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Rola alternatywnych źródeł peptydów antygenowych dla głównego układu zgodności tkankowej klasy I w formowaniu reakcji odpornościowych i tolerancji immunologicznej

The role of alternative sources of antigenic peptides for the major histocompatibility complex class I in the formation of immune responses and immune tolerance

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ABSTRACT

The major histocompatibility (MHC) class I pathway plays a critical role in distinction between healthy cells and those that are malignant or infected by viruses or other pathogens. A key component of the immunosurveillance is the scanning by CD8+ T cells for the presentation of non-self peptides on MHC I molecules. For a long time it was a,ssumed that MHC I immunopeptidome represents peptides derived from proteolytic degradation of so-called retired full-length proteins by the 26S proteasome. However, decades of studies indicated that proteasomal degradation of full length proteins is not the source of MHC I antigenic peptides and other sources have been proposed. The search into antigenic peptide origin has shifted from degradation of full length proteins towards synthesis, of alternative peptides. But how alternative peptide substrates are produced and their physiological role in immune surveillance is still poorly understood. In this study we show that an MHC class I epitope (SL8) inserted in the second intron of the β-globin gene in a C57BL/6 mouse (HBB) generates immune tolerance. Introduction of SL8-specific CD8+ T cells derived from OT-1 transgenic mice in HBB animals resulted in a 3-fold increase in OT-1 T cell proliferation, as compared to wild type animals. The growth of MCA205 sarcoma cells expressing the intron-derived SL8 epitope was suppressed in wild type animals compared to HBB mice. Immunisation with SL8-pulsed and LPS-activated DCs revealed reduced numbers of endogenous SL8-specific CD8+T cells in HBB mice as compared to WT controls. The pre-spliced β-globin message was detected in the light polysomal fraction and introducing stop codons identified a non-AUG initiation site between +228 to +255 nts upstream of the SL8. Isolation of ribosome footprints confirmed translation initiation within this 27 nt sequence. Furthermore, treatment with splicing inhibitor shifts the translation of pre-spliced mRNA to monosomal fractions and resulted in an increase of intron derived peptide substrate as shown by polysome profiling and cell imaging. These results show that non-AUG initiated translation of pre-mRNAs generates peptides for MHC class I immune tolerance and help to explain why the products of alternative tissue specific splicing are tolerated by the immune system.

STRESZCZENIE

Główny układ zgodności tkankowej (MHC) klasy I odgrywa kluczową rolę w procesie odróżniania komórek zdrowych od nowotworowych, zakażonych wirusami lub innymi patogenami. Kluczowym elementem nadzoru immunologicznego jest skanowanie peptydów prezentowanych na cząsteczkach MHC klasy I przez limfocyty T CD8+ w celu odróżnienia peptydów ‘swoich’ od ‘obcych’. Przez długi czas zakładano, że immunopeptydom prezentowany na cząsteczkach MHC I stanowią peptydy pochodzące z proteolitycznej degradacji pełnołańcuchowych białek przez proteasom. Jednak dekady badań wykazały, że proteasomalna degradacja białek nie jest źródłem peptydów antygenowych dla głównego układu zgodności tkankowej klasy I. Pochodzenia peptydów antygenowych zaczęto poszukiwać w syntezie białek, a nie w ich degradacji. Jak dotąd jednak słabo poznano sposób wytwarzania substratów antygenowych i ich fizjologiczną rolę. W tym badaniu pokazujemy, że antygen MHC klasy I (SL8) wprowadzony do drugiego intronu genu β-globiny myszy C57BL/6 (HBB) generuje tolerancję immunologiczną. Wprowadzenie do tych myszy limfocytów T CD8+ pochodzących z transgenicznych myszy OT-1 i specyficznie rozpoznających antygen SL8 spowodowało 3-krotny wzrost proliferacji limfocytów T OT-1 w porównaniu ze zwierzętami typu dzikiego. Wzrost komórek mięsaka MCA205 z ekspresją antygenu SL8 pochodzącego z intronu był hamowany u zwierząt typu dzikiego, ale nie u myszy HBB. Immunizacja myszy za pomocą komórek dendrytycznych stymulowanych syntetycznym peptydem SL8 i aktywowanych przez LPS ujawniła zmniejszoną liczbę endogennych komórek CD8+T specyficznych dla SL8 u myszy HBB w porównaniu z myszami kontrolnymi. Prekursorowe mRNA β-globiny niosące antygen SL8 w intronie zostało wykryte w lekkiej frakcji polisomów, a wprowadzenie kodonów STOP pozwoliło zidentyfikować miejsce inicjacji translacji, inne niż AUG, pomiędzy +228 do +255 nukleotydów powyżej sekwencji kodującej SL8. Izolacja krótkich fragmentów mRNA chronionych przez rybosomy potwierdziła inicjację translacji w obrębie tej 27-nukleotydowejt sekwencji. Co więcej, traktowanie komórek inhibitorem splicingu przesunęło translację prekursorowego mRNA na frakcję monosomalną i skutkowało wzrostem ekspresji substratu

peptydowego pochodzącego z intronu, jak wykazano przez profilowanie rybosomów i analizę mikroskopową komórek. Wyniki te pokazują, że translacja pre-mRNA inicjowana przez kodony inne niż AUG generuje peptydy dla tolerancji immunologicznej MHC klasy I, a także pomagają wyjaśnić dlaczego produkty alternatywnego, tkankowo-specyficznego splicingu są tolerowane przez układ odpornościowy.

LIST OF ABBREVIATIONS

ACT – adoptive T cell transfer

AIRE – autoimmune regulator

APC – Antigen Presenting Cells

APs – antigenic peptides

BH – bleomycin hydrolase

BMDCs – bone marrow derived DCs

CAR – chimeric antigen receptor

CBC – cap binding complex

cDCs – conventional dendritic cells

CLIP – class II – associated invariant chain peptide

CNV – copy number variation

cTECs – cortical Thymic Epithelial Cells

CTLs – cytotoxic T lymphocytes

DCs – dendritic cells

DN – double negative

DP – double positive

DRiPs – Defective Ribosomal Products

EAE – Encephalitis

EBNA1 – Epstein-Barr virus nuclear antigen 1

eEF – eukaryotic elongation factors

eIF – eukaryotic initiation factors

EJC – exon junction complex

ER – Endoplasmic Reticulum

ERAAP – endoplasmis reticulum associated aminopeptidases

ERAP – endoplasmic reticulum aminopeptidases

Fezf2 – FEZ family zinc finger 2

GFP – green fluorescent protein

Hct – hematocrit

Hgb – hemoglobin

HLA – human leukocyte antigen

HMW – high molecular weight

IAV – Influenza A Virus

ICB – immune checkpoint blockade

iDCs – naïve dendritic cells

IFN – interferon

IFNg – interferon gamma

Ifnlr1 – Interferon lambda receptor 1

Ig – immunoglobulin

IL-2 – interleukin 2

ISO – isoginkgetin

JIA – juvenile idiopathic arthritis

LAP – Leucine aminopeptidase LAP

LMPs – low molecular weight proteins

LMW – low molecular weight

LPS – lipopolysaccharide

MAPs – MHC I associated antigenic peptides

MBP – myelin basic protein

MCV – mean corpuscular volume

MHC – Major Histocompatibility Complex

mTECs – medullary Thymic Epithelial Cells

Nlrc5 – NOD like receptor family CARD domain containing 5

NMD – nonsense mediated decay

NP - nucleoprotein

ORF – open reading frame

OVA – chicken ovalbumin

pAPC – professional Antigen Presenting Cells

pDCs – plasmacytoid dendritic cells

PGE – promiscuous gene expression

PLA – proximity ligation assay

PLC – peptide loading complex

pMHC I – peptide-MHC I

PSA – puromycin sensitive aminopeptidase

PTC – premature termination codon

PTPs – Pioneer Translation Products

RAG-1 – recombination activating gene 1

RAG-2 – recombination activating gene 2

RBCs – red blood cells

RBPs – ribonucleoproteins

REG/PA28 – proteasome activator

RF – release factor

SL8 – SIINFEKL

SLPs – synthetic long peptides

snRNPs – small ribonucleoproteins

SP – single positive

Stat1 – signal transducer and activator of transcription 1

TAAs – tumor associated antigens

TAP – transporter associated with antigen processing

TA-PTPs – tumor associated pioneer translation products

TCR – T cell receptor

TECs – thymic epithelial cells

TILs – tumor infiltrating lymphocytes

TNF – tumor necrosis factor

TNFα – tumor necrosis factor alpha

tolDCs – tolerogenic dendritic cells

TPPII – tripeptidyl peptidase II

TRAs – tissue restricted antigens

Tregs – T regulatory cells

UPS – the ubiquitin-proteasome system

Usp14 – ubiquitin-specific protease 14

UTR – untranslated region

INTRODUCTION

Evolution of scientific ideas

The current state of knowledge about major histocompatibility complexes wouldn't be achieved without the evolution of scientific intuition, interdisciplinary approaches and teams who since the beginning of 20th century have been uncovering the more important details regarding how the immune system perceives self versus pathogens. In this chapter, a few of the major scientific discoveries and their impact on immunology and cellular biology and that form the basis for my thesis project will be reviewed.

Immune tolerance has been incessantly a subject of studies since 1945 when Ray Owen observed that dizygotic twins often have two distinct blood groups as an effect of shared blood circulation in early development in utero [1]. The analysis of more than 80 pairs of bovine twins allowed him to observe that cattles share hematopoietic cells that persist into adulthood. Although back then the term immune tolerance was not in use, this observation made significant impact on generation of future concepts on self- and nonself-recognition in central and peripheral positive and negative selection processes. Owen's observations inspired Sir MacFarlane Burnet who hypothesised that during the embryonic development the immune system gradually learns how to recognize self while being exposed to self-defining molecules [2]. This hypothesis was further investigated independently by Peter Medawar who investigated skin graft survival in mice. The studies shown that mice which were exposed to inoculum consisting of cells from different tissues of distinct mice strains during the early stages of foetal development tolerated skin grafts from donor's mice in the adulthood [3]. These studies led to the formation of new concepts of acquired immune tolerance and were awarded a shared Noble Prize to Sir Frank MacFarlane Burnet and Peter Medawar in 1960.

The core observations made in 1950s that led to identification of chromosomal regions comprising histocompatibility genes in mice were made by two independent teams led by the British immunologist Peter Gorer and the American biologist George Snell. Snell based his

hypothesis on the works described by his then supervisor Clarence C. Little whose studies have shown that tumors grow only in selected strain of inbred mice. Snell further investigated the idea and described a method for developing mice strains congenic to the inbred mice tested by his advisor. These new mice strains were differing by a single histocompatibility locus from the inbred mice with *major* or *minor* effects on the tumors transplant survival [4], [5]. The following findings highlighted one chromosomal region that appeared to have a major role in the transplant rejection compared to other minor players in the same process and referred to as the major histocompatibility complex (MHC) [6], [7]. This genomic region was described as encompassing many genes that determine the immunological identity of each individual.

The French immunologist Jean Dausset observed that serum from patients who had received multiple blood transfusions caused marked agglutination of leukocytes. Later on it was revealed that leukocyte-agglutination was mediated by particular antibodies present in the serum. In 1958 he described the first human leukocyte antigen called Hu-1, later renamed to HLA-A2, and suggested that one major chromosomal region comprises the MHC-related genes like in mice [8]–[11]. Later, it was discovered that the human MHC, or HLA, maps to the chromosome 6 short arm and three main regions were described: (1) the class I region containing the HLA-A, -B and -C genes which encode the class I molecule heavy chain (2) the class II region containing the HLA-DR, -DQ and -DP genes, each of which containing A and B genes coding for the α and the β chains respectively, and (3) the class III region containing genes encoding for the complement components. In mice, the ‘classical’ MHC-I molecules were called H2-D, K and L and the MHC-II molecules I-A and I-E. By that time, other important features were starting to be described including the MHC’s highly polymorphic loci.

The MHC and HLA, genes have since been closely linked to immune responses and an important next step was the discovery in the 1960s that T cells mediate HLA recognition and that the presentation of antigenic peptides on HLA molceulse played an important roel in self

vs non-self recognition. However, in order to investigate the detection of peptides presented on HLA molecules by T cells it was important to identify the peptide presented on MHC I and II molecules (pMHC complex). Among many interesting studies, one can cite the work of P.C Doherty and R.M. Zinkernagel on cytotoxic T cell response in mice infected with the lymphocytic choriomeningitis virus (LCMV) which introduced the MHC restriction concept. In brief, they co-cultured mice fibroblasts infected with LCMV and labelled with ^{51}Cr together with spleens cells collected from different strains of LCMV infected mice. By the evaluation of ^{51}Cr release they indirectly evaluated cytotoxic capacities of T cells towards infected fibroblasts. Only cells from mice sharing at least one set of H-2 genes (H-2K) with the target fibroblasts caused high cytotoxicity. On the contrary, cells from spleens of mice with other H-2 haplotype caused only minimal ^{51}Cr release indicating lymphocytes histoincompatibility [12]. These and other studies indicated the ability of T cells to recognize a specific antigen presented by self-MHC class I molecules [12]–[15]. In 1973 the concept of adaptive immune responses became more clear thanks to R. Steinman's discovery of Dendritic Cells (DCs) by then newly established electron-microscopy [16]. Few years after, in 1976 the concept of cross-priming by DCs was introduced by M. J. Bevan [17]. Followed by decades of studies by Steinman's team, and others, on DCs antigen uptake and maturation led to better understanding on DCs contribution to adaptive immune responses and immune tolerance. In 1979 and during following years E. R. Unanue published several studies that contributed to the understanding of T cells interactions with H-2 gene. First, his works were focused on antigens derived from *Listeria monocytogenes* and their processing and presentation by mouse macrophages. To show that H-2 genes are crucial in this interaction he exposed *in vitro* T cells specific to *Listeria* (derived from *Listeria* immunised mice) to macrophages from normal mice exposed to heat-killed bacteria. It was shown that only macrophages bearing H-2 molecule were able to induce specific T cells proliferation [18]. In further works on Hen egg-white lysozyme (HEL) Unanue confirmed the requirement of protein processing by macrophages prior antigen presentation and also observed that two different T cell hybris recognized the same protein fragment. This led to the speculation that the T cells may differ

in affinity of the T cell receptors (TCRs) between the clones and it was now also becoming evident that several types of T cells exists [19], [20]. Work was now focused on investigation of the complexes on antigen presenting cells recognized by TCRs. The first crystal structure of MHC I molecule HLA A2 released in 1987 by Bjorkman et al. revealed a binding site for peptide antigens [21], [22]. This was further investigated by Townsend and colleagues who focused on the recognition of MHC I epitopes derived from cells infected with Influenza A virus (IAV). They indicated that only particular epitopes from nucleoprotein (NP) were able to induce cytolytic T lymphocytes (CTL) response *in vivo* [23]–[25]. These studies made it possible to better understand the characteristics of MHC peptide ligands. It is important to highlight that in the 1980s, the team of T. Boon proposed other sources of antigenic peptides for MHC class I pathway which do not originate from processing and degradation of full-length proteins based on the detection of peptides presented on MHC I molecules from cancer cells transfected with promotorless gene frаments [26]. Those *in vitro* studies have been followed by other teams, mainly Nilabh Shastri's, Jonathan Yewdell's and Jack Richard Bennink's as well as Pierre Coulie's and others and led the direction of research into initiation of mRNA translation having an impact on generation of antigenic peptides, rather than the degradation of full length proteins (APs). In 1993 Shastri's team reported that antigenic peptides can come from incorrect translational reading frames which would not generate full-length proteins [27], [28]. In 1995 Coulie et al. reported the presence of intron-derived peptides on MHC I molecules on human melanoma [29]. Finally in 1996 Yewdell and Bennink introduced the DRiPs (defective ribosomal products) model [30]. At the end of 90s Shastri's team showed a presentation of out-of-frame MHC-I peptide produced by a novel translation initiation mechanism [31]. Even though more evidence was provided about MHC I antigenic peptides originating from outside of canonical main open reading frames it was difficult to understand their physiological function. Yin et al. in 2003 showed in their work that certain viruses can evade immune recognition by suppressing their own mRNA translation and not directly interfering with the degradation of full-length proteins [32]. There is much

more evidence so far described by many teams which shift the focus of research to the initiation of mRNA translation and the true definition of open reading frame.

