D T L K V T Y	Integrin beta-2
A	D	R	D	R	P	S	V	P												A D R D R P S V P	Prot. KIAA0284
L	S	D	G	V	A	V	L	K												L S D G V A V L K	60 KDa heat shock protein, mitochondrial
A	P	P	P	P	P	P	G	H												A P P P P P P G H	Zinc finger prot. ZFPM1
F	P	I	P	D	L	Q	K	Y												F P I P D L Q K Y	Insulin-degrading enzyme

Y P T E D Y K V Y	Trafficking prot. particle complex subunit 2-like prot.
A G L Q F P V G R	Histone H2A type 1-A
L P N D G D E K Y	Programmed cell death prot. 2-like
L P D E K V E L F	Pericentriolar material 1 prot.
T G S R G E L M L E	Kinesin-like prot. KIF12
Y A Q D E H L I T F	U5 small nuclear ribonucleoprot. 200 kDa helicase
Y P D E N G F D A F	Nardilysin
F P E T T S P H E Y	Neuroblastoma-amplified sequence
L A G E S E S N L R	Transitional endoplasmic reticulum ATPase
H P E S S L S S E E	Receptor-type tyrosine-prot. phosphatase
P E P A K S A P A P K	Histone H2B type 1-C/E/F/G/I
P E P V K S A P V P K	Histone H2B type 1-M
T V I D E V R T G T Y	Tubulin alpha-1B chain
P E P T K S A P A P K	Histone H2B type 1-D
L P Y R A T E N D I Y	Heterogeneous nuclear ribonucleoprot.
F P Q E E A I I D K Y	ll-trans-retinol 13,14-reductase
D S Y V G D E A Q S K	Actin, aortic smooth muscle
D S Y V G D E A Q S K	Actin, cytoplasmic 1
A P A V T Q H A P Y F K	Thioredoxin-dependent peroxide reductase
A L S T G E K G F G Y K	Peptidyl-prolyl cis-trans isomerase A
S V Q A T T E N K E L K	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial
P N S S I F L T D T A K	Tryptophan--tRNA ligase, cytoplasmic
A A A E I D E E P V S K	Nascent polypeptide-associated complex subunit alpha
N E D T S H A A T T I P E	Collagen alpha-1(XVIII) chain
A A A A E I D E E P V S K	Nascent polypeptide-associated complex subunit alpha
S N T A G S Q S Q V E T E A	Peptidyl-prolyl cis-trans isomerase FKBP4
H G S Y E D A V H S G A L N D	T-complex prot. 1 subunit alpha
A Q A A A P A S V P A Q A P K	60S ribosomal prot.
R L S A K P A P P K P E P K P K	Non-histone chromosomal prot. HMG-17

H A V S E G T K A V T K Y T S A	Histone H2B type 1-J
A Y V R L A P D Y D A L D V A N K	60S ribosomal prot. L23a
P P A E N S S A P E A E Q G G A E	Nuclease-sensitive element-binding prot. 1
A S S E G G T A A G A G L D S L H K	Actin-related prot. 2/3 complex subunit 1B
K L E K E E E E G I S Q E S S E E E Q	High mobility group prot. HMG-I/HMG-Y
P E N V A P R S G A T A G A A G G R G K	T-complex prot. 1 subunit delta
K A S K T A E N A T S G E T L E E N E A G D	Proliferation-associated prot. 2G4

Low binding peptides associated with HLA-B*35:08 (origin LCL.TPN+ cells)

Peptide position																				Source
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				
F	P	R	C	E	F	L	I													Baculoviral IAP repeat-containing prot. 2
M	V	F	S	E	T	V	I													Dickkopf-related prot. 3
L	P	D	A	H	S	D	Y													RNA-binding prot. 14
L	S	L	A	E	A	S	R													Growth/differentiation factor 15
F	P	N	A	I	E	H	T	L												Ubiquitin-like modifier-activating enzyme 1
T	A	D	V	V	K	V	A	Y												Nuclear pore membrane glycoprot. 210
F	P	S	I	Y	D	V	K	Y												CCR4-NOT transcription complex subunit 8
H	P	I	S	S	E	E	L	L												Prot. C-ets-1
S	P	Q	E	K	E	A	L	Y												ADP-ribosylation factor-binding prot. GGA2
H	P	D	P	I	V	I	N	H												SWI/SNF-related actin-dependent regulator
Y	A	D	P	V	N	A	H	Y												Polypyrimidine tract-binding prot. 3
L	P	F	D	K	E	T	G	F												SRA stem-loop-interacting RNA-binding prot., mitochondrial
N	V	I	R	D	A	V	T	Y												Histone H4
L	P	S	E	I	E	V	K	Y												Anaphase-promoting complex subunit 7
H	P	H	L	V	A	E	A	Y												Speckle-type POZ prot.
F	P	D	K	P	I	T	Q	Y												Dolichyl-diphosphooligosac.glycosyltraf. 48 kDa subunit
I	G	P	L	G	L	S	P	K												60S ribosomal prot. L12
A	A	L	L	K	A	S	P	K												60S ribosomal prot. L14
F	P	A	E	K	E	S	E	W												Calcineurin-like phosphoesterase domain-containing prot.
H	P	N	D	D	D	V	H	F												5'-3' exoribonuclease 1
A	V	D	S	Q	I	L	P	K												60S ribosomal prot. L6
L	P	D	E	K	V	E	L	F												Pericentriolar material 1 prot.
H	A	D	S	I	L	E	K	Y												Inositol 1,4,5-trisphosphate receptor type 1

H A D G T I V R Y	Intraflagellar transport prot. 172 homolog
V I D K E S E V Y	PDZ and LIM domain prot. 1
N P D E A E K A L	RNA-binding prot. with serine-rich domain 1
Y P D E Y H G E Y	Calponin-3
A Y D A T H L V K	S-formylglutathione hydrolase
L P Q E A F E K Y	Structural maintenance of chromosomes prot. 3
F P E I D L E K Y	Dihydrofolate reductase
L P N D G D E K Y	Programmed cell death prot. 2-like
A P E E H P V L L	Actin, cytoplasmic 1
N A Q R Q D I A F	Annexin A2
T A T Q L A V N K	T-complex prot. 1 subunit eta
K L E D G P K F L	Elongation factor 1-alpha 1
T L A A I Q G L L K	Spectrin alpha chain, non-erythrocytic 1
L Q S S R A F T N S	Deoxyribonuclease gamma
L V D I E K A I A H	Alpha-soluble NSF attachment prot.
T P I E E R G D L F	Zinc finger prot. 1 homolog
F P I S E E T I K L	Nucleolar RNA helicase 2
R P I E D D Q E V Y	FYN-binding prot.
H P D E K S I I T Y	Spectrin beta chain, non-erythrocytic 1
L P E V E V P Q H L	Nucleobindin-1
H I A N V E R V P F	Gelsolin
H P S D I E V D L L	Beta-2-microglobulin
P E P V K S A P V P K	Histone H2B type 1
H A T V A T E N E V F	Gamma-interferon-inducible prot. 16
L P Y K A T E N D I Y	Heterogeneous nuclear ribonucleoprot. F
L P Y R A T E N D I Y	Heterogeneous nuclear ribonucleoprot. H
H P E N P K A V E T F	DnaJ homolog subfamily C member 12
S I S E G D D K I E Y	rRNA 2'-O-methyltransferase fibrillarin
T V I D E V R T G T Y	Tubulin alpha-1A chain

F P I P A A E V D R L	Nuclear receptor 2C2-associated prot.
H A Q H E G E S V S Y	Nuclear receptor coactivator 2
F P Q E E A I I D K Y	All-trans-retinol 13,14-reductase
V P E E G G A T H V Y	A-kinase-interacting prot. 1
L K D D E V A Q L K K	L-lactate dehydrogenase B chain
N I D D G T S D R P Y	60S ribosomal prot. L27
A L S T G E K G F G Y K	Peptidyl-prolyl cis-trans isomerase A
A T G P P V S E L I T K	Histone H1.5
V K A E P A K I E A F R	Phosphoglycerate kinase 1
F G K K T G E G F Y K Y	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
A R V I T E E E K N F K	60S ribosomal prot. L13
F A Q I N Q G E S I T H	Adenylyl cyclase-associated prot. 1
D F S E L E P D K F Q N K	Glycogen phosphorylase, liver form
S A D T L W G I Q K E L Q F	L-lactate dehydrogenase A chain
Q G S V Q K V Y N G L Q G Y	DnaJ homolog subfamily B member 11
S P D D P S R Y I S P D Q L	Alpha-enolase
E A M K A Q M A E K E A I L Q	FYVE and coiled-coil domain-containing prot. 1
Y F Q I N Q D E E E E E D E D	60S ribosomal prot. L22
Y G N Q G G G Y G G G Y D N Y	Heterogeneous nuclear ribonucleoprot.s A2/B1
H A V S E G T K A V T K Y T S A	Histone H2B type 1-J
H A V S E G T K A V T K Y T S S	Histone H2B type 1-L
H A V S E G T K A V T K Y T S S	Histone H2B type 1-H
K G D D Q S R Q G G A P D A G Q E	T-complex prot. 1 subunit gamma
G A D F L V T E V E N G G S L G S K	Pyruvate kinase isozymes M1/M2
S V P T S T V F Y P S D G V A T E K	Transketolase
A S S E G G T A A G A G L D S L H K	Actin-related prot. 2/3 complex subunit 1B
K L E K E E E E G I S Q E S S E E E Q	High mobility group prot. HMG-I/HMG-Y
P E N V A P R S G A T A G A A G G R G K	T-complex prot. 1 subunit delta