In last decades the research on sources of antigenic peptides advanced rapidly and what was first awarded a Noble Prize in Physiology and Medicine in 1980 ‘for the discoveries concerning genetically determined structures on the cell surface that regulate immunological reactions’ to George D. Snell, Bary Benacerraf and Jean Dausset, opened the doors to many other fields to investigate further the impact of MHC-peptide complexes on generation of immune tolerance, and responses towards pathogens, cancer and autoimmune diseases. Zinkernagel and Doherty’s research on how the immune system recognizes virus-infected cells was awarded a Nobel prize in 1996. Ralph Steinman’s discovery of tree-like shaped cells followed by decades of studies on DCs maturation, antigen uptake and orchestrating immune responses led to the achievement of Nobel Prize in 2011. Finally, most recent Nobel Prize in immunology was awarded in 2018 to James P. Allison and Tasuku Honjo, who independently described checkpoint immune blockade inhibition as an effective way to boost immune responses towards certain cancers. All the above works, and many others not mentioned here, greatly contributed to current knowledge on immune system without which there wouldn’t be any immune associated therapies towards viruses, bacetria’s, cancers, auoimmune disorders as well as the entire branch of transplantology. The focus of this work has been put on physiological impact of intron-derived antigenic peptides for the MHC I pathway and their contribution to immune tolerance using a newly developed mouse model.

Display windows in cells – a brief introduction to main features of MHC class I and II presentation pathways.

The main role of MHC class I molecules is to display peptide fragments on the surface of cells for recognition by $CD8^+$ T cells. The MHC class I molecules are ubiquitously expressed on all nucleated cells and in normal healthy conditions display cellular self-immunopeptidome in order to give tolerogenic signal to scanning dendritic cells. MHC I molecules also present non-self peptides that come from intracellular viral proteins or those that are generated as a result of somatic mutations during cancer development [33]. According to the recent study of HLA I and II transcripts in different tissues of human body, classical HLA I are more abundantly expressed than HLA II [34]. The highest expression was detected in whole blood followed by spleen and bone marrow. Interestingly, classical HLA I transcripts were particularly abundantly expressed in lung and small intestines [34].

The MHC class II molecules present antigenic peptides for the recognition of CD4+ T helper cells. The expression of MHC class II molecules is restricted to professional antigen presenting cells (pAPCs) like macrophages, dendritic cells, B cells and monocytes, which have the capacity to phagocytose cells invaded with pathogens and present their antigens to CD4+ T cells [35], [36]. In human overall expression of HLA II across different tissues is lower than HLA I, being mostly abundant in lymphoid tissues [34].

Antigen processing and presentation via MHC I and MHC II pathways results from several different routes that can interact with each other. Certain subpopulations of DCs are able to cross-present on their MHC class I molecules peptides that come from extracellular sources [37]. Cross-presentation is particularly important in orchestrating immune responses towards viruses and tumors, nevertheless more and more recent findings suggest DCs role in generating immune tolerance as well. MHC II peptides originate from extracellular material taken up by pAPCs and processed by the lysosomal pathway.

These observations bring even more complexity in the understanding of the pool of antigenic peptides that are expressed by cells, namely the immunopeptidome. In the next chapters, we

will see that our understanding of the MHC molecules structures and of the intracellular pathways, as well as identification of self-, tumor- and viral-associated antigens bring and will continue to bring new insights into the composition of the immunopeptidome and ultimately into the adaptive immune responses or immune tolerance. I will particularly emphasize on the MHC I antigen presentation pathways, the source material that enters this pathway and their role in shaping immune responses.

MHC class I pathway for direct antigen presentation: from what's seen at the surface to the source of presented ligands

The peptide-MHC (pMHC) complex

MHC I molecules present antigenic peptides on the surface of the cells and give information to the adaptive immune system about the current status of the intra- and extra-cellular immunopeptidome. Peptides-MHC (pMHC) complexes presented on the cell surface are stabile structures and it has been proposed that evolutionarily the MHC I molecule derives from MHC II (Fig.1) [38], [39]. The extracellular part of the MHC I molecule, which is distal from the membrane, is the peptide binding domain consisting of a single heavy α chain. It can be separated into 3 regions and the peptide chain is loaded within the groove restricted by two anti-parallel helices (α1 and α2), supported by slightly curved β-sheet base. The length of the peptide that can be loaded within the MHC I groove is restricted by two pockets A and F with peptide C-terminal end docking into the F pocket and N-terminal end docking into the A pocket [39], [40]. Because of that the peptides size is restricted to be between 8-10 residues, also the type of peptide loaded within the groove highly depends on the MHC I allotype, namely their interactions with MHC I side-chains, the geometry, charge distribution and hydrophobicity of the binding groove [41]. The amino acid structure of the peptide-binding domain is the most polymorphic part of the entire molecule and it enables interactions of a wide range of MHC alleles with different pools of peptides. The peptide-binding unit is supported by one immunoglobulin domain and β2-microglobulin protein (β2m) that is associated with heavy α chain by non-covalent binding. The entire peptide-MHC I complex is anchored to the membrane by transmembrane helices of the single heavy α chain [39], [41].

The MHC class II molecules select for longer peptides of 13-25 residues and it comes from the open binding groove, from which the N-terminus of the peptide chain can extrude via P1 pocket. The distal, extracellular domain of MHC II consists of two heavy chains (α and β) and

similarly to the α chain in MHC I each of them is formed by β sheets at a base and α helices on top that make a space to accommodate peptides between them. Both heavy chains are supported by membrane-proximal immunoglobulin domains and they are connected to the membrane also by transmembrane helices [39]–[41].

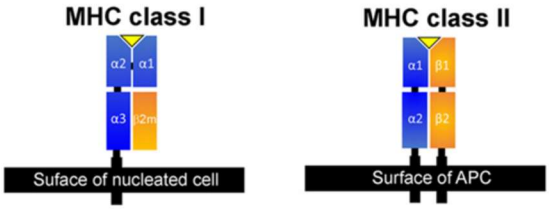


Figure 1. Cartoons represent schematic structure of MHC class I and MHC class II molecules [41].

Source: Wieczorek, Marek et al. “Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation.” *Frontiers in immunology* vol. 8 292. (2017)

Degradation and processing of peptide precursors

Peptides precursors, regardless of their origin, require catalytic cleavage prior to being loaded on MHC molecules. It has been shown that changes in the kinetics of peptide degradation as well as in the cell catalytic activity greatly impact the subsequent cytotoxic T cells response and therefore provide a way for modulating the presented immunopeptidome. One of the early steps in the processing of MHC I peptide ligands is the proteasome system that degrades proteins or bigger polypeptide products into smaller peptide fragments that can be further transported via TAPs into the peptide loading complex (PLC) within the lumen of ER. Within the ER certain peptide ligands undergo further processing to be finally loaded on the MHC I glycoproteins and after successful completion of all the steps the peptide-MHC I complex is transported to the cell surface for the presentation to $CD8^+$ T cells.

The following chapters will elaborate on the important steps within this pathway that affect the generation of MHC I epitopes.

The ubiquitin-proteasome system: dependent or not dependent?

Proteins can be degraded by proteasomes via the ubiquitin-dependent or non-ubiquitin dependent pathways. The first step of the ubiquitin-proteasome system is ubiquitination which involves multiple components to target proteins for degradation via 26S proteasome. It can be divided into three steps: activation, conjugation and ligation in which important part is played by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), respectively [42]. All these enzymes act in concert to achieve a common goal which is the recognition of certain residues on protein substrates and bind it to ubiquitin. Depending on the protein amino acids sequence ubiquitin can bind to lysine, cysteine, serine or threonine or N-terminus of targeted protein via different bond types. However, only a specific type of polyubiquitination is linked with protein degradation by proteasome and is often referred to as ‘molecular kiss of death’ [43]–[46]. The ubiquitin-proteasome degradation pathway is well described in its correlation to the processing of

MHC I peptide precursors. However, beside the UPS system there are other mechanisms under investigation e.g. the requirement of ubiquitination in proteasome dependent antigen presentation. Studies investigating the effect of E1s inhibition only confirmed conflicting results indicating that the need for Ub is highly context dependent [47]. It has been also proposed that ubiquitin independent pathways may supply polypeptide precursors to the proteasome via engaging with ubiquitylated chaperones which in turn, require deubiquitination prior substrate polypeptide release to the 26S proteasome [48]. Hence, deubiquitination may also play an important role in antigen processing. Chemical inhibition of ubiquitin-specific protease 14 (Usp14) which is a proteasome-associated deubiquitinase results in reduction of MHC I direct antigen presentation from non-canonical source [49]. It is well established that 26S proteasome contributes significantly to the generation of MHC I ligands [50]. There are at least two types of the proteasomes that have been described. First are proteasomes that are constitutively expressed by all types of cells. Under certain immune stimuli those proteasomes can change the composition of their 20S catalytic sites and become immunoproteasomes [51]. Immunoproteasomes are the second type of degrading complexes which are more specialized to the MHC I pathway and can be formed also in immune cells (pAPCs) as well as cTECs [52]. The standard structure of 26S proteasome consists of a 20S core barrel and two 19S complexes at the ends of the barrel. Those 19S subunits are responsible for the recruitment and unfolding of ubiquitylated substrates as well as for the activation of 20S barrels [53]. The constitutive 20S barrels consist of the catalytic beta-subunits which are characterised by their different properties (chymotrypsin, trypsin or caspase-like activities) to cleave polypeptide substrates after their C-terminal amino acids. Importantly, the type of cleavage is strictly correlated with the type of beta-subunit within the barrel. Another important aspect that correlates the proteasomal degradation with MHC I pathway is the C terminal cleavage that prepares the smaller peptide fragments ready to be docked at the F pocket of the MHC I groove. Interestingly, upon the stimulation by proinflammatory cytokines like interferon gamma (IFNg) or tumor necrosis factor (TNF) certain beta-subunits are exchanged within the 20S barrels by functionally different

counterparts – low molecular weight proteins (LMPs) or βi [54], [55]. The processes of exchanging particular β subunits are particularly important in another type of proteasome – thymoproteasomes [56], [57]. These types of 26S proteasomes are expressed mainly in cTECs where it has been speculated that their catalytic activities enable stronger binding of peptides to the MHC I groove [58]. Deficiencies in β5t subunits in cTECs result in substantial defects in positive selection of CD8+ T cells. Approximately 20% of CD8+ T cells are selected upon these conditions and even they have been described to have diminished responsiveness to infections [59]–[62]. In the situations when both the immunoproteasome and thymoproteasome subunits are deficient and polypeptide precursors are degraded only by constitutive proteasomes, only around 10% of mature CD8+ T cells are developed [63]. Hence, it is clear that the generation of peptide antigens for MHC I pathway and immune tolerance is proteasome dependent. However, peptides attachment to MHC I molecules is not restricted only to the C-terminal ends but also to following residues which would stabilise low affinity peptide ligands within the MHC I groove. Beside the 19S-20S-19S (26S) proteasome other catalytic structures have been described for example asymmetric, single 19S capped 26S proteasome or proteasome containing both 19S and PA28 complexes [64], [65]. The REG/PA28 family are other types of regulators crucial in proteasome activation and several have been described to impact the production of antigenic peptides for the MHC I pathway. They have a particular contribution in changing cleaving activity within the 20S core and are associated with proteolysis of shorter peptide precursors rather than full-length proteins, which in effect yields distinct products [66]. It has been also indicated that REGγ (also abbreviated as PSME3) – a member of REG/PA28 regulators, is overexpressed in several types of cancer, e.g. breast cancer, leading to abnormal degradation of MHC I antigens and tumors' escape from hosts' immunosurveillance [67].

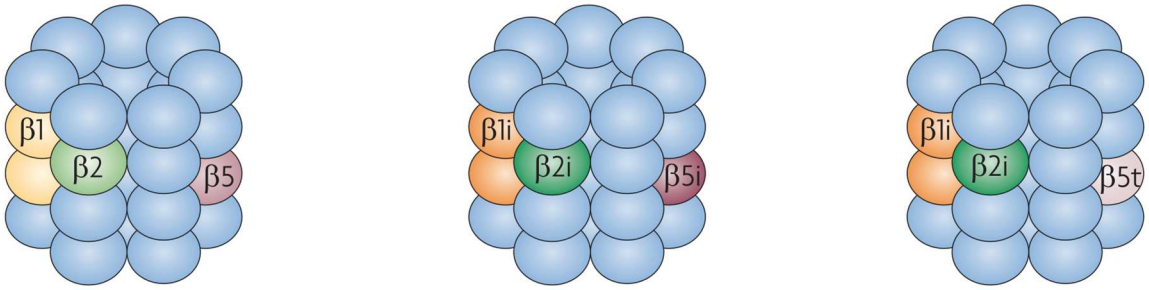


Figure 2. Models of different types of proteasomal 20S proteolytic barrels. (A) Constitutive proteasome. (B) Immunoproteasome with exchanged β-subunits to β1i, β2i and β5i. (C) Thymoproteasome with exchanged β5 subunit into β5t. Immunoproteasome features distinct caspase-like and chymotrypsin proteolytic activities from constitutive proteasome, whereas thymoproteasome exhibits decreased chymotrypsin activity [51].