High binding peptides associated with HLA-B*35:08 (origin LCL.TPN+ cells)

Peptide position 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 Ligands	Source
D E G W F L I L	Activating signal cointegrator 1 complex subunit 3
F P E D E G I H Y	B-cell receptor CD22
F P Q E G M H Q M	Probable global transcription activator SNF2L2
I A D K P M T Q Y	Survival of motor neuron-related-splicing factor 30
M P A A T D N R Y	Aminoacylase-1
L P Q K G D V E M	DNA (cytosine-5)-methyltransferase 1
S P Q E K E A L Y	ADP-ribosylation factor-binding prot. GGA2
F P Q Y P D K E L	CCR4-NOT transcription complex subunit 1
L P F D K E T G F	SRA stem-loop-interacting RNA-binding prot., mitochondrial
F P E P E H S S F	Transmembrane and coiled-coil domain-containing prot. 4
T A A A P M I G Y	Probable ATP-dependent RNA helicase DDX5
F P D V E K A E W	Extended synaptotagmin-1
H P D V A K V S F	4-trimethylaminobutyraldehyde dehydrogenase
Y P D S K D L T M	Dual specificity tyrosine-phosphorylation-regulated kinase 4
H P N D D D V H F	5'-3' exoribonuclease 1
M P E K N E H S Y	Pentatricopeptide repeat-containing prot. 3, mitochondrial
F P L T T E S A M	60S ribosomal prot. L23a
T A D P S H Q T M	SPATS2-like prot.
Y P N E E K D A W	Disintegrin metalloprot.ase domain-containing prot. 17
M P M V T D N K M	Serine/threonine-prot. kinase ATR
M A D Y S D P S Y	Cell division cycle 5-like prot.

Q A I E D M V G Y	26S proteasome non-ATPase regulatory subunit 1
M P I G P D V S L	Spermatogenesis-associated prot. 5-like prot. 1
F P D K P I T Q Y	Dolichyl-diphospho--prot. glycosyltransferase
M P D V V V R S F	Mediator of RNA polymerase II transcription subunit 27
H A D G T I V R Y	Intraflagellar transport prot. 172 homolog
F P D E T H E R Y	Glycosylphosphatidylinositol anchor attachment 1 prot.
F P E E L T Q T F	Elongation factor 1-gamma
H A D K P V A T Y	ATP-binding cassette sub-family A member 13
Y A D P V N A H Y	Polypyrimidine tract-binding prot. 3
L P D E K V E L F	Pericentriolar material 1 prot.
L P D T L K V T Y	Integrin beta-2
E A A P H D I G Y	N-acetyl-D-glucosamine kinase
Q A V A D A V T Y	Proteasome subunit beta type-6
F P E I D L E K Y	Dihydrofolate reductase
F P M P G F D E H	Serine hydroxymethyltransferase, mitochondrial
M P D E A T P H Y	Alpha-mannosidase 2
Y P N G V V V H Y	Vam6/Vps39-like prot.
M A A D D V E E Y	Programmed cell death prot. 10
N V E E A D A A M	Heterogeneous nuclear ribonucleoprot. A0
N V I R D A V T Y	Histone H4
M P M G P D Q K Y	Nuclear receptor coactivator 3
T P E E K E Q V Y	Homologous-pairing prot. 2 homolog
L P S E I E V K Y	Anaphase-promoting complex subunit 7
L P D T R S E A Y	Cyclin-T1
M T A D R D P V Y	DNA repair prot. XRCC4
F P A E K E S E W	Calcineurin-like phosphoesterase domain-containing prot. 1
F P S S N V H V Y	DnaJ homolog subfamily B member 12
F P N A I E H T L	Ubiquitin-like modifier-activating enzyme 1
M A D Y P D Y K Y	Transcription factor SOX-4

L P V A K D V S Y	UTP--glucose-1-phosphate uridylyltransferase
N A D P Q A V T M	Melanoma-associated antigen D2
N A A Q T S V A Y	Arginine--tRNA ligase, cytoplasmic
L P D E I Y H V Y	N-terminal Xaa-Pro-Lys N-methyltransferase 1
F P E E D K K T Y	NADH dehydrogenase [ubiquinone] 1 subunit C2
L P N D G D E K Y	Programmed cell death prot. 2-like
F P M T M D E K Y	Cullin-3
T P D E I D H V F	Poly [ADP-ribose] polymerase 14
T A D V V K V A Y	Nuclear pore membrane glycoprot. 210
F P I P G E P G F	Actin-related prot. 2/3 complex subunit 3
M P L E E G D T F	Splicing factor 3B subunit 3
H P D A P M S Q V Y	Mortality factor 4-like prot.
N P D D I T N E E Y	Heat shock prot. HSP 90-alpha
M P N S A S R D E F	Dedicator of cytokinesis prot. 11
F P E E F D K T S F	N-acetylglucosamine-1-phosphotransferase subunits alpha/beta
N P D D I T Q E E Y	Heat shock prot. HSP
Y A Q D E H L I T F	U5 small nuclear ribonucleoprot. 200 kDa helicase
H P D G P E G Q A Y	Lysine--tRNA ligase
M P V D P N E P T Y	Inhibitor of growth prot. 4
F P D V P D K E N F	Prot. CASC5
R P I E D D Q E V Y	FYN-binding prot.
I P I A G R D I T Y	Actin-related prot. 3B
L P I E N D T Y K Y	Syntaxin-binding prot. 3
M P P Q D A E I G Y	Dual specificity prot. kinase TTK
F P E T T S P H E Y	Neuroblastoma-amplified sequence
M P G E G E V V R Y	Prot. RRNAD1
Y P D P S K Q K P M	Tyrosine--tRNA ligase, cytoplasmic
L P I E N D V Y K Y	Syntaxin-binding prot. 1
Y P E G F E I H S M	Long-chain-fatty-acid--CoA ligase 4

F P D T G S D H S Y	THAP domain-containing prot. 11
M P I K N T N Q D I Y	Peptidyl-prolyl cis-trans isomerase-like 4
H P D T G I S S K A M	Histone H2B type 1-A
N A E P A R P D I T Y	Coiled-coil-helix domain-containing prot. 2, mitochondrial
L P Y R A T E N D I Y	Heterogeneous nuclear ribonucleoprot. H
F P Q E E A I I D K Y	All-trans-retinol 13,14-reductase
H P A E D T E G T E F	Centromere prot. F
H P E D S E Y E A E M	Ubiquitin carboxyl-terminal hydrolase 16
S I S E G D D K I E Y	rRNA 2'-O-methyltransferase fibrillarin
V P E E G G A T H V Y	A-kinase-interacting prot.
L P D A H S D Y A R Y	RNA-binding prot. 14
M P S K E D A I E H F	Poly [ADP-ribose] polymerase 1
M P S Q V V K G G A F	EH domain-containing prot. 1
N P D E H S D S E M Y	B-cell linker prot.
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.
E P D S S T D M E Q Y	Apoptosis-enhancing nuclease
L P Y K A T E N D I Y	Heterogeneous nuclear ribonucleoprot. F
N A E D A D G K D V F	C1GALT1-specific chaperone 1
F P Q S E L G R A E A Y	DNA-directed RNA polymerase I subunit
L P N D E T R V N A T M	Ubiquitin conjugation factor E4

Low binding peptides associated with HLA-B*35:62 (origin LCL.TPN- cells)