Source : Groettrup, Marcus et al. "Proteasomes in immune cells: more than peptide producers?." *Nature reviews. Immunology* vol. 10,1 (2010)

As described above, proteasomes have the capacity to cleave and generate proper C terminus of MHC I peptides. Such generated peptides are of different amino acids length and often require further trimming on the N terminal side. This process is mediated by varied aminopeptidases both in cytosol or ER [68]. Amongst the cytosolic aminopeptidases tripeptidyl peptidase II (TPPII) which has the capacity to trim N-extended peptides regardless of their initial length [69]. TPPII inhibition reduced the MHC I antigen presentation of certain viral epitopes like influenza virus nucleoprotein or EBV LMP1 [70], [71]. Other cytosolic aminopeptidases have been also reported (Leucine aminopeptidase LAP, bleomycin hydrolase BH, puromycin sensitive aminopeptidase PSA), however their role in peptide cleavage is not clear. The difficulty of drawing clear cut conclusions from the studies describing these aminopeptidases is related to the experimental systems in use. Data from ex cellulo studies indicated their capacity of trimming N-terminus of peptides precursors however, as shown in *in vivo* studies in LAP-/-, BH-/- and PSA-/- mice their effect on MHC I antigen presentation was not evident [72]–[74]. It is important to take into account that many

other cytosolic aminopeptidases may play a role in this process and it is not due to a single enzyme to generate particular epitopes [75].

Another cellular compartment in which the N-terminal trimming takes place is ER to which peptide precursors are transported in a TAP-dependent or independent manner. ER aminopeptidases ERAPs (also called ER- associated aminopeptidase; ERAAP) have been associated with trimming of the N-terminus of peptides so that they are of proper size to fit in the MHC I binding groove [76]. In mice there is only one type of ERAP1, however in humans and few other mammals 2 forms of this enzyme have been described [77]. Out of the two ERAP1 has a dominant role in peptide trimming and it's been shown with the use of mice that its knockout had an effect on the generation of overall MHC I immunopeptidome and $CD8^{+}$T cells repertoire [78], [79].

Beside the UPS degradation pathway there are other mechanisms involved in proteolysis that are independent from ubiquitin or complement 26S proteasome in case of its dysfunctions [80], [81]. In fact, it has been argued that relying on the ubiquitin pathway for generating antigenic peptides would provide an opportunity for viral immune evasion. In fact, our lab has unpublished data showing that deletion of every lysine residues in the chicken ovalbumin that can act as ubiquitination targets does not affect antigen presentation. Another study also showed that targeting a protein for the 26S proteasome does not affect the presentation of antigenic peptides for the MHC I pathway [85]. The non-ubiquitin pathways contribute to the generation of a variety of MHC I peptide pools and have been extensively described elsewhere [82]–[85]. Importantly, they indicate multiple peptide routes prior to supplying MHC I complexes and often are substrate dependent, which was also described by our team in terms of autophagy role [86]. However, it has been proved that MHC I antigenic peptide precursors originating from alternative sources including non-coding genomic regions are degraded via the canonical UPS pathway [87]–[90].

Loading and formation of the complex

As mentioned above, partially trimmed peptides are loaded on chaperoned empty MHC I heterodimers within the ER lumen which is strictly correlated with PLC (Fig. 3). PLC is a multisubunit complex consisting TAP1 and TAP2 translocons, the oxidoreductase ERp57 and chaperons tapasin and calreticulin [91]. All subunits play important roles in orchestrating peptide translocation and loading as well as MHC I stabilisation. Deficiencies in particular components of PLC in mice result in severe immunodeficiencies or cause embryonic lethality [92]–[94]. For example the lack of TAP1 in mice drastically reduces the levels of MHC I complexes on the cell surface as well as overall levels of CD8+ T cells [95]. After the peptide-MHC I complex is formed it dissociates from the TAP and is selectively exported by the Golgi apparatus to the cell surface for the recognition by $CD8^+$ T cells or scanning DCs [96].

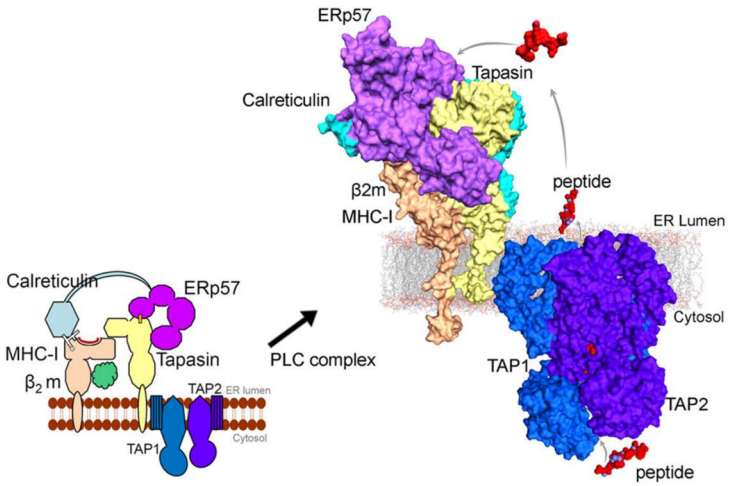


Figure 3. Cartoon and structural model representing peptide-loading complex (PLC) crucial for MHC I antigen processing. Model represents proposed route of peptide transport from the cytosol to the ER lumen via TAP. Once in the ER, peptides undergo further trimming of the N-terminus and loading to stabilized by tapasin, calreticulin and ERp57, partially folded MHC I molecules [40].

Source: Padariya, Monikaben et al. “Viruses, cancer and non-self recognition.” Open biology vol. 11,3 (2021)

Sources of peptides presented by MHC class I pathway.

According to the canonical MHC class I pathway, peptides presented on MHC I molecules are derived from degraded full-length proteins. In this model, proteins are targeted for proteasomal degradation and transported via TAP-dependent mechanism to the lumen of endocytic reticulum (ER), where they are further processed and loaded to awaiting, partially folded MHC class I molecules. In this scenario however, the final peptide:MHC class I repertoire presented on the cell surface would be limited to the final products of functional protein expression mechanisms. Studies have since challenged this dogma. Several studies on viral and cancer immune evasion mechanisms significantly contributed to the overall knowledge regarding crucial components of the MHC I pathway and enabled further investigation of this hypothesis by many teams over the last 40 years [97], [98]. It has been shown that many proteins have a half-life of days or even weeks and in those circumstances infected cells wouldn't be readily recognized by the immune system if it was due to a full-length protein degradation mechanism to generate MHC I epitope. Furthermore, it has been shown the kinetic discrepancy between the half-life of metabolically stable proteins and rapid antigen presentation of related epitopes [87], [99], [100]. Hence, the questions that have been asked are: (1) what are the polypeptide sources prior to entering the 26S proteasome? (2) where are they synthesised? (3) what would be their physiological function? The first two questions have been addressed by many, including our team [30], [89], [101]–[106]. However, this remains a controversial topic and one of the issues relates to peptides originating from main open reading frames but it has to be kept in mind that even main open reading frames can be translated via alternative mechanisms of synthesis. Antigenic peptide substrates derived from alternative sources such as alternative translation products was introduced by Thierry Boon as a 'pepton hypothesis' in late 1980s. This hypothesis directly stated that peptides for MHC I pathway are produced from "short genetic regions located around the sequence for the peptide [that] can be transcribed autonomously" [26]. It's been also argued that these short precursors would be translated independently from the

translation of main open reading frames. These studies led a way to other hypothesis and in 1996 Yewdell and Bennink argued that the major source of the peptides for MHC I are in fact products generated as a result of point mutations, premature stop codons or in general would be rather dysfunctional polypeptides that are quickly degraded [30]. However, the major difference between the Pepton and the DRiPs hypotheses is related to the translation mechanism involved in the generation of these products [90]. With the emergence of new technologies and tools valuable in the recognition of MHC I epitopes on the cell surface new studies gave more insight into both scenarios.

In 2011 and continued in following years the team of Robin Fahraeus introduced another explanation for the peptides that are correlated with non-protein coding genomic regions or cryptic translation products. It has been shown that the translation of full length proteins and antigenic peptides are spatio-temporarily different mechanisms [87], [88]. More to that, the introduction of highly antigenic SIINFEKL (SL8) encoding sequence in different positions of introns or exons of beta-globin gene along with premature termination codon (PTC) did not affect antigen presentation to specific $CD8^+$ T cells. These and many other studies led to the formation of the hypothesis that antigenic peptides for the MHC I pathway are generated from pre-spliced mRNAs as Pioneer Translation Products (PTPs) with implications that this process is happening in the nucleus. This idea is more close to the peptons hypothesis as well as the studies introduced by Shastri's team, however by no means exclude DRiPs. It is plausible that several different mechanisms contribute to the global immunopeptidome depending on the conditions the cells are under e.g. stress response, viral infection, somatic mutations.

By this moment it is known that antigenic peptides (AP) can be produced from cryptic and non-conventional translational products including 5' and 3' untranslated regions, intron and intron/exon junctions [107]–[111]. APs can be also derived from transfected promoterless genes containing frame shifts or stop codons upstream the peptide coding regions [26]. Based on works related to Epstein-Barr virus, it has been shown that MHC class I immune

surveillance is directly correlated with the mechanism that regulates protein synthesis [112]. Together with other results they highlight the importance of pre-mRNA and mRNA processing in providing antigenic peptides for MHC class I surveillance in the form of PTPs and DRiPs.

Recent advances in the analysis of MHC I immunopeptidome and the importance of precise peptides prediction

Emerging Mass Spectrometry (MS) analyses and its correlation to the genomic sequences revealed significant contribution of non-canonical translation of above described regions in the generation of MHC I immunopeptidome [113], [114]. It is particularly important in predictions of tumor associated antigens (TAAs) that would be suitable for targeting with new therapies. However, most of the predictions currently in use omit the non-coding space which can have a major impact on the drug discovery field. The study of MHC I associated immunopeptidome (MAPs) in 18 B lymphoblastoid cell lines revealed identification of 25270 peptides on 27 HLA-A and HLA-B allotypes [115]. Detected MAPs represented 59% of genes expressed and covered only 10% of exonic sequences expressed in those cell lines. Another study on human B cells revealed that around 10% of MAPs derived from non-coding genomic sequences or exonic out-of-frame translation [113]. Another recent study indicated 15% of peptides originating from cryptic translation products [116]. Recent analysis of MAPs in glioblastoma detected HLA ligands derived from non-canonically translated proteins and that over 80% of tumor exclusive peptides successfully primed specific CD8+ T cells [117]. Taking into account the intricacies around all the mechanisms involved in the synthesis of polypeptide precursors as well as cancer immune evasion mechanisms it is important to fully understand the noncanonical translation processes. Identification of TAAs that precisely distinguish malignant cells from other healthy tissues is particularly important in the development of new cancer immunotherapies as well as improvement of those that already exist and exploit CD8+ T cell mediated responses [118], [119]. These include: DCs cancer vaccines, adoptive T cell transfers (ACT) that comprise both native cytotoxic T lymphocytes (CTLs) or tumor infiltrating lymphocytes (TILs) as well as engineered T cells expressing tumor antigen specific TCR or chimeric antigen receptors (CARs) [120]–[122]. Another promising immunotherapy that aim to boost CD8+ T cell mediated anti-tumor response comprise of immune checkpoint blockade (ICB) such as anti-PD-1, anti-PD-L1 and anti-

CTLA-4. Combination therapies of ICB and CAR proved particular clinical efficacy and led to many drugs being approved by FDA [123]. However, not all cancer patients can benefit from these treatments and do not respond well to ICB or CAR. Antigen presentation by MHC I pathway has been considered as one of the very important factors influencing the fate of cancer cells. Many efforts have been dedicated to improve both direct antigen presentation of TAAs to effector CD8+ T cells as well as cross-presentation of TAAs by DCs in order to prime naïve CD8+T cells [121]. However, it is still of utmost importance to also recognize the pathways involved in the generation of TAAs themselves and correlate the sequences of these TAAs with the rest of the immunopeptidome to be able to predict treatment specificity as well as the context in which those antigens are cross-presented by DCs.

The role of pre-mRNA processing in peptides generation

In eukaryotes, a variety of different mechanisms play an important role in modulation of gene expression mechanisms ranging from transcription, mRNA splicing, mRNA translation to protein turnover. These mechanisms are often performed in a temporal and cell-specific manner depending on the physiological cues. Here, I will focus on the impact of pre-mRNA maturation processes on the synthesis of antigenic peptide precursors. The processing of pre-mRNA is complex and requires multiple factors involved in mRNA capping, splicing and polyadenylation. The pre-mRNA 5' end capping by m7GpppN takes place co-transcriptionally already during the synthesis of nascent transcript by RNA polymerase II [124]. This process plays multiple roles as it protects the 5' end from the exonuclease cleavage and mediates subsequent steps in pre-mRNA maturation by recruiting factors responsible for splicing, polyadenylation and nuclear export. It also provides foundation for cap-dependent translation by the recruitment of the nuclear cap binding complex (CBC) CBP80-CBP20 heterodimers which are replaced by eukaryotic initiation factor 4E (eIF4E) during translation initiation process in the cytoplasm [125]. Constitutive splicing requires a large multicomponent spliceosome composed of five small ribonucleoprotein particles (snRNPs; U_1, U_2, U_3, U_4, U_5 and U_6) as well as several non-snRNP proteins [126]. Its main responsibility is to recognize essential conserved sequences that are mainly encoded within intron/exon junctions both at the 5' and 3' splice sites the branch side and the polypyrimidine tract). After spliceosome recruitment it is engaged in two transesterification reactions that lead to the formation of intron lariat structure, splicing of exons and lariat release. Majority of mRNA splicing is performed co-transcriptionally [127]. At the end of the process, after the sequence of spliceosome rearrangements and dissociation, mRNA is released in the form of messenger ribonucleoprotein (mRNP) whose major component is exon junction complex (EJC). EJC functions as a molecular guide, whose responsibility is to couple mRNA splicing to the subsequent post transcriptional processes. It is recruited during spliceosome assembly and stably bound to the mRNA 24 nucleotides upstream of exon-exon junctions [128]. After

splicing nuclear-cytoplasmic shuttling proteins facilitate the transport of newly formed mRNP to cytoplasm through nuclear pores. Great majority of human transcripts (>90%) are generated via alternative splicing in a tissue or developmental specific manner [129]. Alternative splicing involves different mechanisms leading to full or partial intron or exon skipping or retention. It allows encoding for multiple proteins that vary in their sequence, function and activity in a single pre-mRNA transcript and has a great impact on the generation of proteome diversity. The **β**-globin gene has been used as a model system to study mRNA splicing [130]. Particularly impactful were discoveries related to the mutations within intron 2 as they are responsible for abnormal alterations in splicing and by this distorted translation of final protein product. As a result these mutations are well linked to multiple forms of beta-thalassemia and anemias in mice and humans [131]–[133]. Since any alterations within the splicing of the **β**-globin gene or the synthesis of related full-length protein can be readily identified we have used it as a model template for studies on the generation of antigenic peptides for MHC I pathway [134].

Last but not least, splicing termination is marked by 3' end processing that consists of two tightly correlated reactions: hydrolysis of a phosphodiester bond and polyadenylation. After the completion of the transcription, mature mRNPs complexes are exported to the cytoplasm with the support of a large repertoire of ribonucleoproteins (RBPs) which are essential in this step. The mRNAs with defects in maturation are retained and degraded in the nucleus. Several studies described the link between splicing inhibition and MHC I antigen presentation, including studies on TAAs. Cell treatment with well described splicing inhibitor Isoginkgetin resulted in the increase of antigen presentation from introns [88]. Mice carrying tumors expressing intron derived antigens were responsive to treatment with peptide vaccinations of correlated sequence and showed the importance and relevance of underappreciated non-coding genomic regions in generation of potential anti-cancer therapeutics. More to that, Isoginkgetin derivatives were used in evaluation of potential small molecule anti-cancer treatments as modulators of immune responses and showed promising

results by increasing the presentation of TAAs [135]–[137]. In combination with good predictive algorithms these data give more insight into the potential immune response to tumor specific neoantigens. Unfortunately, many of the vaccination approaches towards potential neoantigens are not specific due to poor understanding of sequences from which the actual immunopeptidome is derived and of their intracellular processing. Here we wanted to approach this problem and study how the hosts' adaptive immunity reacts against intron derived antigenic peptides from within a known genomic context. This would better explain what happens under physiological conditions and raise the awareness about the importance of the peptide prediction specificity.

Potential translation mechanisms of peptides precursors

The canonical translation mechanism involves ribosome complexes moving along mature mRNAs that have been exported to the cytoplasm. More precisely, ribosomal complexes are responsible for recognition of mRNA codons with the help of cognate tRNA, peptidyl transfer and mRNA-tRNA translocation. Eukaryotic mRNAs usually encode single polypeptide chains, however there have been examples of polycistronic mRNAs which encode for multiple polypeptides. mRNA translation starts at the particular initiation sites of 5' end usually with the recognition of AUG-Met codon and stops towards the 3’ end of mRNA at one of three termination codons (UAA, UAG or UGA). This mRNA region is surrounded by 5’ and 3’ ‘untranslated regions’ (UTRs), respectively. mRNA translation can be divided in 3 steps: initiation, elongation and termination. At first small ribosomal subunit binds to specific methionyl tRNA and the mRNA, followed by joining large ribosomal subunit forming a functional 80S ribosome able to proceed with elongation of nascent polypeptide chain. Multiple non-ribosomal proteins are required in each step like eukaryotic initiation factors (eIFs) which recognize both the 5’ and 3’ ends of mRNAs that are capped or polyadenylated, respectively. In next stages eukaryotic elongation factors (eEFs) and release factors (RFs) as well as other proteins are involved. Several active ribosomal complexes can be attached to the target mRNA at up to 200 nt intervals, forming polysomes [138].

The conventional notion regarding protein translation states that transcription is uncoupled from translation and these processes happen in the nuclear and the cytoplasmic compartments, respectively. However, several studies have indicated alternative translation mechanisms including cap-independent, non-AUG translation initiation, nuclear co-transcriptional translation [139]. The topic regarding nuclear translation remains controversial. Nevertheless, an alternative translation mechanism could provide a better explanation into how non-protein coding regions of the genome gives rise to peptide precursors. In favour of a non-canonical translation event for the production of antigenic peptide substrates are the observations that splicing inhibition increases MHC I antigen

presentation and that disruption of eIF4E interaction with the cap structure prevents the generation of full-length proteins but the synthesis of MHC I antigenic peptides [87]. Furthermore, the team of Nilabh Shastri provided experimental data that indicated alternative translation mechanisms initiating from CUG codon and dependent on eIF2A but not eIF4E that provides peptide precursors for the MHC I pathway [140]. Yewdell's team they identified several ribosomal factors that are correlated with the MHC I expression and antigen presentation [141]. The depletion of certain ribosomal protein subunits modulated MHC I antigen presentation. For example the depletion of 40S ribosomal protein S28 (RPS28) led to the increase of overall antigen presentation of cellular peptides. However the depletion of 60S ribosomal protein L6 (RPL6) or RPL28 played opposite roles in modulating the presentation of viral antigens. RPL28 depletion increased the levels of antigens presented from Influenza A virus, and the depletion of RPL6 had an opposite effect [141].

These results led to the formation of the hypothesis that peptide precursors from 'non-coding' regions may be translated during the ribosomal scanning for potential PTCs of pioneer round of mRNA translation [142]. So far it has been commonly accepted that the pioneer round of translation serves as an mRNA surveillance mechanism that directs mRNAs to destruction by NMD if PTCs are detected [142], [143]. This mechanism has been dissociated from the possibility of generation or mediating generation of any polypeptide precursors. However, more studies have indicated the possibility of alternative translation mechanisms that would in fact synthesise short polypeptide precursors from PTC encoding mRNAs targeted for NMD pathway and that those polypeptides would be rather unstable and rapidly degraded [87], [89]. That in turn was in agreement with Thierry Boon's Pepton hypothesis as well as Nilabh Shastri's work on non-AUG dependent translation mechanisms. However, further work is needed in order to fully understand the processes involved in the synthesis of polypeptide precursors originating from non-conventional genomic regions.

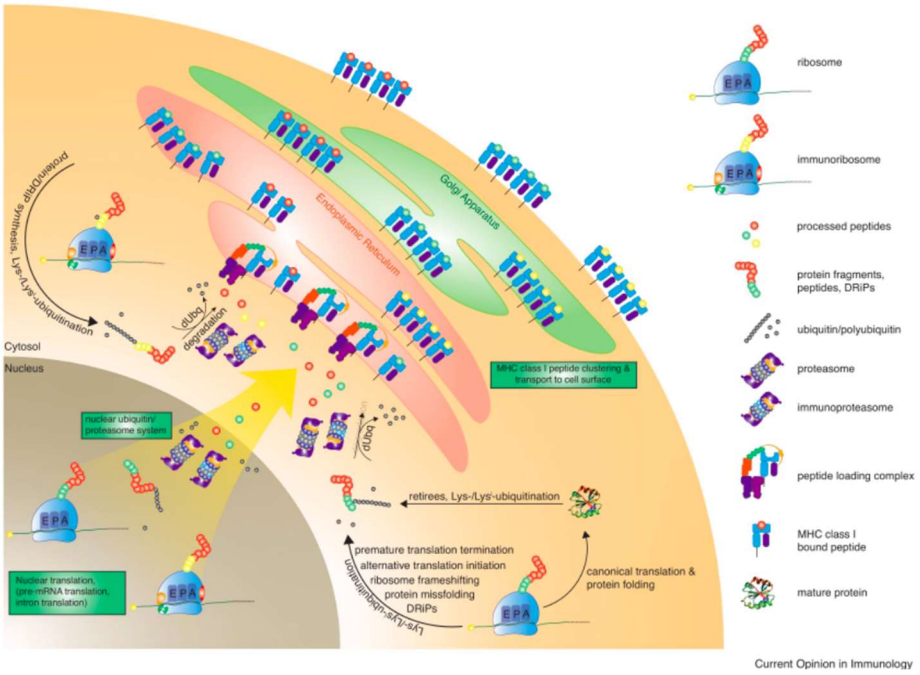


Figure 4. Model proposed by Jonathan Yewdell representing canonical as well as most recently proposed mechanisms involved in the production and processing of antigen peptide precursors for direct MHC I pathway. Wide spectrum of pathways orchestrates generation of endogenous MHC I peptides for immunosurveillance, including alternative mechanisms translating theoretically 'non-coding' genomic regions like introns or pre-spliced mRNAs and identified as Pioneer Translation Products (PTPs). Another pool of peptides precursors have been described as Defective Ribosomal Products (DRiPs) which are linked to translation and processing events occurring in the effect of cellular errors in splicing or ribosome frameshifting or protein misfolding. Polypeptide precursors undergo proteasome degradation that can be ubiquitin dependent or independent, depending on the polypeptide source. Further they are transported to the ER via TAP transporter where the epitopes are loaded onto stabilized MHC I molecules and as a complex transported via Golgi Apparatus to the cell surface for the recognition of CD8+ T cells [90].

Source: Yewdell, Jonathan W, and Jaroslav Hollý. "DRiPs get molecular." *Current opinion in immunology* vol. 64 (2020)

MHC II pathway – a brief description of antigen processing and presentation

Antigen presentation by MHC class II pathway is more cell specific than MHC class I and restricted to pAPCs. pAPCs can internalise extracellular antigens (pathogens, proteins) into their endocytic vesicles via different routes (phagocytosis, micropinocytosis, autophagy) [144]. Endocytosed proteins are degraded by activated proteases within the endolysosome. Cytosolic proteins or damaged organelles are processed for MHC class II presentation via autophagy and their degradation also takes place within the acidified microenvironment of lysosomes. MHC class II molecules are also stored in the ER. However, in this case MHC class II keep their peptide binding groove blocked by class II -associated invariant chain peptide (CLIP) of MHC II-associated invariant chain (Ii, CD74) in order to prevent premature binding of peptides or misfolded proteins[25]. MHC class II:Ii complex is further targeted to low-pH endosomes where CLIP is exchanged for other peptides, process mediated by HLA-DM and HLA-DO. MHC class II molecule with well bound peptide is translocated onto the cell surface and presented to $CD4^+$ T cells.

Currently there have been several pathways described already that show intracellular proteins being a source of APs for the MHC II pathway. For example, it has been also shown that B cells infected with Epstein-Barr virus present antigens from endogenously expressed EBNA1 protein [145]. EBNA1 is delivered by autophagy to the lysosomes where it can enter the MHC II pathway, leading to the activation of $CD4^+$T cells [146]. MHC II presentation of endogenous antigens has been also shown on DCs transfected with modified Ovalbumin [147]. Interestingly, there are examples of autophagy playing a role in tolerance-avoidance mechanisms by $CD4^+$T cells contributing to several autoimmune diseases like encephalitis (EAE) and juvenile idiopathic arthritis (JIA) [148]. It's been proven that inhibition of autophagy pathway in those mouse models or in patients, reduces the severity of spinal cord damage and restores tolerance in T cells, respectively.

MHC I cross-presentation by Dendritic cells.

As mentioned above, certain subpopulations of dendritic cells (e.g. CD24+ DCs, Ly6C+TremL4- DCs, splenic CD8α+CD24+DCs, CD103+ DCs, Langerhans cells) are able cross-present on their MHC class I molecules peptides that come from extracellular sources. Cross-presentation is particularly important in orchestrating immune responses towards viruses and tumors, nevertheless more and more recent findings suggest DCs role in generating immune tolerance as well. Antigens are delivered into DCs via multiple routes, including micropinocytosis, endocytosis or phagocytosis. Intensive scope of research has been devoted to the analysis of molecular mechanisms underlying antigen uptake and cellular internalization by both mice and human DCs. However, there is much to be learnt about the sources and processing of natural peptides within pAPCs. There are two main intracellular routes that have been reported for cross-presentation – vacuolar and cytosolic. Cytosolic pathway is sensitive to proteasome inhibitors which suggests that antigens are internalized to the cytosol and trimmed by proteasome. Interestingly, it has been reported that they can be further processed both in TAP dependent or independent manner, which forces to pose questions on where and how such antigens can be loaded on MHC class I molecules. Vacuolar pathway is sensitive to inhibitors of lysosomal proteolysis (e.g. Cathepsin S) and independent of transporter associated with antigen processing (TAP), which suggests that peptide processing and loading to MHC class I molecules may occur in the endocytic compartment [149]. There have been many speculations around multiple proposed cross-presentation mechanisms, however here we would like to highlight the importance of processing and presentation of peptide antigens derived from underappreciated genomic regions like introns. It has been shown before that processing of full-length proteins by cross-presenting DCs does not contribute a good source of antigens for MHC I pathway [150]. Instead, far better sources, as proven by several studies, are synthetic long peptides (SLPs) that activate functional CD8+ T cell responses to viruses and tumors [151], [152]. SLPs are usually of 20 to 35 amino acids length and up until now have

been tested as potential therapeutic anti-cancer vaccines, including personalised vaccines based on neoantigens derived from melanoma and glioblastoma patients [153]–[155]. Taking into account studies on alternative sources of antigenic peptides for the MHC I pathway Apcher's team took the concept of PTPs to the next level and investigated whether they constitute a good source of antigens for cross-presentation. More of that, they have particularly evaluated the role of tumor-associated PTPs (TA-PTPs) in inducing specific CD8+ T cells responses [156]. They have shown that TA-PTPs contribute to the overall pool of cross-presented antigens by DCs and elaborated on exosomes being the antigen delivery route from tumors to DCs. After the antigen internalisation, TA-PTPs are processed by cytosolic pathway as they require proteasome and TAP for efficient cross-presentation. Those TA-PTPs were also subjected for testing as potential anti-cancer vaccines in mice and proved to efficiently suppress tumor growth.

Self and non-self recognition

Immune tolerance has been incessantly a subject of studies since Ray Owen's observations in 1945 of mixed blood groups in dizygotic cattle twins [1], [157]. This observation made significant impact on generation of future concepts on self- and nonself-recognition in central and peripheral positive and negative selection processes. Central tolerance consists of sequential processes that lead to the deletion of developing, autoreactive T or B lymphocytes in thymus or bone marrow, respectively. Developmental processes of each of those cell types have been thoroughly studied and the most important aspects of lymphocytes' positive and negative selection will be revised in the following chapters in order to highlight the need for further investigation of sources of antigenic peptides involved in self and nonself-recognition.

The development of B lymphocytes starts in the endosteum which is the lining of the inner cavity of the long bones (femur, tibia) and also the place where the earliest stem cells receive signal from transcription factors Ikaros, E2A and EBF. Those factors induce the expression of key proteins involved in gene rearrangement, including components of the V(D)J recombinase (RAG-1 and RAG-2) [158]–[161]. In the presence of another protein Pax5 they also ensure that pro-B cells will initiate the pathway necessary for B-lineage cell development. As the cells successfully finish the rearrangement of the heavy-chain locus, pre-B-cell receptor signalling enforces their allelic exclusion and the rearrangement of light-chain locus as well as cell-surface immunoglobulins. Due to the big variety of generated receptors they undergo examination towards reactivity with self-antigens and the affinity of B cell receptor (BCR) binding to antigens determines B cells fate. Those that bind strongly may undergo programmed cell death or apoptosis and those with a moderate affinity will be subjected to receptor editing or become anergic. Throughout the entire process, developing B-cells remain in contact with reticular-stromal cells in the trabecular spaces and as they mature they move toward the central sinus of the marrow cavity. From this place cells that successfully pass the selection process and do not recognize self-antigens are moved to

peripheral lymphatic organs (mainly spleen) where they undergo a second round of control [162]. However, in the circumstances when developing B cells turn to be autoreactive within bone marrow they will undergo processes resulting in clonal deletion through programmed cell death, anergy induction or receptor editing [162].