Peptide position

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Ligands	Source
F P Q V I K S K	Fructose-bisphosphate aldolase A
M P S L P S Y K	Transketolase
Q A I D K M Q M	COP9 signalosome complex subunit 3
D Q N N K L S K	Myosin-11
L G L L M G T G	Anaphase-promoting complex subunit 2
S P G S V V F R	Renin receptor
I A P G D E T A	Uncharacterized family 31 glucosidase KIAA1161
D V S D L L H Q Y	Proteasome subunit beta type-8
D S S T V T H L F	Proteasome subunit alpha type-6
A Y D A T H L V K	S-formylglutathione hydrolase
T A A D I F K Q Y	Cohesin subunit SA-2
A P V K K L V V K	60S ribosomal prot. L22
Y I D Q E E L N K	Heat shock prot. HSP
A A N P H S F V F	Suppressor of SWI4 1 homolog
S G S D I V K L Y	Erythroid differentiation-related factor 1
E A D K T I K V Y	Pleiotropic regulator 1
D I D T R S E F Y	Actin-related prot. 2
K Q L N L L F A K	Zinc finger SWIM domain-containing prot. 6
S P M D R N S D E Y	CCAAT/enhancer-binding prot. gamma
G V R G A S K E V V	Kelch repeat and BTB domain-containing prot. 13
K F D Q L L A E E K	Myosin-9
T P M E D V L H S F	Aspartate aminotransferase, mitochondrial

S V S D N D I R K Y	Transitional endoplasmic reticulum ATPase
R A V D L I Q K H K	Flap endonuclease 1
S V E T L K E M I K	Pyruvate kinase isozymes M1/M2
E A A V E D L H H Y	Phosphatidylserine decarboxylase proenzyme
K F D Q L L A E E K	Myosin-14
S K K G I E E S L R	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
D V Y D D G K H V Y	Ribosomal prot. S6 kinase alpha-1
L L D V V H P A A K	T-complex prot. 1 subunit eta
L A L L S L S G L E	Beta-2-microglobulin
T F H T I G F C P Y	Zinc finger prot. 36, C3H1 type-like 2
D A L D D Y E H H Y	Zinc finger prot. 511
H P L V L Q E C V S D	Lysine-specific demethylase 4A
N T V G Q N E L K I T	Centrosome and spindle pole-associated prot. 1
S A I N E V V T R E Y	60S ribosomal prot. L31
V L M T Q Q P R P V L	H/ACA ribonucleoprot. complex subunit 3
E I T A L A P S T M K	Actin, cytoplasmic 1
A E L L D N E K P A A	Calcyclin-binding prot.
F Y N Q V S T P L L R	Inter-alpha-trypsin inhibitor heavy chain H2
P M F I V N T N V P R	Macrophage migration inhibitory factor
A A E I D E E P V S K	Nascent polypeptide-associated complex subunit alpha
P E P V K S A P V P K	Histone H2B type 1-M
A M E A V A A Q G K A	Phosphoglycerate mutase 1
G P S S V E D I K A K	Nucleophosmin
T A I I E E Q P K N Y	Prot. Farnesyl/geranyl transferase type-1 subunit alpha
S K P V F S E S L S D	Mitochondrial import inner membrane translocase subunit Tim8
D A L K D S D L L H W	Anaphase-promoting complex subunit 5
N A S E D E I K K A Y	DnaJ homolog subfamily C member 7
H I T D C R L T N G S R	Ribonuclease pancreatic
A D L A E T R P D L K N	Layilin

D N I Q G I T K P A I R	Histone H4
A L R Y P M A V G L N K	60S ribosomal prot. L36
L T S D D V K E Q I Y K	40S ribosomal prot. S13
A L S T G E K G F G Y K	Peptidyl-prolyl cis-trans isomerase A
E P L P S E V T S N H F	DNA polymerase theta
D A L E T L G F L N H Y	Heterogeneous nuclear ribonucleoprot. L
R Q Q P G P S E H I E R	Vasodilator-stimulated phosphoprot.
S V Q A T T E N K E L K	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial
P Q E Q A D A A K F M A	Brain-specific angiogenesis inhibitor 3
T F N P G A G L P T D K	U2 small nuclear ribonucleoprot. A'
G T V S V A D T K G V K	Low-density lipoprot. receptor
A L G Q N P T N A E V L K	Myosin light polypeptide 6
H A Q G E K T A G I N V R	T-complex prot. 1 subunit delta
S V L I S L K Q A P L V H	Clathrin light chain A
A T D F V A D R A G T F K	Isocitrate dehydrogenase [NADP], mitochondrial
N G V M P S H F S R G S K	40S ribosomal prot. S19
L K L Q E E Q F V N A V E	Peptidase M20 domain-containing prot. 2
V A K V S Q G V E D G P D	SUMO-activating enzyme subunit 1
N S V S Q I S V L S G G K	Actin-related prot. 2/3 complex subunit 1B
T V V N K D V F R D P A L	60S ribosomal prot. L27
S V S L T G A P E S V Q K	Far upstream element-binding prot. 2
A Q P A Q P A D E P A E K	Proteasome subunit alpha type-1
K F K Y P Q A P V I M G N	Aldehyde oxidase
Q A G W T A C G A V D M N	Leucine carboxyl methyltransferase 2
D S D G D E D Q G E G E A	Leucine-rich repeat and guanylate kin. domain prot.
L G G S A V I S L E G K P L	Cofilin-1
V P S G Q N A D V Q K T D N	N-alpha-acetyltransferase 50
G A V D G G L S I P H S T K	60S ribosomal prot. L5
G M G T V E G G D Q S N P K	Treacle prot.

A A V A G K K P V V G K K G	60S ribosomal prot. L4
I V S G K D Y N V T A N S K	L-lactate dehydrogenase A chain
I V A D K D Y S V T A N S K	L-lactate dehydrogenase B chain
A V R V F E F G G P E V L K	Quinone oxidoreductase
I F V G G L S P D T P E E K	Heterogeneous nuclear ribonucleoprot. D0
V A K V T G G A A S K L S K	60S ribosomal prot. L35
F S E S R A D E V A P A K K	ATP-citrate synthase
K L G G F S D D L H K T P G	Outer dense fiber prot. 3
T V T P A K A V T T P G K K	Nucleolin
I F F T D L D S F L I T S M A	Olfactory receptor 1J2
H G S Y E D A V H S G A L N D	T-complex prot. 1 subunit alpha
A Q A A A P A S V P A Q A P K	60S ribosomal prot. L29
L G D V Y V N D A F G T A H R	Phosphoglycerate kinase 2
S Q V I S N A K N T V Q G F K	Heat shock 70 kDa prot. 4
A S G N Y A T V I S H N P E T	60S ribosomal prot. L8
L V G S Q K E P S E V P T P K	High mobility group prot. HMG-I/HMG-Y
E A M K A Q M A E K E A I L Q	FYVE and coiled-coil domain-containing prot. 1
S E A V A D R E D D P N F F K	Glutamate dehydrogenase 1, mitochondrial
I R L T I D T T Q T I S E D T D	Prot. FAM188B
R P G C Q G G S E R G S I P L L	Uncharacterized prot. C2orf71
G G K P E P P A M P Q P V P T A	40S ribosomal prot. S3
A Y V R L A P D Y D A L D V A N	60S ribosomal prot. L23a
H A V S E G T K A V T K Y T S A	Histone H2B type 1-K
P D P A K S A P A P K K G S K K	Histone H2B type 1-H
S T A T D I T G P I I L Q T Y R	Putative neutrophil cytosol factor 1C
A Q L G G P E A A K S D E T A A	Heat shock prot. beta-1
K L T G K D V N F E F P E F Q L	40S ribosomal prot. S7
K R K D T T S D K D D S L G S Q	Nuclear autoantigen Sp-100
G A P G P G P A D A S K V V A K	Filamin-A

A K G K P D A A K K G V V K A E	High mobility group prot. B1
N D Q A N Y S L N T D D P L I F K	Adenosine deaminase
H A V S E G T K A V T K Y T S S K	Histone H2B type 1-C/E/F/G/I
K E E S E E S D D D M G F G L F D	60S acidic ribosomal prot. P1
A T S N V F A M F D Q S Q I Q E F K	Myosin regulatory light chain 12A
A A N D A G Y F N D E M A P I E V K	3-ketoacyl-CoA thiolase, mitochondrial
F R I L C T T E D G L L R F V S P V	WD repeat-containing prot. 87
A G L C T E G A L L L L E M L K A T M S	WD repeat- and FYVE domain-containing prot. 4
E I L L K K C T N I L N S N G E L R G F	Plasma membrane calcium-transporting ATPase 3
T L V N L H L A A L T L L G S E N P S K	Endonuc./exonuc./phos. family domain prot.
K A P G F G G F G S S A V S G G S T A A	Coatomer subunit delta
P M K K K I L A A K G G R V I I E C K P K	Contactin-1
S L D R N L P S D S Q D L G Q H G L E E D F M	Serglycin
Y L K P F R V L I S L L D K P E I G P Q V V G	Prot. dopey-2

High binding peptides associated with HLA-B*35:62 (origin LCL.TPN- cells)