The development of T cells also starts from multipotent hematopoietic stem cells and despite some similarities in the developmental stages, their progenitors unlike B cells migrate via blood to the thymus where within the thymic stroma they find an unique microenvironment for further development and selection [163]. Thymus is situated in the upper anterior thorax above the heart and behind the sternum. It consists of numerous lobules divided into an outer cortex mostly consisting of immature thymocytes and scattered macrophages, and inner medulla containing more mature thymocytes, along with professional antigen presenting cells (dendritic cells, macrophages and some B cells). In order to begin their development, T-cell precursors require a signal from thymic epithelial cells that is transduced through Notch1 receptor [164]. TCF-1, GATA3 are key proteins involved at that stage and they initiate the expression of several genes encoding components of the CD3 complex, as well as RAG-1, required for T-cell receptor rearrangement. In RAG-1 and RAG-2 mutant mice, T-cell differentiation is blocked at the CD4-CD8- double negative (DN) stage in the thymus and B-cell differentiation is blocked at the B220+CD43+ pro-B cell stage in the bone marrow. However, introduction of rearranged and functionally assembled TCR or Ig transgenes restores T- and B-cells differentiation and maturation in mice with RAG-deficient background [158], [162]. Another important factor is Bcl11b that ensures the precursor's commitment to T-cell lineage [163], [165]. The development process of thymocytes seems to be more stringent than in B cells taking into account that only ~2% of those cells will pass the positive and negative selection after gene rearrangement and formation of T-cell receptors. However, more recent studies have shown that even around 4% of peripheral CD8+ T cells are self-specific [166].

The role of the peptide: MHC I and II complexes in central and peripheral immune tolerance

As soon as α:β receptor is expressed on thymocytes their structure undergo stringent examination during interaction with peptide:MHC ligands expressed by cTECs and mTECs [167], [168]. In the thymus, developing CD4+CD8+ double positive (DP) thymocytes undergo positive and negative selection once exposed to self-peptide:MHC complexes on cTECs and mTECs as well as some antigen presenting cells (e.g. macrophages and dendritic cells) [165]. During this process the interaction between TCR and peptide-MHC complex on cTECs mediates also DPs commitment to particular CD4 or CD8 lineage. Thymocytes that first are able to bind to the complex are positively selected as they are specific enough to recognize a peptide on a self MHC receptor. However, those that bind too strongly are induced to die [169]. It has been found that Foxn1 expression in cTECs orchestrates the expression of CD83 and Psmb11 which are responsible for MHC II stabilisation and thymoproteasome expression, respectively. Hence, cTECs are directly correlated with positive selection of both CD4 single positive (SP) and CD8 SP thymocytes [170], [171]. Positive selection triggers the upregulation of certain C chemokine receptors (CCR4 and CCR7) which in turn play a key role in directing developing thymocytes towards medulla, which is a home for many pAPCs, e.g. DCs and where further negative selection begins [172]–[175]. This negative aspect of selection faces stringent recognition of self-peptide:MHC complex and when thymocytes αβ receptors have high affinity to the complex they will undergo clonal deletion [176]. In particular circumstances certain developing thymocytes can be directed to a regulatory lineage and become Tregs that play an important role in maintaining peripheral tolerance [177]–[179].

There is still much to be discovered about the way tissue-specific self-peptides are expressed and presented by MHC I molecules in the thymus and in particular by mTECs which express up to 100-fold more MHC I molecules on their surface than extrathymic epithelial cells (ECs) from the skin, colon and lung. Also the distortion of the level of MHC I

molecules in TECs can lead to severe forms of several autoimmune diseases, stressing the importance of MHC I antigen presentation pathways in T cells selection in the thymus. The MHC I expression is regulated at two levels: the transcription of MHC I genes and the generation of MHC-associated peptides that stabilize peptide-MHC complexes. It has been shown that the expression of MHC I on TECs was driven mainly through constitutive secretion of type III interferon (IFN) by mTECs and that genes like Aire, *Ifnlr1*, *Stat1*, or *Nlrc5* play important roles in this process. AIRE (autoimmune regulator) is expressed in mTECs and promotes ectopic expression of peripheral tissue-restricted antigens (TRAs). Mutations of deficiencies in AIRE result in multi-organ autoimmune disorders and impair mechanisms of clonal deletion in the thymus. Together with other factors it plays a significant role in transcription regulation as it seems to lengthen transcripts that would otherwise terminate earlier or their expression would not begin. AIRE interacts with a large number of proteins involved in pre-mRNA processing, transcription and nuclear export [180]. AIRE makes an impact not only in maintaining central tolerance but it also acts peripherally via blood and lymph node-derived DCs, macrophages and epithelial cells [181], [182]. Interestingly, it's been shown in mice that AIRE's expression in the neonatal period can prevent autoimmune symptoms in otherwise AIRE knockout mice [183]. Other factors involved in promiscuous gene expression (PGE) in thymus have been identified, e.g. Fezf2. Although its role in antigen presentation has not been precise it is clear that Fezf2 deletion causes severe autoimmune syndrome in mice [184]. Another aspect in shaping immune tolerance by mTECs relates to the generation of T regulatory cells (Tregs) on both CD8+ and CD4+ lineages. It has been shown by deep sequencing analysis of TCRα chains that the Treg TCRs were underrepresented in AIRE-deficient mice. In fact, they were amongst the pool of conventional T cells [185]. Although the AIRE plays a very important role in both clonal deletion in Treg induction for both CD4+ and CD8+ thymocytes, it has been shown that clonal deletion of T cells specific towards self-antigens and TRAs is not complete [186], [187]. More to that in certain tissues like in lungs or intestines the tolerance to self antigens required functional Tregs [188]. Interestingly, the induction of particular tolerance

mechanisms by thymic cells may not be solely due to particular MHC ligands presented by thymic APCs but may depend on the relative peptide expression levels within the thymus.

Hence, it is clear that AIRE has multiple roles in directing T cells fate, however, the exact mechanisms of which antigens are to be selected for presentation are yet to be discovered. CD4+ or CD8+ single positive cells that successfully pass positive and negative selection are ready to undergo final maturation process and leave as naïve populations into the periphery where they can be still eliminated by mechanisms of peripheral tolerance in case of self-recognition with simultaneous lack of inflammation or costimulatory signals through the cell-surface proteins CD28 [189].

As I mentioned in the previous sections, different types of DCs have great impact on the generation of immune tolerance. In fact, they are involved not only in the thymus but also efficiently keep in guard adaptive immune responses in the periphery. In the thymus, three major DCs types (resident DCs, migratory DCs and plasmacytoid DCs, pDCs) are responsible for the presentation of self-antigens and cross-presentation of blood-derived self-antigens from the periphery [190], [191]. Each of the DC types differs in a role they play in tolerance induction. For example resident DCs consist of more than 50% of the entire DCs pool in the thymus and are localised in the medulla [192], [193]. Hence, they significantly contribute to the presentation of self-antigens as well as cross-presentation of blood-derived antigens and tissue-specific antigens from mTECs. On the other side migratory DCs and pDCs are located mainly in the corticomedullary perivascular space which allows them to efficiently present blood-derived antigens and contribute to immune tolerance mechanisms by the induction of thymocytes negative selection. In particular that relates to the thymocytes with high affinity for self-antigens. As it's been already mentioned the DCs play an important role also in orchestrating tolerance mechanisms in the periphery and by this maintaining immune homeostasis throughout the life. Particularly important in these mechanisms are Tregs of thymic origin and tolerogenic DCs (TolDCs) in the periphery. TolDCs consist of naïve DCs (iDCs) and semi-mature DCs incapable to mature under the signal inducing

stimuli [194]. These types of DCs express low levels of costimulatory molecules as well as MHC II complexes, and therefore they do not induce adaptive immune responses. However, they have a great capacity to constantly sample foreign antigens and apoptotic cells by phagocytosis and endocytosis. This feature of iDCs in combination with their immature phenotype is associated with tolerance induction through T cell deletion, anergy or polarization toward a regulatory phenotype. Interestingly, several studies have also shown that naïve T cells that were stimulated repetitively with iDCs can convert their phenotype to Tregs [195].

AIMS OF STUDIES

Objectives of these studies were divided into two parts based on experimental models used for evaluation of intron derived antigenic peptides – *in vitro* and *in vivo*.

In vitro studies – evaluation of the β-globin-SL8 construct which encodes for SIINFEKL (SL8) peptide in the intron 2 of the β-globin gene.

- Evaluation of the expression of intron-derived antigenic peptide in the context of the β-globin gene.
- Investigation if the intronic insertion had an effect on the expression or splicing of full-length β-globin in comparison to previous studies from our group.
- Assessment of antigen presentation of SIINFEKL from β-globin-SL8 construct.
- Evaluation of translational status of intronic regions from β-globin-SL8 construct.
- Assessment of the effect of splicing inhibition on the expression of polypeptide precursors.
- Identification of reading frame from which SIINFEKL sequence can be translated into polypeptide precursor and investigation of its potential translation initiation site.

In vivo studies - establishment of novel mouse model encoding SIINFEKL sequence in the intron 2 of β-globin gene and investigation of the physiological role of intron-derived antigenic peptides for the MHC I pathway.

- Establishment of homozygous strain of mice carrying SIINFEKL in the intron 2 of the β-globin gene (HBB mice).
- Evaluation of knock-in specific pre-mRNA levels in different organs and tissues.
- Investigation of the effect of intronic insertion on β-globin protein expression and erythropoiesis.
- Evaluation of intron-derived SIINFEKL presentation
- Evaluation of the effect of adoptive T cell transfers on antigen expressing tissues.

- Assessment of endogenous SIINFEKL specific CD8+T cell levels and responses to immunizations with peptide-loaded DCs.
- Assessment of the effect of intron-derived MHC I epitope on the development of tumors expressing self antigens from the known genomic context.
- Establishment of mice strains carrying both β-globin-SL8 knock-in as well as SL8-specific transgenic CD8+T cells as future tools for the evaluation of molecular mechanisms behind potential immune tolerance or autoimmunity mechanisms induced by intron derived MHC I antigen.

MATERIALS AND METHODS

I. Generation of constructs for studying intron derived SIINFEKL

A. pcDNA3

β-globin-SL8 in pcDNA3 was a gift from Dr. Rodrigo Martins and used as a template in further cloning. All plasmids were generated using standard procedures. Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from New England Biolabs. Purified synthetic oligonucleotides were obtained from ThermoFisher and Eurofins Genomics. Routine plasmid maintenance was carried out in DH5α bacteria strain.

B. Site-Directed Mutagenesis

All substitutions to the β-globin-SL8 in pcDNA3 were performed by site-directed mutagenesis using the PfuTurbo DNA polymerase (Agilent) and the oligonucleotide pairs listed in the table number 1.

Conctruct name	Position upstream of SIINFEKL	Mutation		SDM primers sequence (5'-3')
1	+12	TAA	sense	cctgagaacttcagggtgagtctgTAAggcacctccagtataatcaac
			antisense	gttgattatactggaggtgccTTAcagactcaccctgaagttctcagg
2	+15	TAA	sense	cctgagaacttcagggtgagtTAAatgggcacctccagtataatcaac
			antisense	gttgattatactggaggtgcccatTTAactcaccctgaagttctcagg
3	+21	TAA	sense	cctgagaacttcaggTAAagtctgatgggcacctccagtataatcaac
			antisense	gttgattatactggaggtgcccatcagactTTAcctgaagttctcagg
4	+42	TAA	sense	gctccactgtgacaagctgcatTAAgatcctgagaacttc
			antisense	gaagttctcaggatcTTAatgcagcttgtcacagtggagc
5	+213	TAA	sense	ccttggacccagcggTAAtttgatagctttggagacc
			antisense	ggtctccaaagctatcaaaTTAccgctgggtccaagg
6	+228	TAA	sense	ccctttttaggctgctggttgtctacTAAtggacccagcggtactttgatagc
			antisense	gctatcaaagtaccgctgggtccaTTAgtagacaaccagcagcctaaaaaggg
7	+255	TAA	sense	gtgtcccctgtctatgtttAActtttttaggctgctggttgtctaccccttgg
			antisense	ccaagggtagacaaccagcagcctaaaaagTTaaacatagacaggggacac
A	+12 and +15	CUG to CUC; AUG to AUC	sense	cctgagaacttcagggtgagtctCatCggcacctccagtataatcaac
			antisense	gttgattatactggaggtgccGatGagactcaccctgaagttctcagg
B	+48	CUG to CUC	sense	gctccactgtgacaagctCcatgtggatcctgagaacttc
			antisense	gaagttctcaggatccacatgGagcttgtcacagtggagc
C	+111	CUG to CUC	sense	ctgcctttaacgatggcctCaatcacttggacagcctcaaggg
			antisense	cccttgaggctgtccaagtgattGaggccatcgttaaaggcag
D	+171	AUG to AUC	sense	cctatcctctgcctctgctatcatCggtaatgccaaagtgaagg
			antisense	ccttcactttggcattaccGatgatagcagaggcagaggatagg
E	+240; +243; +255	CUG to CUC; CUG to CUC; TAA	sense	cccctgtctatgtttAActtttaggctCctCgttgtctaccttggacccagcgg
			antisense	ccgctgggtccaagggtagacaacGagGagcctaaaaagTTaaacatagacagggg

Table 1. List of mutations introduced to β-globin-SL8 construct by site-directed mutagenesis (SDM). Table indicates constructs names relevant to Figure 13 as well as position of mutated codon upstream SIINFEKL sequence (nts), type of substitutions made and sequences of primers used for PCR reaction.

II. Generation of stable cell lines for tumor tolerance assay

A. pLVX-IRES-ZsGreen1

pcDNA3 with β-globin-SL8 and pLVX-IRES-ZsGreen1 plasmids were digested with EcoRI HF and NotI HF (Units/reaction) for 3h, 37°C and subjected gel electrophoresis in 1% agarose gel (50min at 120V). DNA of correct size was extracted with the use of Qiagen gel extraction kit according to manufacturer's protocol and quantified by nanodrop. pLVX-IRES-ZsGreen1 linearized vector was dephosphorylated with Antarctic phosphatase (5 units/1 pmol of DNA) and incubated 1h, 37°C. Enzyme was inactivated at 65°C. pLVX-IRES-

ZsGreen1 vector was subjected to agarose gel electrophoresis, extraction and quantification with the same conditions as above. β-globin-SL8 and pLVX-IRES-ZsGreen1 were ligated in 3:1 molar ratio. Vector and insert volumes were calculated in the NEBiocalculator and samples were incubated for 2h at RT and further O/N at 4°C. Competent DH5α cells were transformed according to the standard protocol and grew O/N on LB-agar ampicillin plates.

B. **psPAX2** - lentiviral packaging plasmid

C. **pCMV-VSV-G** - envelope protein for producing lentiviral particles. To be used in conjunction with packaging plasmid psPAX2.

D. **Generation of lentiviral particles**

HEK293T cells were seeded in corning tissue culture dish 100x20 mm style (3.5 mln cells/dish) and cultured in standard medium and conditions for 24h or untill they reached 80-90% confluency. Cells were transfected with psPAX2 (6 µg), pCMV-VSV-G (2 µg) and plvx-IRES-ZsGreen with β-globin-SL8 (2 µg) or empty plvx-IRES-ZsGreen (2 µg) to create particles carrying β-globin-SL8 or empty particles, respectively. Plasmids of interest were mixed with 1ml optiMEM glutamax (Life Technology) and 20 µl X-treme transfection reagent (Roche). Samples mixed and incubated at RT for 20 min. Added 9 ml of optiMEM glutaMAX to each, a total 10 ml of transfection solution was added to wash with DPBS(1x) HEK293T cells and incubated for 4h, 37°C 5%CO_2. Then the transfecting medium was exchanged to 13 ml of standard HEK293T medium. After 48h of incubation, supernatants were filtered through a sterile syringe filter w/0.45 µm cellulose acetate membrane (VWR international). Viral titer was assessed with the use of Lenti-X GoStick App.

E. **Lentiviral transduction and FACS sorting**

MCA205 cells were seeded on 6-well plates (1x10^5/well) the day before transduction. After approximately 24h cells were washed with PBS and the transduction mix consisting of 1,5 ml of fresh medium, 750 µl of viral particles with constructs β-globin-SL8 or EV; polybrene

1:1000 was added to each well. Cells were centrifuged 70 min at 20°C, 4000rpm and after 24h incubation at 37°C 5%CO_2 and the medium was changed to the standard MCA culture medium. Cells were prepared for FACS sorting on the same day. Each well was washed with PBS and trypsinized for 2 min. at RT. Cells of the same constructs were pooled together, centrifuged 5 min 1300rpm at RT and resuspended in PBS. Transduction efficiency and population purity was assessed by FACS with GFP marker with the use of ARIA II flow cytometer (Figure 27).

III. Cell culture and transfection

H1299 - is a human non-small cell lung carcinoma cell line derived from the lymph node and has a homozygous partial deletion of the TP53 gene. Cells were used as a control in *in vivo* antigen presentation and as antigen presenting cells in *in vitro* antigen presentation experiments and proximity ligation assay (PLA). Cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS, 2% L-glutamine and 1% Penicillin/Streptomycin at 37°C 5% CO_2.

MCA205 mouse sarcoma cell line was cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino-acids and 1% penicillin/streptomycin at 37°C 5%CO_2. MCA205 cells were used to generate cell lines stably expressing β-globin-SL8 or empty vector (EV).

The 293T human embryonic kidney (HEK293T) cell line was used to generate lentiviral particles carrying β-globin-SL8 construct or EV and in ribosome profiling experiments. Cells were cultured in DMEM (1x, Life Technologies) supplemented with 10% FBS, 1.4% L-glutamin, 1.4% Penicillin/Streptomycin and 2% HEPES at 37°C under 5%CO_2.

Transfection conditions: All cell transfections were performed with genejuice, following manufacturer's protocol.

IV. *In vitro* antigen presentation

Naive $OVA_{257–264}$ specific $CD8^+$ T cells were isolated by negative selection from peripheral and mesenteric lymph-nodes of 12 weeks old female OT-1 mice using the $CD8^+$ T cell isolation kit (Miltenyi Biotec, Germany). Afterwards, $CD8^+$ T cells were stained with CellTrace™ Violet (Thermo Fisher Scientific, USA) according to the manufacturer's protocol and mixed with H1299 cells co-transfected with mouse Kb expression vector and the indicated constructs. For all the assays, $1.25x10^5$ H1299 cells were harvested 24 h after transfection and co-incubated with 5×10^5 $CD8^+$ T cells at 37°C in humidified air/CO_2 atmosphere in RPMI medium containing 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM HEPES and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). For IL-2 release analysis, supernatants were collected after 24h of co-incubation and IL-2 levels were measured employing the IL-2 ELISA MAX™ Standard kit (Biolegend, USA) according to manufacturer's instructions.

V. Ribosome fractionation

Five–fifty percent wt/vol linear sucrose gradients were freshly casted on SW41 ultracentrifuge tubes (Beckmann) using the Gradient master (BioComp instruments) following the manufacturer’s instructions. HEK293T cells were transfected and then treated or not on the same day with Isoginkgetin at [10 µM]. Twenty-two hours post treatment, cells (with 80% confluency) were treated with cycloheximide 100 µg/ml for 5 min at 37 °C and then washed twice with 1× PBS (Dulbecco modified PBS, GIBCO) containing cycloheximide 100 µg/ml. Cells were then scrapped, lysed with polysome lysis buffer (100mM KCL, 50mM HEPES KOH, 5mM MgCl2, 0.1% NP-40, 1 mM DTT, cycloheximide 100 µg/ml, pH 7.4) and spin at 2348xg for 10 minutes at 4°C. Lysates were then loaded on a sucrose gradient and centrifuged at 222228×g for 2 h at 4 °C in a SW41 rotor. Samples were fractionated using Foxy R1 fraction collector (Teledyne ISCO) at 0.5 min intervals . RNA purifications from fractions were performed using ethanol precipitation combined with RNeasy Mini Kit

(Qiagen). RT-qPCR were performed as described below using primers listed in Table 7. The relative distribution of target mRNA was calculated using fraction 1 as reference according to Panda et al. (2017) [196]. The analysis was performed in collaboration with Maria Tovar Fernandez from our group.

VI. Adapted ribosomal profile

Cells were lysed under conditions to maintain the ribosome in position using Harringtonin treatment followed by cycloheximide [197]. RNAse and DNAse were added to generate ribosome footprint and the ribosomes were isolated following ultracentrifugation at 36000 rpm for 22 hours. The ribosome-protected RNA fragments were isolated as described and stem loop (SL) primers were fused to the 3'. Stem loop primers were designed according to the protocol [198]. RT-qPCR was performed using primers corresponding to the 5' of the predicted 27 nt. initiation sequence and the SL with the use of the thermocycler StepOne Real-Time PCR system (Applied Biosystems). The list of primers described in Tables 2 and 3. Analysis was performed in collaboration with Dr. Chrysoula Daskalogianni from our group.

Stem loop primers	Sequence (5'-3')
1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTAGAC
2	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGTA
3	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAAGG
4	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTCCA

Table 2. Sequences of reverse transcription stemloop (SL) primers used for analysis of translation initiation of SIINFEKL precursor. Out of 4 SL primers tested, number 3 worked (refers to Fig.17).

Adapted ribosomal profile	Sequence (5'-3')
Fw1	CTTTTTAGGCTGCTGGTT
Fw2	CACGCACTTTTTAGGCTG
Rv	CCAGTGCAGGGTCCGAGGTA

Table 3. Sequences of primers used for qPCR analysis of the presence of RNA footprint released from initiating ribosomes (refers to Fig.17). Out of 2 forward (Fw) primers tested Fw1 gave positive qPCR signal in pair with the reverse primer corresponding to the common sequence of SL RT primers 1-4.

VII. Proximity ligation assay

H1299 cells were grown on coverslips and transfected with indicated constructs for 24 h and treated with 30 uM Isoginkgetin (Merck Millipore) for 22 hours. The cells were fixed in 4% paraformaldehyde for 20 min before being permeabilized in PBS and 3% BSA containing 0,1% saponin. Custom made primary antibodies- Rabbit anti-SIIN, Goat anti-FEKL (Eurogentec) were incubated in the same buffer overnight. After the cells were washed, PLA probes were added, followed by hybridization, ligation and amplification according to the manufacturer's protocol (Duolink, Thermo Fisher). Then, immunofluorescence was performed using primary mouse anti-β globin antibody and secondary anti-mouse Alexa488. Coverslips were mounted on slides using a SlowFade diamond antifade mounting medium (Thermofisher) with Hoescht. Slides were analysed by fluorescence microscopy. The number of PLA dots were quantified in H1299 cells with or without β-globin-SL8 immunofluorescence signal by a custom-made automated script in FIJI. Proximity ligation assay was performed in collaboration with Maria Tovar Fernandez from our group.

VIII. Mice

OT-1 CD45.2 mice were used as donors of CD8+ T cells with TCR recognizing SIINFEKL-MHC(Kb) complex in *in vivo* antigen presentation assays. Mice are deficient of RAG and were provided by Institut Curie and bred at Platforme Saint Louis animal facility.

C57BL/6 HBB CD45.2 mice were generated in Ciphe's laboratories, Marseille by introduction of SIINFEKL DNA sequence to intron 2 of β-globin gene in chromosome 7 via homologous recombination. Heterozygous ES cells were selected with neomycin and injected to blastocysts used in mice *in vitro* fertilization. Chimeric animals were bred into WT C57BL/6 CD45.2 and heterozygotes were backcrossed 7-9 rounds. The strain was later crossed with C57BL/6-Ly5.1 mice in order to obtain animals with CD45.1 alloantigen expressed on CD8+ T cells. Mice were backcrossed and bred at St Louis animal facility.

All animal experiments were carried out in compliance with French and European laws and regulations.

IX. Blood collection and mice phenotyping

Blood samples of 250 ul were collected from the submandibular vein. Samples were lysed with Red Blood Cell Lysing Buffer Hybri-Max and stained for FACS analysis with Fixable Viability eFluor 780 (APC-Cy7); anti-CD3e (APC) and anti-CD19 (BV650). Cells were gated for live APC-Cy7 negative events (100 000 events collected) and data were analysed using FlowJo software version 8 (Tree Star).

X. Blood analysis

Blood samples were collected from submandibular vein and complete blood count was assessed with the MS9-5V analyser (Melet Schloesing Laboratories).

XI. Genomic DNA isolation and analysis

Genomic DNA from HBB mice was isolated from tail biopsies and PCR performed with the use of Phire Tissue Direct PCR Master Mix according to the manufacturer's protocol. Primers used are specified in table number 5.

Genomic DNA from HBBx OT-1 mice was isolated from tail biopsies with the use of PureLink Genomic DNA Mini Kit according to the manufacturer's protocol. Duplex qPCR analysis was performed with the use of Kapa Probe Fast qPCR kit Rox Low according to the

manufacturer's protocol. Sequences of primers and probes are listed in the tables number 6 and 7.

Primers PCR	Sequence (5'-3')
HBB Forward	GGCAGAGCATATAAGGTGAG
HBB Reverse	ACTGGAAATTAAAAGCTTAC

Table 4. Sequences of primers used for detection of HBB gene and discrimination between heterozygous and homozygous mice by PCR and subsequent agarose gel electrophoresis.

Primers duplex qPCR	Sequence (5'-3')
OT1 Tcra Tg Forward	CAC ATC ACA GAC TCT CAG CC
OT1 Tcra Tg Reverse	GAG CCC CAG ATC AAC TGA TAG T
OT1 Tcrb Tg Forward	TCA CCA GTC ATT TCT GCC TTT G
OT1 Tcrb Tg Reverse	TTA CCT AAA ACC GTG AGC CTG
Control Forward	CAC GTG GGC TCC AGC ATT
Control Reverse	TCA CCA GTC ATT TCT GCC TTT G

Table 5. Sequences of primers used for detection of OT-1 transgenic T cell receptor alpha (Tcra) or beta (Tcrb) chains along with primers detecting control gene by duplex qPCR and subsequent copy number variation analysis (CNV).

Probes duplex qPCR	Sequence (5'-3')
OT1 Tcra Tg	[6-FAM] CTC AGC TAC CTA CTT CTG TGC AGC AA [BHQ1a-Q]
OT1 Tcrb Tg	[6-FAM] ACT GTT CAT AAT TGG CCC GAG AGC TG [BHQ1a-Q]
Control	[Cy5] CCA ATG GTC GGG CAC TGC TCA A [BHQ2a-Q]

Table 6. Sequences of probes used in duplex qPCR for detection of OT-1 Tcra or Tcrb chains along with control gene.

XII. Gel electrophoresis

PCR products from HBB mice genotyping as well as selected qPCR products from **β**-globin-SL8 analysis were subjected agarose gel electrophoresis. For this 2% agarose gel has been utilized and gDNA or cDNA distribution has been performed under conditions of 120V; 50min. Gels were exposed to UV light and images were taken with the use of MY ECL Imager (Thermo Scientific).

XIII. RNA analysis

A. Total RNA Isolation

a) Cell lines

Cell samples collected after transient transfections were pelleted by centrifugation and frozen at -80oC. Total RNA was isolated with the use of Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol. 600 µl RLT plus 1% βME was added to each sample and followed by cell lysis/ homogenisation by pipetting and passing through the needle 21 g connected to the syringe of 1 ml capacity. Eluted RNA was gDNA free as the kit contained a gDNA elimination step. RNA was quantified by nanodrop and 500 ng of each RNA sample was reverse transcribed in order to generate cDNA.

b) Thymus, spleen, lymph nodes, liver

HBB and WT mice of 10 weeks old were sacrificed and thymus, spleen, lymph nodes, liver were collected, cut into 2-3 even parts and snapped frozen at liquid nitrogen. RNA extractions were performed according to the protocol provided by Qiagen RNeasy Mini Kit. 600 µl RLT plus 1% βME was added to each sample and followed by cell lysis with a bead homogenizer. Eluted RNA (50 µl) was treated with 50 µl DNase I mix (10 µl RDD; 2.5 µl DNase I; 38 µl H_2O) for 30 min at RT followed by the purification with RNA clean up kit RNeasy QIAGEN. Total RNA was quantified by nanodrop and 2000 ng of each RNA sample was reverse transcribed in order to generate cDNA.

c) Blood and Bone marrow

Blood samples of 250 ul were collected from the submandibular vein of HBB and WT mice that were 6 months old. After collection, mice were sacrificed by cervical dislocation and bones (femurs and tibias) were collected and submerged in the medium. In aseptic conditions all the bones were cut open at the edges and bone marrow flashed out with the use of syringe-needle filled with medium. All the BM was collected by pipetting and passed

through the strainer 0.70 μM to the falcon tube. Cells were divided in 2 and RNA from one part was extracted with the use of TRIZOL reagent following the manufacturer's protocol. RNA from blood samples were extracted with Trizol LS according to the manufacturer's protocol. Total RNA was quantified by nanodrop and 2000 ng of each RNA sample was reverse transcribed in order to generate cDNA.

d) OT-1 CD8+ T cells

RNA from stimulated or not OT-1 CD8+T cells was isolated with the use of Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol. 600 μl RLT plus 1% βME was added to each sample and followed by cell lysis/ homogenisation by pipetting and passing through the needle 21 g connected to the syringe of 1 ml capacity. Eluted RNA was gDNA free as the kit contained a gDNA elimination step. RNA was quantified by nanodrop and 500 ng of each RNA sample was reverse transcribed in order to generate cDNA.

B. Reverse Transcription

RNA samples were made up to 20 μl volume with nucleic acid-free water following with addition of master MIX (20 μl) consisting of buffer 5x, dTT, dNTP, Random Hexamers, RNaseOUT, M-MLV enzyme and DNase, RNase free water.

For the analysis of activation status in OT-1 cells, reverse transcription reaction was performed with oligoDTs primers.

C. qPCR

All single-plex qPCR reactions were performed with the use of Perfecta SYBR Green Fast mix according to the manufacturer's instructions. cDNA obtained after the RT-PCR was mixed with Perfecta SYBR Green Fast mix, DNase and RNase treated water and both sense and antisense target specific primers. The list of all primers used in these studies is enclosed in the table number 7. Actin (ACTB) was chosen as a housekeeping gene in all reactions. All single-plex qPCRs were performed with the use of the thermocycler StepOne Real-Time PCR system (Applied Biosystems).