Peptide position

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Ligands	Source
Y V R D I S A Y	Serine/threonine-prot. kinase Nek10
M K E V T R T W	Trimeric intracellular cation channel type B
G H T H D G G Y	Manganese-dependent ADP-ribose/CDP-alcohol diphosphatase
N P N G G F R M Y	Plasma membrane calcium-transporting ATPase 4
F P Q D Q I R L W	Ubiquitin carboxyl-terminal hydrolase 7
F P N A L V T K L	Prot. fem-1 homolog B
T P S P S P H A W	Ninein
S P D A T I R I W	Pre-mRNA-processing factor 19
T P A S A G H V W	Transcription factor SOX-9
A A A D S I K I W	WD repeat-containing prot. 3
D P S G T Y H A W	Proteasome subunit alpha type-7-like
F P A K V T A H W	Trafficking prot. particle complex subunit 5
A V S A V V H E Y	Sterol O-acyltransferase 1
T A A D I F K Q Y	Cohesin subunit SA-2
T P S A V F R V W	Aladin
D T S D I V H I W	Centromere prot. U
H V I L G T Q Q F	Eukaryotic translation initiation factor 3
D V N S L L K Y F	NADH dehyd.[ubiquinone] 1 alpha subcomplex assembly factor 4
D P V D I Y K S W	Ras GTPase-activating-like prot. IQGAP1
E G N P D T H S W	Ubiquitin-associated prot. 2-like
D V S D L L H Q Y	Proteasome subunit beta type-8
E L F E G V Q K W	Prot. regulator of cytokinesis 1

S A M D V A K A Y	Ankyrin repeat and EF-hand domain-containing prot. 1
T P M F V V K A Y	Elongation factor 2
D V T E S N A R W	Parkinson disease 7 domain-containing prot. 1
N V S K V S T T W	F-box only prot. 5
L P S E L E R S Y	Cullin-1
D A G P P T H A F	E3 ubiquitin-prot. ligase pellino homolog 2
E A D G G L K S W	F-actin-capping prot. subunit alpha-1
D V T G V V R Q W	Transforming growth factor beta-1
D V D G V I K V W	WD repeat-containing prot. 91
S A S E V L K E W	Spermatogenesis-associated serine-rich prot. 2
D I S E K E Q R W	Hematopoietic lineage cell-specific prot.
D A N P L K T L W	Leucine-rich repeat-containing prot. 33
D A I G P R E Q W	Prot. FRG1B
N A T L S V H Q L	Tubulin beta-6 chain
H P Q D G R S A W	Sterol regulatory element-bind. prot. cleavage-activ. prot.
M P A D T N K A F	ER membrane prot. complex subunit 3
Q A T P T F H Q W	Ena/VASP-like prot.
Q P S Q A Q R M Y	Tetratricopeptide repeat prot. 19
H P I Q T Q A Q Y	La-related prot. 4
M A I E A Q Q K F	Histone acetyltransferase type B catalytic subunit
H A S D R I I A L	Transketolase
A P D N I I K F Y	GON-4-like prot.
D A I N D A N L L	Farnesyl pyrophosphate synthase
G V A E S I H L W	WD repeat-containing prot. 18
N P Q P M T P P W	Prot. Asterix
E V S F V I H N L	Selenide, water dikinase 2
E A I K Q A S E W	Poly [ADP-ribose] polymerase 4
E A M R L G P G W	Prot. syndesmos
Y P N V N I H N F	Spectrin beta chain, non-erythrocytic 1

N V I D S K E L W	Mdm2-binding prot.
T P G E S L H G Y	Transmembrane prot. 214
E A T A L V H T L	Putative GTP-binding prot. 6
S A S P H A A Y Y	Malignant fibrous histiocytoma-amplified sequence 1
E P K A L V S E W	DNA damage-binding prot. 1
D Q M I S R I E Y	Casein kinase I isoform epsilon
Y P F S S E Q K W	Calcium-transporting ATPase type 2C member 1
H V S T V N P N Y	Homeobox prot. Hox-C4
D V M A H V H T F	Adenylosuccinate lyase
T A A D V V K Q W	RalA-binding prot. 1
L A N D V A E Q W	Ubiquitin-conjugating enzyme E2 N
S A S P T S P D Y	SAM and SH3 domain-containing prot. 3
Q A A E R P Q E W	NF-kappa-B inhibitor alpha
E A I Y E T H T W	Plasma alpha-L-fucosidase
A A N P I S G H Y	DNA replication licensing factor MCM6
S N S A S P H R W	E3 SUMO-prot. ligase RanBP2
T A N G T P K V Y	Pro-interleukin-16
A A N P H S F V F	Suppressor of SWI4 1 homolog
N P A A S S N H W	Dual specificity prot. kinase TTK
A A Q D F F Q R W	AP-2 complex subunit alpha-1
E P S E S N M R W	Mediator of RNA polymerase II transcription subunit 12
D P N G A G S E W	KRAB domain-containing prot. ZNF747
E P L P R N G D Q W	Wee1-like prot. kinase
N A M D N T V R V W	U5 small nuclear ribonucleoprot. 40 kDa prot.
S P M D R N S D E Y	CCAAT/enhancer-binding prot. gamma
F G T A G L R S A M	lucose 1,6-bisphosphate synthase
E A A T A Q R E E W	Heterogeneous nuclear ribonucleoprot.
D A M I V N K A S W	DNA-directed RNA polymerase I subunit RPA2
E A Q D A G Y R M Y	Ser/thr.-prot. Phosph. 2B catalytic subunit beta isoform

E V S P A T E R Q W	RNA-binding prot. 12
T P M E D V L H S F	Tight junction prot. ZO-2
A A S G N A V R M W	Kinesin-like prot. KIF21A
N A I Q D S L T R W	Valine--tRNA ligase
T A Q S D N K I T W	V-type proton ATPase catalytic subunit A
H A A D P I I T R W	ADP-ribose pyrophosphatase, mitochondrial
E A A V E D L H H Y	Phosphatidylserine decarboxylase proenzyme
A H L Y F Q A H G S	Pleckstrin homology domain-containing family N member 1
E A Q A V T T S K W	U2 snRNP-associated SURP motif-containing prot.
G F G D Y V A G A D	Potassium channel subfamily K member 4
D A Q Q S L Q S F W	Nuclear transcription factor Y subunit gamma
A P S P Q D R P S F	Tyrosine-prot. kinase JAK3
D V M A P D V E V S	Prot. AHNAK2
S P I T S S P P K W	Prot. lin-52 homolog
F L S P E Q H A C S	Fibroblast growth factor-binding prot. 3
A A A G G G R S P E	PH domain leucine-rich repeat-containing prot. phosphatase
P D P G G K S Q D A	Transmembrane and coiled-coil domains prot. 1
V C P E C A K I S V	Ubiquitin carboxyl-terminal hydrolase 15
P A G N C T D E E G I	Mucin-5B
S A S D D G T V R I W	WD repeat-containing prot. 26
T A A A D F T A K V W	Serine-threonine kinase receptor-associated prot.
E A I S D S L L R K Y	Putative ATP-dependent RNA helicase DHX33
D Q M P Q G A P R L Y	Phosphatidylinositol 3,4,5-trisphosphate-dep. Rac exch.prot.
T A I I E E Q P K N Y	Prot. farnesyl/geranylgeranyl transferase subunit alpha
S P A P A G E R R I Y	Zinc finger prot. 511
N V I K E A E A Q L W	Uncharacterized prot. C21orf59
T P L E D V G K Q V W	Methyltransferase-like prot. 22
G E I E S P A S S F H	Integrin beta-5
D A M M A N A A Q K F	Asparagine synthetase [glutamine-hydrolyzing]

D V L S D P Q E R A W	DnaJ homolog subfamily C member 21
T A S E D G S V R L W	Telomerase prot. component 1
H A S D V L E T S G W	Speckle-type POZ prot.
S A S S V T V T R S Y	Prelamin-A/C
T A S A D G T I K L W	Transducin beta-like prot. 3
G E I G E K G Q K G E	Collagen alpha-1(IV) chain
S A A A D S A V R L W	Elongator complex prot. 2
S A T N D A S L H V W	WD repeat-containing prot. 73
S A A A D E T L R L W	Cell division cycle prot. 20 homolog
A P S G Y V A G H G W	Paired box prot. Pax-9
S E M E V Q D A E L K	Proliferation-associated prot. 2G4
T C L E P T N V V A W	Tetratricopeptide repeat prot. 18
E A L G D N V K Q Y W	Transcriptional adapter 1
T A S D D A T I K A W	F-box/WD repeat-containing prot. 5
Y P M E N G I V R N W	Actin-related prot. 2
S A I N E V V T R E Y	60S ribosomal prot. L31
A P T A E A P P P S V	Probable ATP-dependent RNA helicase DDX17
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.
D A L D V D D Y R F V	JmjC domain-containing prot. 4
T V D G P S G K L W R	Glyceraldehyde-3-phosphate dehydrogenase
S R K D P S G A S N P S	Hepatocyte nuclear factor 3-alpha
T P L P E P D L T R L Y	Prot. asteroid homolog 1
S N S G A G V L P S P A	Mediator of RNA polymerase II transcription subunit 13-like
C I C L F D I T K L E D	DNA-directed RNA polymerase III subunit RPC8
Y A M D N S G E H V T W	Cation-independent mannose-6-phosphate receptor
M G G K V P P A T Q K A	Hsc70-interacting prot.
V G G T S D V E V N E K	60 kDa heat shock prot., mitochondrial
G M K A A L Q V S M N D	Anthrax toxin receptor 1
A G N L G G G V V T I E R	60S ribosomal prot. L22