Primers qPCR	Sequence (5'-3')
Spliced Forward	ccctgggcagGCTGCTGG
Spliced Reverse	gcccaggagCCTGAAGTTCTC
I2Neo Forward	CTCGAGCTCGCGAAAGCTAGC
I2Neo Reverse	CCTCGAGATCTAGATATCGATGAATTG
I2SL8 Forward	GGGCACCTCCAGTATAATCAACT
I2SL8 Reverse	CTTTCGCGAGCTCGAGTTTC
Ex1 Forward	GGTGAACGCCGATGAAGTTG
Ex2 Reverse	CAGCTTGTCACAGTGGAGCTC
Ex2 Forward	CCTCTGCCTCTGCTATCATGGG
Actin Forward	TCACCCACACTGTGCCCATCTACGA
Actin Reverse	TGAGGTAGTCAGTCAGGTCCCG
ki67 Forward	AATCCAACTCAAGTAAACGGGG
ki67 Reverse	TTGGCTTGCTTCCATCCTCA
OT1 Tcrb Forward	ACGTGTATTCCCATCTCTGG
OT1 Tcrb Reverse	CTGTTCATAATTGGCCCGA
IL-2 Forward	TCCTCACAGTGACCTCAAGTCC
IL-2 Reverse	TGACAGAAGGCTATCCATCTCC
FasL Forward	TCAATGAAACTGGGCTGTACTTT
FasL Reverse	AGAGTTCCTCATGTAGACCTTGT
Perforin Forward	TCATCATCCCAGCCGTAGT
Perforin Reverse	ATTCATGCCAGTGTGAGTGC
GranzymeB Forward	ATCAAGGATCAGCAGCCTGA
GranzymeB Reverse	TGATGTCATTGGAGAATGTCT

Table 7. Sequences of primers used in qPCR reactions.

XIV. Western blot

Immunoblotting on mouse primary cell lysates was performed according to standard protocol on PVDF membranes with the use of primary rabbit polyclonal antibodies to Hemoglobin subunit beta/ba1 and secondary goat anti-rabbit HRP antibodies. Membranes were developed with the use of Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and images were taken with the use of MY ECL Imager (Thermo Scientific).

XV. *In vivo* antigen presentation

OT-1 CD45.2 mice were sacrificed by cervical dislocation. Lymphoid organs were homogenized on a 70 μM cell strainer with PBS FBS. $CD8^+$ T cells were purified with Mouse $CD8^+$ T cell Isolation Kit (MACS, Miltenyi Biotec) according to the manufacturer's instructions. Cell viability and quantification were assessed with Trypan Blue and Countess (Invitrogen).

$CD8^+$ T cells were labelled with cell-trace violet (CellTrace™ Cell Proliferation Kits, Invitrogen) according to the manufacturer's protocol regarding alternate methods to label cells in suspension (5 μM for 10 minutes at 37ºC in PBS). Cells were injected intravenously (i.v.) to C57BL/6-Ly5.1 HBB and WT mice (2x10^6 T cells/animal).

For positive control, 3 hours after i.v. injection, C57BL/6-Ly5.1 WT mice were injected intraperitoneally with 5x10^6 H1299 cells transiently transfected with Kb and OVA.

After 3 days animals were killed by cervical dislocation. $CD8^+$ T cells were isolated from lymphatic organs as above following with FACS analysis on a CANTO II flow cytometer (BD Biosciences, USA) towards markers: CD45.2 (PE-Cy7, BD Pharmigen), Fixable Viability Dye eFluor 780 (Affymetrix eBioscience; APC-Cy7), Cell Trace Violet (Life technologies, DAPI). Data was acquired using DIVA software and analyzed using FlowJo software version 8 (Tree Star). The percentage of proliferating T cells was considered for statistical analysis [199].

XVI. T cell adoptive transfer and histopathology analysis

C57BL/6 WT and HOM HBB mice on the Ly5.1 background were injected i.v. with 2x10^6 naive OT-1 CD8+ T cells each, followed by 3 subsequent injections of *ex vivo* activated OT-1 CD8+ T cells, as stated on the diagram (Fig. 25). On day 45 animals were killed by cervical dislocation. Blood and spleen were collected for analysis. Immediately after excision, spleens were fixed in PFA 4% for 24h. Next, the tissues were dehydrated in ETOH 70% and embedded into IHC-grade paraffin. Splenic microdissections and Hematoxylin and Eosin staining were performed at Institut Cochin, France. Image analysis was performed with the use of CaseViewer software by Dr. Jacek Kowalski (ICCVS).

XVII. Tumor tolerance assay

WT or HBB mice were injected subcutaneously with 5x10^4 MCA205 mouse sarcoma cells stably expressing β-globin-SL8 or cells stably expressing empty vector (EV) were used as control. Tumor growth was measured over time at days 9, 17 and 21 or until an ethical point

was reached. Mice were sacrificed by cervical dislocation, tumors extracted and measured post-mortem.

XVIII. BMDCs generation and Immunisation assay

Dendritic cells were differentiated from bone marrow collected from C57BL/6N WT bones (femurs and tibia) as described above. Cells were suspended in 20 ml of medium (IMDM supplemented with 12ml J558 supernatant as a source of GM-CSF, 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 50μM βME) and transferred to a 20mm Petri dish with low adherence. On day 4 cells were passed to new dishes in confluence 10x10^7 in 20 ml of complete medium per dish. BMDCs were harvested in the same way and used for experiments on day 8.

BMDCs were pulsed with SIINFEKL peptide in concentration 0.5 μg per 10^6 of cells in 1 ml along with LPS (1μg/10^6/1ml) for 2h at 37ºC. Control cells were pulsed with LPS only in the same concentration. Cells were suspended in PBS prior i.v. injections to WT and HBB mice (1x10^6 cells/100ul) on days 0 and 7. On day 10 blood samples (100 μl) were collected from the facial vein of experimental and RBC digested with RBC lysis buffer (Sigma Life Sciences) according to the manufacturer's protocol. Cells obtained were stained with MHC-I Tetramer and antibodies for FACS analysis as described below. On day 12 all mice were sacrificed by cervical dislocation and lymph nodes and spleens collected. Single cell suspensions of 5x10^6 cells were prepared for FACS analysis as described below.

XIX. FACS staining and analysis

Lymphoid organs (lymph nodes and spleen) were homogenized on a 70 μM cell strainer with 5% FBS. Cell viability and quantification were assessed manually under the microscope with Trypan Blue and a hemocytometer. FACS analysis was performed with: iTAGTM MHC Tetramer H-2Kb OVA SIINFEKL (MBL;PE) further referenced as Kb-SL8 Tetramer, anti-CD8 clone KT15 (MBL; FITC), anti-CD4 (MACS Miltenyi Biotec; VioBlue), anti-CD3e (MACS Miltenyi Biotec; APC), Fixable Viability Dye eFluor 780 (Affymetrix eBioscience; APC-Cy7).

All samples were fixed with eBioscience™ Fixation/Permeabilization reagents following manufacturer's protocol, stored at 4°C and analysed on CantoII flow cytometer (BD Biosciences, USA) on the following day. Cells were gated for live $CD3^+$ cells (100 000 events collected) and data were analyzed using FlowJo software version 8 (Tree Star). The percentage of tetramer positive CD8+T cells was considered for statistical analysis.

RESULTS

I. Establishment of model construct encoding SIINFEKL sequence in the intron2 of β-globin gene (β-globin-SL8).

In order to take forward knowledge from published *in vitro* studies regarding Pioneer Translation Products and study their mechanism of expression we have created a construct in which SIINFEKL is encoded in the Intron 2 of the mouse **β**-globin gene (**β**-globin-SL8).

A. Construct

The **β**-globin-SL8 construct has the SIINFEKL encoding sequence in intron 2 of the mouse **β**-globin gene (Fig. 5 A) as well as the part of NeoR cassette that was important during the generation of transgenic mice described in next chapters. All *in vitro* experiments were performed with the use of this construct which was a gift from Dr. Rodrigo Prado-Martins.

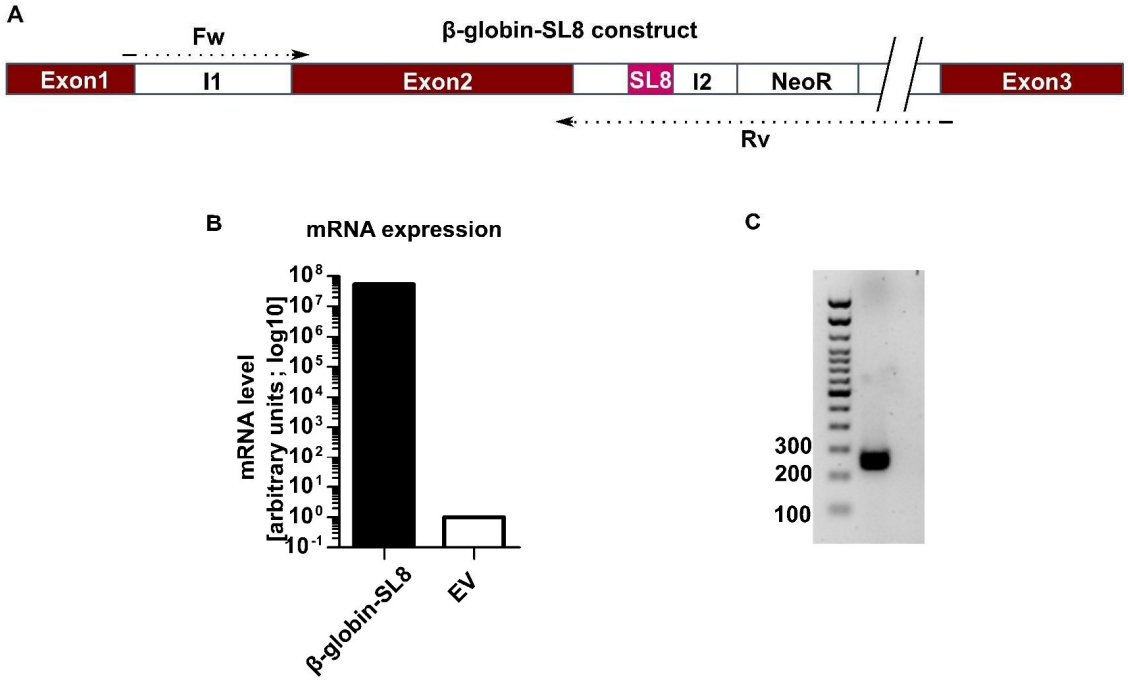


Figure 5. Cartoon (A) represents the **β**-globin-SL8 construct gene with the insertion of sequence encoding for SIINFEKL (pink bar) in the intron 2. **β**-globin consists of 3 exons and 2 introns and the remaining part of NeoR marker within the intron 2 is indicated. Dashed arrows indicate positions of primers used for the analysis of splicing by qPCR. Primers

hybridization sites are indicated by continuous lines at the beginning and end of the arrow. The qPCR analysis of mRNA isolated from transiently transfected cells indicated high levels of spliced products (B) as oppose to the empty vector control (EV). The agarose gel electrophoresis of qPCR amplicons confirmed products of correct size around 242bp (C).

B. Analysis of the expression of β-globin-SL8 construct

In order to assess the expression of spliced **β**-globin-SL8 mRNA we performed RT-qPCR analysis on RNA extracted from transiently transfected H1299 cells and with primers as stated in the Table 7 and assigned in the Figure 5 A. The primer oligos were designed in the way that they can only hybridize to the cDNA template if **β**-globin exons are correctly spliced. RT-qPCR analysis show clear positive signal from **β**-globin-SL8 construct as compared to empty vector (EV) control (Fig. 5 B). In order to verify the length of the product detected by qPCR we performed agarose gel electrophoresis, which showed the single band of size around 242bp (Fig. 5 C). Next, we assessed by RT-qPCR if the intron-derived SIINFEKL encoding sequence is also expressed. Comparative CT analysis was performed with the use of primers indicated in Figure 6 A and Table 7. Data was normalized against the actin housekeeping gene and compared to levels in cells transfected with empty vectors. Both intronic and exonic regions were detected (Fig. 6). The analysis shows a high expression level of pre-spliced RNA detected by primers hybridizing to insert specific regions in the intron 2 (NeoR). Similar level was detected by primers specific to exon 2 and SIINFEKL in the intron 2 (Ex2-I2SL8). The analysis of exon 2 specific regions showed its higher level (Ex2/Ex2) as compared to pre-spliced regions (NeoR/NeoR and Ex2/SL8).

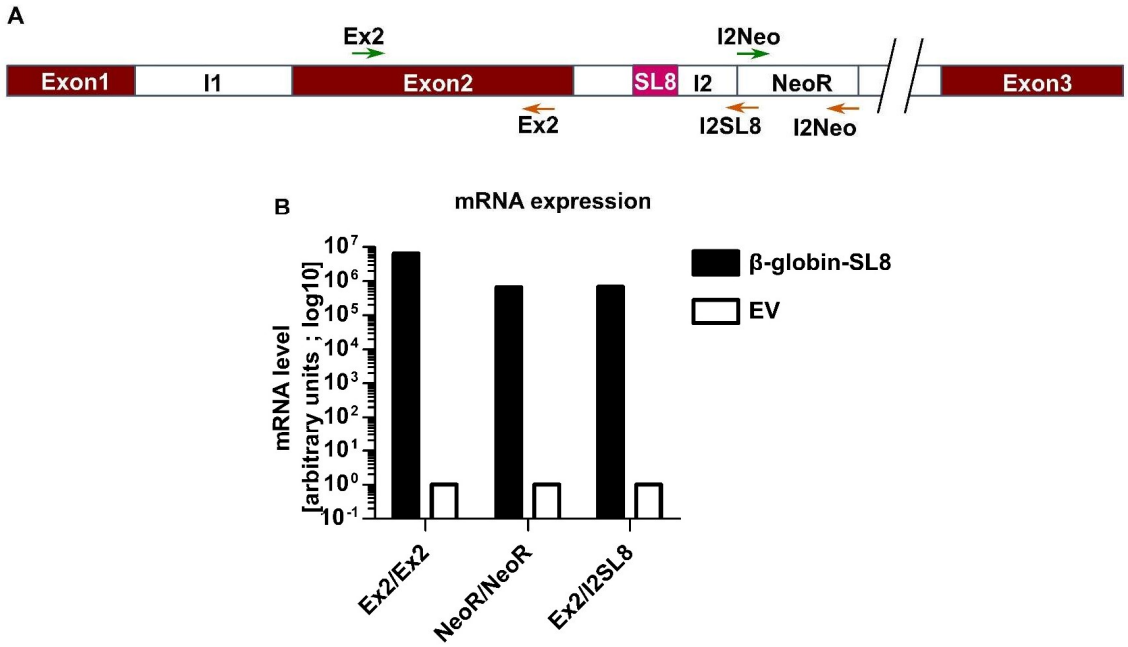


Figure 6. Cartoon (A) represents **β**-globin-SL8 construct and the sites of forward and reverse primers annealing are indicated by green and orange arrows, respectively. qPCR analysis performed with three sets of primers indicate high levels of pre-/mRNA expression in transiently transfected cells as compared to empty vector controls (EV).