T A S G V D V G G Q H E W	Transcription elongation factor SPT5
M L P H P T P I S T E A N	Neurocan core prot.
L D L G P S M L D A V L G V M	Cdc42 effector prot. 5
L I A P V A E E E A T V P N N K L	lactate dehydrogenase B chain

Low binding peptides associated with HLA-B*35:62 (origin LCL.TPN+ cells)

Peptide position

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 181 19 20

Ligands	Source
G A L A L E E K	Myosin-9
P V G A A N F R	Alpha-enolase
R L A S S V L R	60S ribosomal prot. L19
R V I G S G C N	L-lactate dehydrogenase B
A P I I A V T R	Pyruvate kinase isozymes M1/M2
S P L H E R I Y	Proteasome subunit beta type-9
F E M L S F W L	Unconventional myosin-Vc
E S S E S L P K	Activating transcription factor 7-interacting prot. 1
Y L T D P V L F	Latrophilin-2
V P N P D P V T M	Rac GTPase-activating prot. 1
F P N A I E H T L	Ubiquitin-like modifier-activating enzyme 1
I G P L G L S P K	60S ribosomal prot. L12
L P S E I E V K Y	Anaphase-promoting complex subunit 7
M P A E I V E L H	Transcriptional repressor prot. YY1
S P I D V V E K Y	Kelch-like prot. 12
T P A G V V N K Y	7-dehydrocholesterol reductase
N A M N M Q E T Y	Sorting nexin-4
F P V I Y D V K Y	CCR4-NOT transcription complex subunit 7
I P A A V K L T Y	E3 ubiquitin-prot. ligase UBR5
S N L E N I D F K	Adenylosuccinate lyase
A I A T G G A V F	60 kDa heat shock prot., mitochondrial
H P N D D D V H F	5'-3' exoribonuclease 1
N P F E K G D L Y	DnaJ homolog subfamily A member 2

M P N S P A P H F	Absent in melanoma 1 prot.
N A I K E S Y D Y	Polycomb prot. EED
L P P G V H I S Y	Integrin beta-7
I P N E I I H A L	Heterogeneous nuclear ribonucleoprot. M
Y P V D L G D K F	DNA-directed RNA polymerases I, II, and III subunit RPABC3
M P M G P D Q K Y	Nuclear receptor coactivator 3
M A Y G H I D S Y	E3 ubiquitin-prot. ligase Praja-1
L P Q E A F E K Y	Structural maintenance of chromosomes prot. 3
M P A D T N K A F	ER membrane prot. complex subunit 3
L P N D G D E K Y	Programmed cell death prot. 2-like
M P I S S H V D L	Spermatogenesis-associated prot. 5-like prot. 1
N P N S P S I T Y	Enhancer of rudimentary homolog
Q P I N L I F R Y	Small nuclear ribonucleoprot. E
F P S I Q A V K I	Carboxypeptidase A1
L P A K I L V E F	ZW10 interactor
F P E I D L E K Y	Dihydrofolate reductase
L P H A P G V Q M	Histone deacetylase 2
A V S A V V H E Y	Sterol O-acyltransferase 1
M P Y S H P S S Y	Nipped-B-like prot.
S A V E E K V S Y	KIF1-binding prot.
Y P N G V V V H Y	Vam6/Vps39-like prot.
D P F V D R I G Y	Nucleoporin NUP188 homolog
V P P S S P Q E L	Prot. FAM186A
N V A D L H E K Y	Proteasome subunit beta type-5
F P S I Y D V K Y	CCR4-NOT transcription complex subunit 8
M P A V K A I I Y	Squalene synthase
V V A P I T T G Y	Calcyclin-binding prot.
E A F R E H Q Q Y	Epithelial-stromal interaction prot. 1
L P L E E A Y R F	EH domain-containing prot. 4

E D K Q P C Y I L	Twinfilin-1
M A A D D V E E Y	Programmed cell death prot. 10
L P S N D S S K F	Nucleolar transcription factor 1
L P G E S L T F M	Seizure 6-like prot.
T G V R Q V P G F	Dermokine
H P L F T Q S Q E S	Centrosomal prot. of 97 kDa
L P S L G L S S L D	Transmembrane prot. 63B
L P I E N D T Y K Y	Syntaxin-binding prot. 3
I P S L N V D C A V	Citrate lyase subunit beta-like prot., mitochondrial
L P A D I T E D E F	Splicing factor, proline- and glutamine-rich
I P S E V D V E K Y	Prot. FAM48A
A S S P S S A H S A	Neuron navigator 2
Y P Y N A P T V K F	Ubiquitin-conjugating enzyme E2 C
Q A F Q E R L N S Y	Nuclear pore complex prot. Nup155
T P I Y E G R T Y Y	Plakophilin-4
N L Q T V N V D E N	60S ribosomal prot. L31
M P F P T E E E S V	Proto-oncogene vav
L P I E N D V Y K Y	Syntaxin-binding prot. 1
I P I A G R D I T Y	Actin-related prot. 3B
M P V D P N E P T Y	Inhibitor of growth prot. 4
M P F Q R A G V N F	SWI/SNF-related reg. of chromatin subfamily A-like prot. 1
M A A T N R P N S I	Transitional endoplasmic reticulum ATPase
V A S L S S Q L Q D	Myosin-11
A G F A G D D A P R	POTE ankyrin domain family member I
L P S P V T A Q K Y	Elongation factor 2
F P M T H G N T G F	Poly(rC)-binding prot. 2
T A A L I K G E L Y	Intraflagellar transport prot. 172 homolog
Y A Q D E H L I T F	U5 small nuclear ribonucleoprot. 200 kDa helicase
Q D V N G T L V S I	Leucyl-cystinyl aminopeptidase

Y P I D P V T Q E F	Lymphoid-specific helicase
S P I E F L E N A Y	Lanosterol 14-alpha demethylase
E V S S A T N A L R	U1 small nuclear ribonucleoprot. A
T P I E E R G D L F	Zinc finger prot. 1 homolog
E A F N M I D Q N R	Myosin regulatory light polypeptide 9
P G P L G P S A F F	Zinc finger prot. 469
G V K A A L L Q L L	Cyclin-dependent kinase 13
L P L G N G K A A E E	Sodium- and chloride-dependent GABA transporter 3
A L D L Y P E P A F L	Putative uncharacterized prot. C17orf82
V L S I S P N C G Y I	Macrophage-stimulating prot. receptor
L A L I K I F G A L I	Adenosine 3'-phospho 5'-phosphosulfate transporter 2
P T T A E G T S M P I	Mucin-17
Q P L D E E L K D A F	Sodium/potassium-transporting ATPase subunit alpha-1
A P F Q T S A A M H H	Homeobox prot. DLX-5
L P V P N L D P D T Y	Sulfite oxidase, mitochondrial
M P V R G P D V E A Y	Transmembrane prot. 9B
F S N V M I H V V Q Y	Polyprenol reductase
M P K S E V A S S V F	Oxysterol-binding prot.-related prot. 11
E A F D E L L A S K Y	Regulator of G-prot. signaling 2
N P F D S Q E A K P Y	COP9 signalosome complex subunit 2
M A P E R I S G E Q Y	Dual specificity mitogen-activated prot. kinase kinase 5
S P L G M P D P H L Y	Fc receptor-like A
F P I I I H D E P T Y	Pseudopodium-enriched atypical kinase 1
Q P A P S S T S G S Y	RNA-binding prot. FUS
Q A A D I D T R S E F	Actin-related prot. 2
V P E E G G A T H V Y	A-kinase-interacting prot. 1
M P I R E G D T V T L	B-cell receptor CD22
F P Q E E A I I D K Y	All-trans-retinol 13,14-reductase
L I C N V G A G G P A	60S acidic ribosomal prot. P1

M D S T E P P Y S Q K	Elongation factor 1-alpha 1
L P F P D E T H E R Y	Glycosylphosphatidylinositol anchor attachment 1 prot.
T P D P K M N A R T Y	Splicing factor 3B subunit 1
P M F I V N T N V P R	Macrophage migration inhibitory factor
F A N E E G E A Q K F	Antigen peptide transporter 1
N P A D S I S H V A Y	Transformation/transcription domain-associated prot.
L P I G D V A T Q Y F	T-complex prot. 1 subunit eta
L P F D G S P K I T Y	HERV-V_19q13.41 provirus ancestral Env polyprot. 1
M P V G P D A I L R Y	Large proline-rich prot. BAG6
V V D C T L K L D P I	Heterogeneous nuclear ribonucleoprot. D0
T P I Q D N V D Q T Y	Germinal center-associated signaling and motility prot.
N F G I G Q D I Q P K	60S ribosomal prot. L7a
L P Y K I T A E E M Y	Pre-mRNA branch site prot. p14
E P I Y P E V V H M F	Ser/thr phosphatase 2A 56 kDa regulatory subunit gamma
Q P L L I I G K G A A Y	2-hydroxyacyl-CoA lyase 1
M V P P T S G T S T P R	Collagen alpha-1(XXVII) chain
M P T G K Q L A D I G Y	Cytochrome c oxidase assembly prot. COX14
D V V G A A T A G Q T Y	Cyclic nucleotide-gated cation channel beta-1
A S G P P V S E L I T K	Histone H1.3
I L G T A G T E E G Q K	Quinone oxidoreductase
L P S Q E D M P H N Q F	Lysosomal-associated transmembrane prot. 5
N N A S T D Y D L S D K	60S ribosomal prot. L3
F P A V G E P N I Q Q Y	UDP-N-acetylgluc.peptide N-acetylglu.transf. 110 kDa subunit
S A A A L D V L A N V Y	Transportin-1
Y P Y D G I H P D D L S F	Tyrosine-prot. kinase Lyn
A G K S G S A L E L S V E	Prot. phosphatase 1B
M V I D E E L L G D G H S Y	Proteasome subunit beta type-4
A Q A A A P A S V P A Q A P K	60S ribosomal prot. L29
Y F Q I N Q D E E E E E D E D	60S ribosomal prot. L22

H K E L A P Y D E N W F Y T R	40S ribosomal prot. S19
K L E K E E E E G I S Q E S S E E E Q	High mobility group prot. HMG-I/HMG-Y
S P A T A T G A M A T T T G A L P A Q	Neurogenic locus notch homolog prot. 3

High binding peptides associated with HLA-B*35:62 (origin LCL.TPN+ cells)

Peptide position																				
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ligands																				Source
E	A	F	Q	L	F	D	R													Myosin light polypeptide 6
I	P	D	W	F	L	N	R													40S ribosomal prot. S18
A	D	N	L	C	R	K	L													YEATS domain-containing prot. 2
H	P	T	I	I	S	E	S	F												T-complex prot. 1 subunit delta
V	L	L	A	E	V	Q	Q	H												WD repeat-containing prot. 72
I	P	N	E	I	I	H	A	L												Heterogeneous nuclear ribonucleoprot. M
I	G	P	L	G	L	S	P	K												60S ribosomal prot. L12
A	L	Q	F	L	E	E	V	K												T-complex prot. 1 subunit zeta
I	A	I	Y	E	L	L	F	K												40S ribosomal prot. S10
M	P	A	E	I	V	E	L	H												Transcriptional repressor prot. YY1
T	G	F	Q	A	V	T	G	K												Eukaryotic translation initiation factor 2 subunit 2
N	A	F	K	E	I	T	T	M												Transcription initiation factor IIB
N	S	T	F	S	E	I	F	K												Transketolase
Y	P	V	D	L	G	D	K	F												DNA-directed RNA polymerases I, II, and III subunit RPABC3
L	P	F	D	K	E	T	G	F												SRA stem-loop-interacting RNA-binding prot., mitochondrial
K	N	S	S	D	L	K	Y	R												Proto-oncogene tyrosine-prot. kinase ROS
V	P	K	P	D	L	D	S	Y												DNA replication complex GINS prot. SLD5
F	D	Q	L	L	A	E	E	K												Myosin-14
A	Y	D	A	T	H	L	V	K												S-formylglutathione hydrolase
H	P	I	R	I	A	D	G	Y												T-complex prot. 1 subunit epsilon
P	A	A	P	I	T	E	I	V												NACHT, LRR and PYD domains-containing prot. 4

I A S N A G S I A	60S ribosomal prot. L23
P G D P A S D E G D	RUN domain-containing prot. 1
S I P G G Y N A L R	Ubiquilin-4
Q L S K E A L L K L	Ras GTPase-activating prot. SynGAP
E V S S A T N A L R	U1 small nuclear ribonucleoprot. A
Y I K G G N S E I K	HERV-K_6q14.1 provirus ancestral Gag-Pol polyprot.
T P E T L C H V G V	Myelin gene regulatory factor
D C I L D E D H S G P	Lymphocyte antigen 75
M G G D I A N R V L R	Proliferation-associated prot. 2G4
R T I A Q D Y G V L K	Peroxiredoxin-1
P M F I V N T N V P R	Macrophage migration inhibitory factor
I P V N E K D T L T Y	Histone deacetylase complex subunit SAP30
Y Q A V T A T L E E K	Putative 60S ribosomal prot. L13a-like MGC87657
E D E D D E E D F E D	Neural Wiskott-Aldrich syndrome prot.
K P M V V L G S S A L Q	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial
K L I S D T I S D A L L	WD repeat-cont. planar cell pol.eff prot. fritz homolog
R K P D T I E V Q Q M K	Moesin
T P L H E A A A K G K Y	Tankyrase-2
S V Q P T S E E R I P K	Septin-9
R I E P A D A H V L Q K	N-alpha-acetyltransferase 50
K Q M V I D V L H P G K	40S ribosomal prot. S24
A A A L E A M K D Y T K	Stress-induced-phosphoprot. 1
H P I S S E E L L S L K	Prot. C-ets-1
R T A A T L A T H E L R	Leucine-rich repeat-containing prot. 47
K F Y N Q V S T P L L R	Inter-alpha-trypsin inhibitor heavy chain
N N A S T D Y D L S D K	60S ribosomal prot. L3
G Y V V R I S G G N D K	40S ribosomal prot. S6
L P S Q E D M P H N Q F	Lysosomal-associated transmembrane prot. 5
Y Y K V D E N G K I S R	Ubiquitin-40S ribosomal prot. S27a

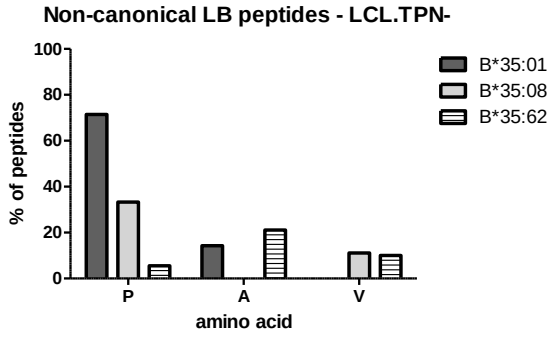
G K G F G F I K L E S R	Splicing factor, proline- and glutamine-rich
L P A G W I L S H L E T Y	Cytochrome c oxidase subunit 8A, mitochondrial
M L L H S E Q H P G Q L K	Pyrroline-5-carboxylate reductase 1, mitochondrial
M E K D D S A Q T R Y I K	E3 ubiquitin-prot. ligase RING2
S A Y E F S E T E S M L K	Leukotriene A-4 hydrolase
G F V K V V K N K A Y F K	60S ribosomal prot. L5
A T D F V A D R A G T F K	Isocitrate dehydrogenase [NADP], mitochondrial
V E E I A P D P S E A K R	E3 ubiquitin-prot. ligase RAD18
T K T P G P G A Q S A L R	40S ribosomal prot. S14
A S M Q Q Q Q Q L A S A R	Chromatin target of PRMT1 prot. 1
A Q V A R P G G D T I F G K	Histidine triad nucleotide-binding prot. 1
K K Y E E M N A E I S Q F K	Guanylate-binding prot. 6
H G V V P L A T Y M R I Y K	60S ribosomal prot. L21
V V K V A N V S L L A L Y K	40S ribosomal prot. S23
S L E S I N S R L Q L V M K	60S ribosomal prot. L30
S P V A K D V D L E F L A K	Transitional endoplasmic reticulum ATPase
A A L R P L V K P K I V K K	60S ribosomal prot. L32
Q I S R L E E R E A E L K K	C-Jun-amino-terminal kinase-interacting prot. 4
N Q Q I T H A N N T V S N F K	Heat shock prot. 105 kDa
G K V R D K L N N L V L F D K	40S ribosomal prot. S25
S K M T T D E L S V S E N I L	Prot. eyes shut homolog
S I Y G E K F E D E N F I L K	Peptidyl-prolyl cis-trans isomerase A
S E A V A D R E D D P N F F K	Glutamate dehydrogenase
D T G K T P V E P E V A I H R	40S ribosomal prot. S20
P Q D S P G Q A L A G Q A T P E	CMT1A duplicated region transcript 15 prot.-like prot.
E V Y Q Q Q Q Y G S G G R G N R	Heterogeneous nuclear ribonucleoprot. A/B
N K D I R K F L D G I Y V S E K	60S ribosomal prot. L9
T G A A P I I D V V R S G Y Y K	60S ribosomal prot. L27a
G G K P E P P A M P Q P V P T A	40S ribosomal prot. S3

A Q L G G P E A A K S D E T A A	Heat shock prot. beta-1
K L T G K D V N F E F P E F Q L	40S ribosomal prot. S7
S Q S A A V T P S S T T S S T R	Proteasomal ubiquitin receptor ADRM1
L V L V G D G G T G K T T F V K	GTP-binding nuclear prot. Ran
S T A V K A L T G G I A H L F K	Dihydrolipoyl dehydrogenase, mitochondrial
K I L D S V G I E A D D D R L N K	60S acidic ribosomal prot.
A A K V L E Q L T G Q T P V F S K	60S ribosomal prot. L11
S A I N E V V T R E Y T I N I H K	60S ribosomal prot. L31
A L L E R T G Y T L D V T T G Q R K	Heterogeneous nuclear ribonucleoprot. Q
T A D T I L N T L Q N I S E G L V V	Adenylosuccinate lyase
T V A G G A W T Y N T T S A V T V K	60S ribosomal prot. L37a
N L A S R P Y S L H A H G L S Y E K	Coagulation factor V
K A V P K E D I Y S G G G G G G S R	Heterogeneous nuclear ribonucleoprot. A0
K L N I A R N E Q D A Y A I N S Y T R	Acetyl-CoA acetyltransferase, mitochondrial
G R S I S L Y Y T G E K G Q N Q D Y R	Nucleolin
N K P G P N I E S G N E D D D A S F K	Eukaryotic translation initiation factor 5B
S A A Q A A A Q T N S N A A G K Q L R	Plasminogen activator inhibitor 1 RNA-binding prot.
N Q Q E I P S Y L N D E P P E G S M K	UPF0444 transmembrane prot. C12orf23
A M E G I F I K P S V E P S A G H D E L	Stromal cell-derived factor 2-like prot. 1
E A H Q L F L E P E V L D P E S V E L K	Flap endonuclease 1
N G T Q V H G T I T G V D V S M N T H L K	Small nuclear ribonucleoprot. Sm
A Q A V S E D A G G N E G R A A E A E P R	Eukaryotic translation initiation factor 3

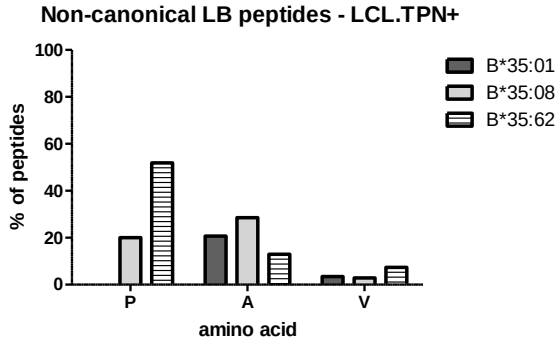
Appendix VI

p2 anchor motif of non-canonical HLA-B*35/156 restricted peptides

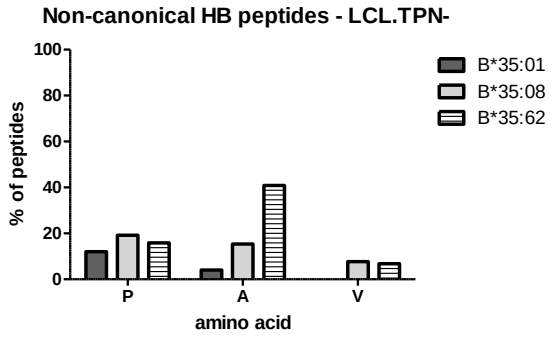
A



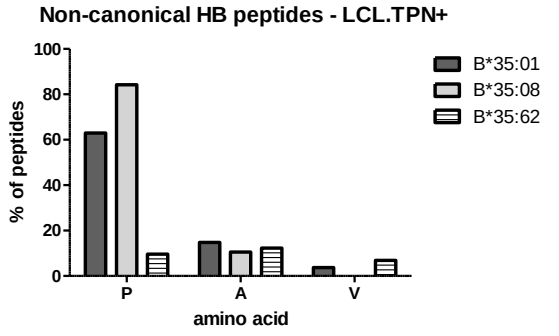
B



C



D



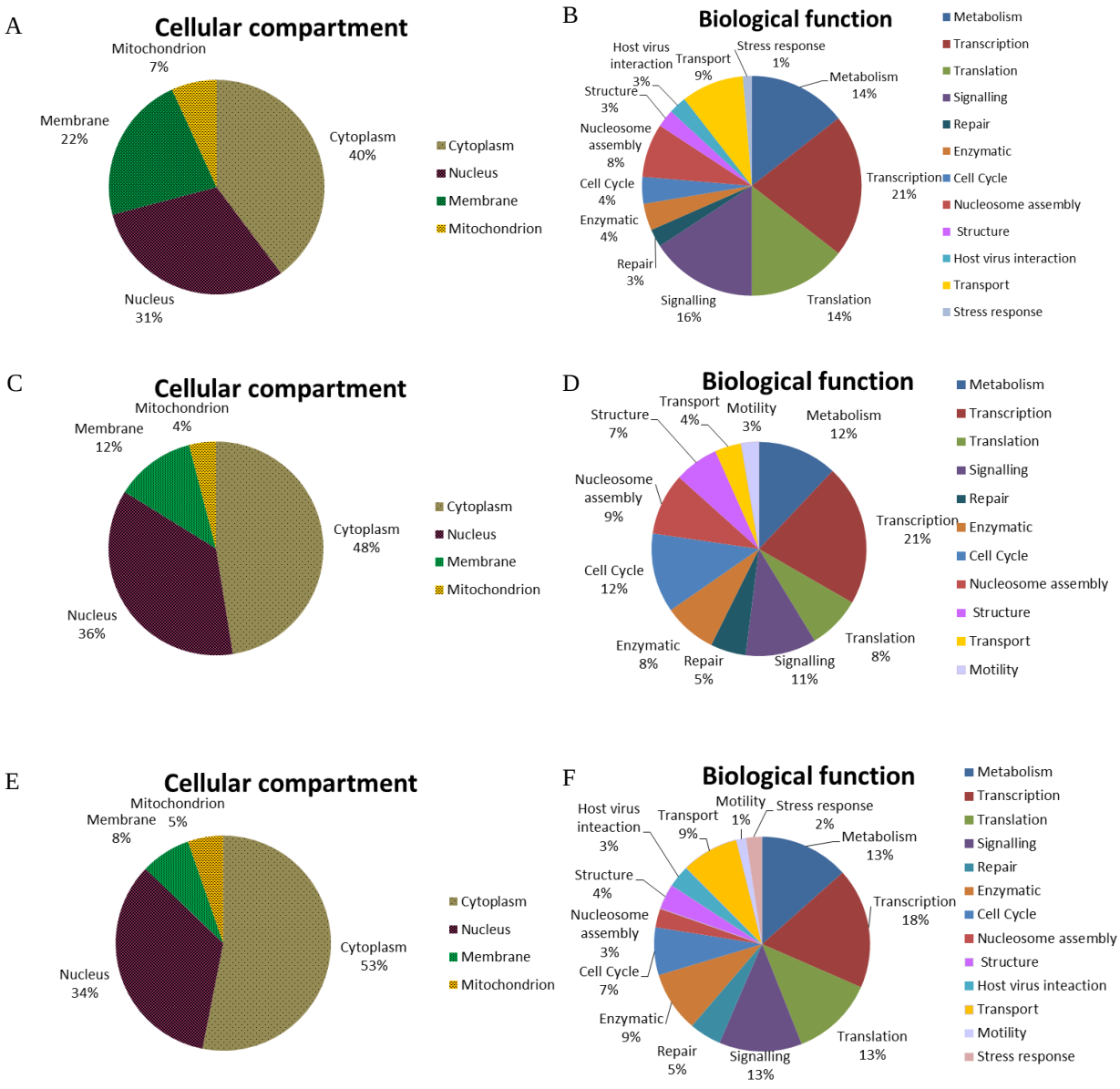
Frequencies of AAs occurring at peptide position p2 of non-canonical peptides (>10 AA long). The x axis represents AA residues occurring at p2. The y axis represents the percentage prevalence of individual AAs at p2. Black, grey or crossed bars represent the alleles HLA-B*35:01, B*35:08 or B*35:62. A) and B) Frequency of the AAs occurring at p2 among non-canonical sHLA-B*35/156 restricted LB peptides. C) and D) Frequency of AAs occurring at p2 among non-canonical sHLA-B*35/156 restricted HB peptides. In the presence of TPN, a significant percentage of HB peptides restricted by HLA-B*35:01 or B*35:08 showed the occurrence of Pro at p2. However, in the absence of TPN, the preference for Pro at p2 was diminished for HB peptides. The majority of HLA-B*35:62 restricted HB peptides acquired in the absence of TPN showed the occurrence of Ala at p2 position.

Appendix VII

Analysis of peptide sources

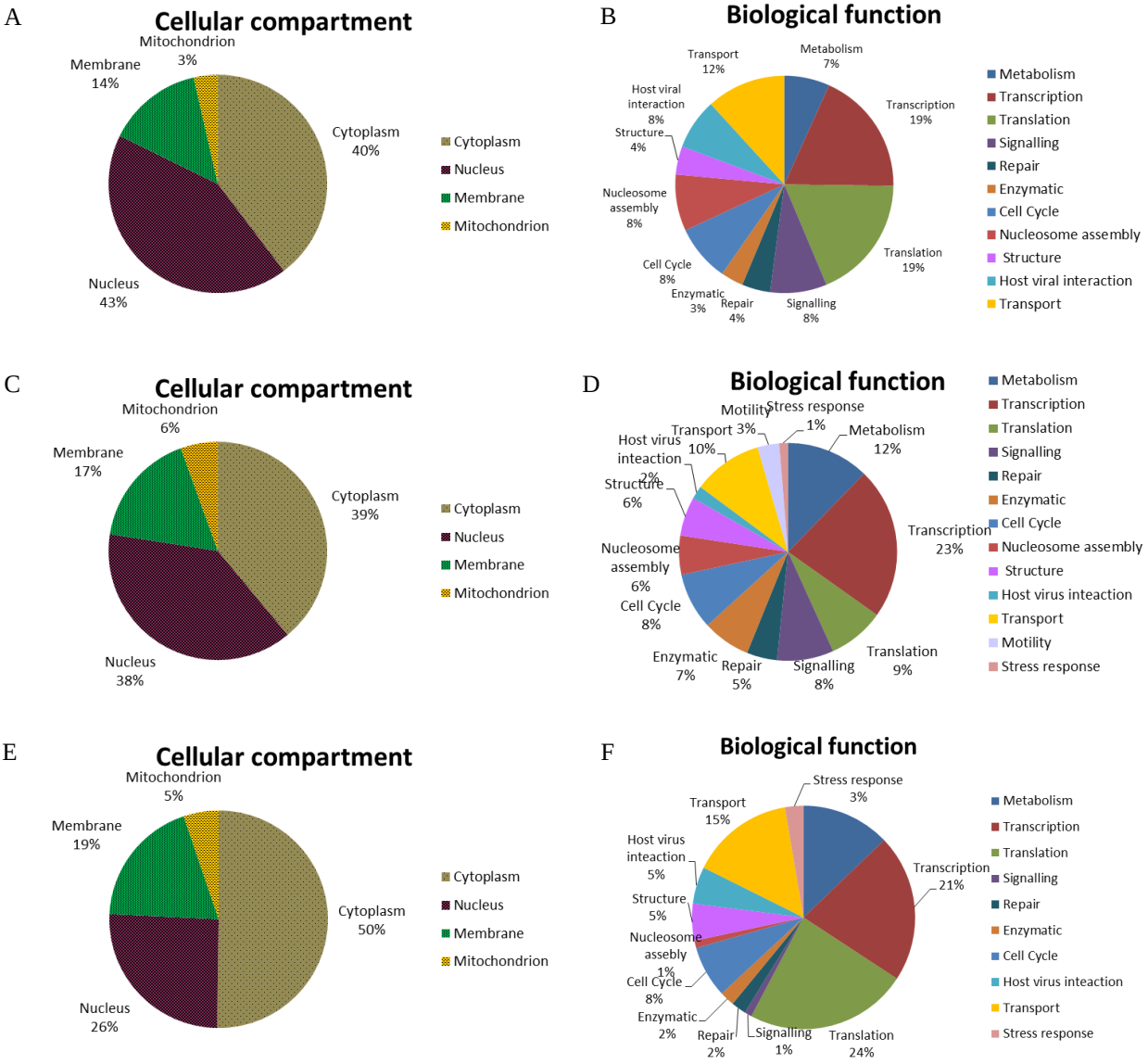
Uniprot database was used to further investigate the gene ontologies of the peptide sources. Analysis of the subcellular compartment and biological functions of the proteins revealed no significant differences between the sources of eluted peptides. However, a subtle difference was noticed regarding the subcellular compartment and biological functions among the sHLA-B*35:62 restricted peptides compared to B*35:01 or B*35:08 restricted peptides. Data obtained here revealed a subtle difference between the sources of peptides obtained from LCL 721.221 cells transduced with constructs encoding for sHLA-B*35:62 molecules from those of B*35:01 or B*35:08.

Protein sources of peptides acquired in the absence of TPN



Cellular compartment and biological function of peptide sources. A) and B) sHLA-B*35:01 restricted peptides. C) and D) sHLA-B*35:08 restricted peptides. E) and F) sHLA-B*35:62 restricted peptides.

Protein sources of peptides acquired in the presence of TPN



Cellular compartment and biological function of peptide sources. A) and B) sHLA-B*35:01 restricted peptides. C) and D) sHLA-B*35:08 restricted peptides. E) and F) sHLA-B*35:62 restricted peptides.

CURRICULUM VITAE

Personal information

First name(s) / Surname(s)	Trishna Manandhar
Date of birth	28.12.1980
Place of birth	Kathmandu, Nepal
Nationality	Nepalese

Education and training

Dates	2009 - 2011
Title of qualification awarded	Master's Degree in Applied Biological Sciences: Environmental Health (CGPA 3.86 out of 4.0)
Title of M.Sc thesis	Role of an *hpd* gene in oxidative stress response of a human pathogenic bacterium *Pseudomonas aeruginosa*
Thesis Supervisor	Dr. Mayuree Fuangthong, Chulabhorn Gradulate/Research Institute, Thailand
Name and address of organisation providing education and training	Chulabhorn Graduate/Research Institute, Thailand

Dates	2002 - 2005
Title of qualification awarded	Master of Science in Microbiology (75.00 %)
Title of M.Sc thesis	Antibiotic susceptibility profile of bacterial pathogens in urinary tract infections with special reference to Extended Spectrum Beta Lactamase (ESBL) producing strains
Thesis Supervisors	Professor Dr. Bharat Mani Pokhrel, Tribhuvan University, Nepal Dr. Prakash Ghimire, Tribhuvan University, Nepal
Name and address of organisation providing education and training	Central Department of Microbiology, Tribhuvan University, Nepal

Dates	2000 - 2002
Title of qualification awarded	Bachelor of Science in Microbiology (72.95 %)
Name and address of organisation providing education and training	Tri-Chandra Multiple Campus, Tribhuvan University, Nepal

Work experience

Dates	2012 - 2014
Occupation or position held	PhD Researcher
Name and address of employer	Institute for Transfusion Medicine, Hannover Medical School, Germany
Type of sector	Research/ Educational Academy
Supervisors	Dr. Christina Bade-Döding, Hannover Medical School, Germany Professor Dr. Rainer Blasczyk, Hannover Medical School, Germany

Dates	2007 - 2009
Occupation or position held	Research Associate
Name and address of employer	Nepal Academy of Science and Technology (NAST), Nepal.

Dates	2006 - 2009
Occupation or position held	Lecturer
Name and address of employer	Microbiology Department, Tri-Chandra College, Tribhuvan University , Nepal

Awards / Grants

2013: Bursary for 21st Annual Meeting of the German Society for Immunogenetics (DGI), Heidelberg, 5-7 September, 2013

2013: Deutscher Akademischer Austausch Dienst (DAAD) scholarship; Research grant for doctoral candidates, young academics and scientists

2009: Thai International Postgraduate Programme (TIPP) scholarship awarded by the Royal Thai Government's Thailand International Development Cooperation Agency (TICA), Ministry of Foreign Affairs, Thailand

2006: Research Assistantship Grant awarded by Nepal Academy of Science and Technology, Nepal

Membership

Member German Society for Immunogenetics (Deutsche Gesellschaft für Immungenetik, DGI)

I *hereby declare that information furnished in the above datasheet is true, complete and correct to the best of my knowledge and belief.*

Ort, Datum:_____ Unterschrift: _____