C. Functional analysis of β-globin-SL8 construct

We performed an *in vitro* antigen presentation assay to assess if the intron-derived SIINFEKL encoding sequence can generate antigenic peptides and be presented on the Kb MHC class I molecules on cell surface. For this purpose transiently co-transfected H1299 cells with **β**-globin-SL8 construct and Kb were co-cultured with CD8+ T cells expressing SIINFEKL specific TCR (OT-1). After 24 h the medium supernatants were collected for further analysis of Interleukin 2 (IL-2) release levels. It's known that when CD8+ T cells recognize the peptide-MHC-I complex they proliferate and during the first 24 h release IL-2 [200]. Hence, we used this phenomenon in our approach of assessing whether an intron-derived sequence can generate a SIINFEKL peptide and be recognized by OT-1 cells *in vitro*. IL-2 levels were assessed by ELISA and the data showed mean 68 pg/ml IL-2 released to the supernatants from samples with **β**-globin-SL8 construct and -1 pg/ml base mean detected in empty vector (EV) control (Fig. 7). The difference between **β**-globin-SL8 and EV is statistically significant

(** p>0.005). Chicken Ovalbumin (OVA) encodes SIINFEKL sequence in the main open reading frame (ORF) and it was used as a positive control. Mean IL-2 concentration in OVA samples is 454 pg/ml.

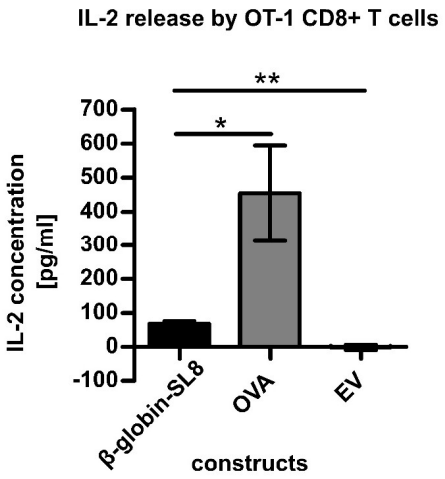


Figure 7. IL-2 release by OT-1 CD8+ T cells in response to transiently co-transfected H1299 cells with Kb and **β**-globin-SL8 or Ovalbumin (OVA) constructs or empty vector (EV). IL-2 concentration in the medium from co-cultured cells was analysed by ELISA. Data in the graph represent 3 independent experiments.

II. Analysis of β-globin-SL8 translation *in vitro*

A. Polysome fractionation

We next addressed the question of the RNA source from which the intron-derived SL8 peptide is translated. The polysome fractionation was carried out on transiently transfected HEK293T cells in order to identify the β-globin-SL8 mRNA distribution profile (Fig. 8). The diagram shows the UV absorbance detected by a spectrophotometer at 254nm and peaks indicate the distribution profile of mRNAs according to their increasing molecular weight in 10-50% sucrose gradient [196].

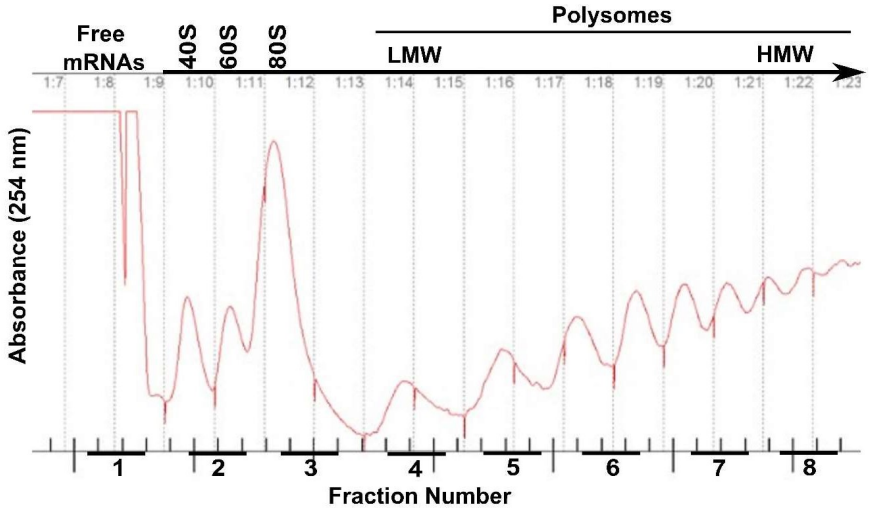


Figure 8. Graph shows the polysome profile from the analysis performed on transiently transfected HEK293T cells with β-globin-SL8 construct. It indicates the distribution of mRNA in 10-50% sucrose gradient. Free mRNAs with no ribosomes bound appear first on the diagram, followed by heavier fractions in which 40S, 60S ribosomal subunits start to attach. mRNAs with fully formed 80S monosome bound are represented by the subsequent peak. Further low molecular (LMW) polysomes start to bind to template mRNAs and depending on the translation rate as well as the length, sequence and structure of mRNA more ribosomes can attach forming high molecular weight polysomes (HMW).

B. Analysis of β-globin-SL8 mRNA levels after polysome profiling

The RNA from different fractions after polysome profiling was isolated and spliced or pre-spliced RNAs were detected using RT-qPCR with a combination of primer pairs corresponding to exons 1 and 2 as well as to sequences within intron 2 (SL8 and NeoR) (Fig. 9 A and Table 7). To confirm the size of achieved amplicons we performed agarose gel electrophoresis with the use of pooled qPCR products from fractions number 3-8 (Fig. 9 B). We see clear single bands of size corresponding to the primers restricted sequences in lines 1-2, 4 and 6. In line number 5 there are two bands detected of size around 341bp and 228bp.

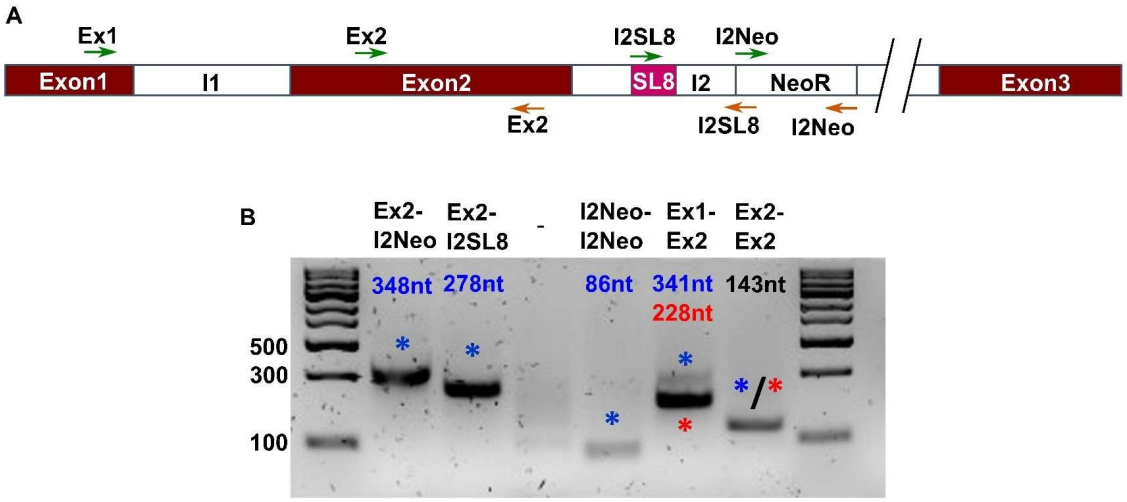


Figure 9. Cartoon (A) represents the **β**-globin-SL8 construct. Primers used for RT-qPCR are indicated. Forward primers indicated by green and reverse primers by orange arrows. (B) Fractions 3 to 8 were pooled and RT-qPCR was performed using indicated primers and 5 separation on agarose gel shows the estimated size of the of PCR products. Blue asterisk indicates pre-spliced products and red spliced. Primers specific to exon 2 (Ex2-Ex2) do not distinguish spliced from pre-spliced mRNA and, thus, the band is marked with blue and red.

The qPCR analysis of mRNAs in individual fractions revealed the highest levels of regions detected by primers hybridizing to exon 2 (forward) or NeoR and SIINFEKL or NeoR in the intron 2 (reverse) in fractions number 3 and 4. The levels of the same amplicons were decreased in fractions 1 and 6-8 (Fig. 10 A). Simultaneously, the analysis of fragments corresponding to exon 1 and exon 2 were of the lowest levels in fractions 1-3, higher in fractions 4-6 and the highest in last two fractions 7-8 (Fig. 10 B). Treating cells with pre-mRNA splicing inhibitor Isoginkgetin (ISO) prior the polysome fractionation resulted in an increase of mRNA fragments detected by primers hybridizing to intron 2 (I2SL8/I2SL8) in fractions 3 and 4 as compared to DMSO controls. Simultaneously, the analysis revealed higher levels of exon 2 mRNAs in fractions 4 and 5 and further its decrease in fractions 6 and 7. Overall, we see a shift of intronic and exonic mRNA fragments towards the lighter fractions in samples treated with ISO as compared to DMSO controls (Fig. 10 C). Also, we see a noticeable decrease of exonic mRNA fragments in heavier fractions 6-7 in samples treated with ISO as compared to DMSO control.

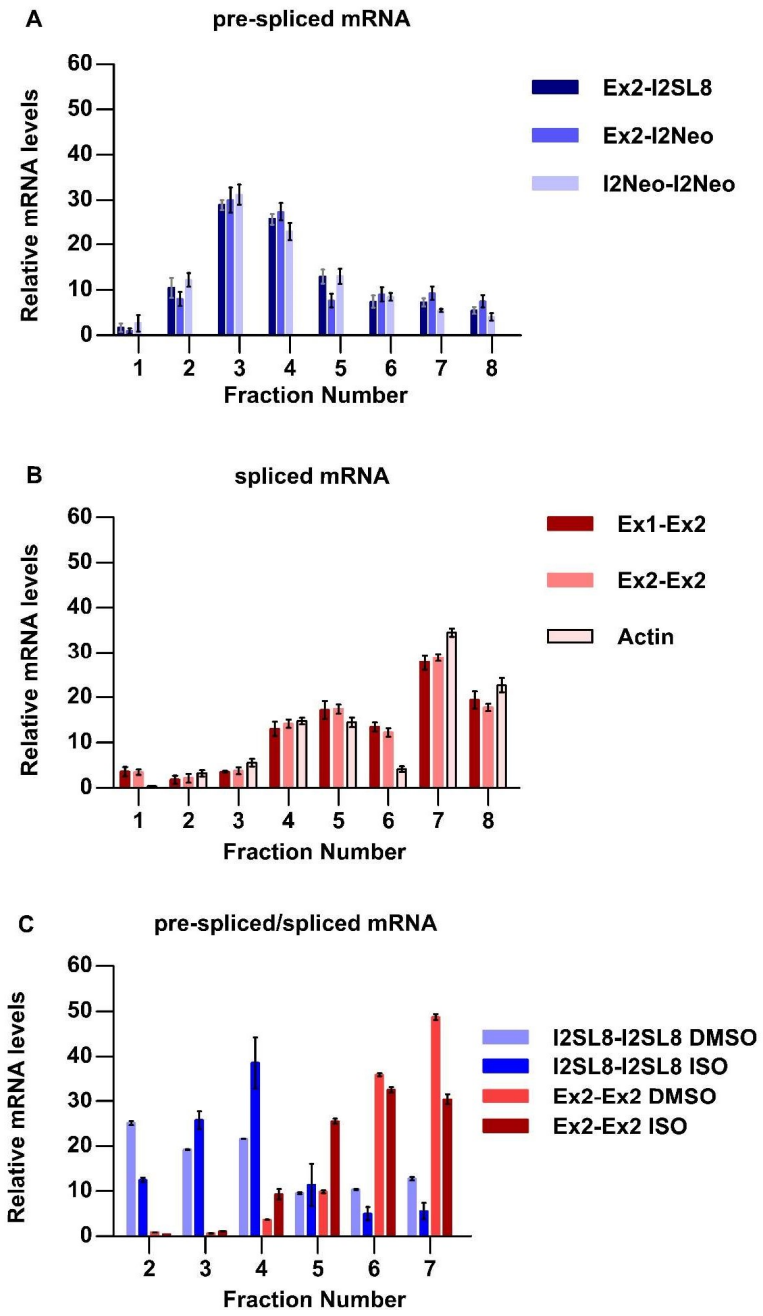


Figure 10. RT-qPCR analysis of RNAs isolated from selected fractions after polysome profile. (A and B) The relative levels of pre-spliced (A) or spliced (B) mRNAs using indicated primer pairs. (C) Treatment with splicing inhibitor Isoginkgetin (ISO) shows decrease of mRNAs in heavy polysome fractions and an increase of pre-spliced mRNA in lighter fractions.

C. Analysis of SIINFEKL peptide expression by Proximity Ligation Assay (PLA)

In order to visualize the presence of intron-derived peptide products we used the proximity ligation assay (PLA) and a combination of rabbit and goat purified polyclonal antibodies raised against the N- or the C- terminal sequences of the SL8 peptide plus flanking intron sequences (Fig. 11). Antibodies were produced, purified and validated by Eurogentec.

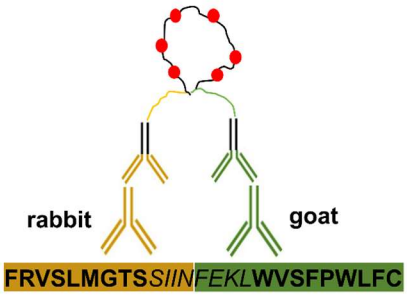


Figure 11. Rabbit and goat antibodies were generated using indicated peptides that include the N-terminal half of SIINFEKL plus flanking intron sequence, or the C-terminal half plus flanking sequence. These custom antibodies were used in PLA assay to visualise the expression of the intron-derived SL8 substrate in H1299 cells expressing the β-globin-SL8 construct.

Treatment of transiently transfected H1299 cells with ISO resulted in a significant increase in the amount of SL8-carrying peptide substrate as compared to the cells treated with DMSO (Fig. 12). Simultaneously, immunohistochemistry analysis performed with antibodies detecting **β**-globin protein showed less GFP signal in cells treated with ISO (Fig. 12 A). The quantification of detected PLA signals indicate highly significant increase of SL8 peptide precursor in cells transfected with **β**-globin-SL8 construct (GFP positive) and treated with ISO as compared to DMSO controls (Fig. 12 B). The automated quantification allowed us also to determine the signal detected in non-transfected cells and there was no difference between DMSO or ISO treated samples. The difference in the PLA signal detected in DMSO treated

cells between transfected (GFP positive) and non-transfected (GFP negative) was also non-significant statistically.

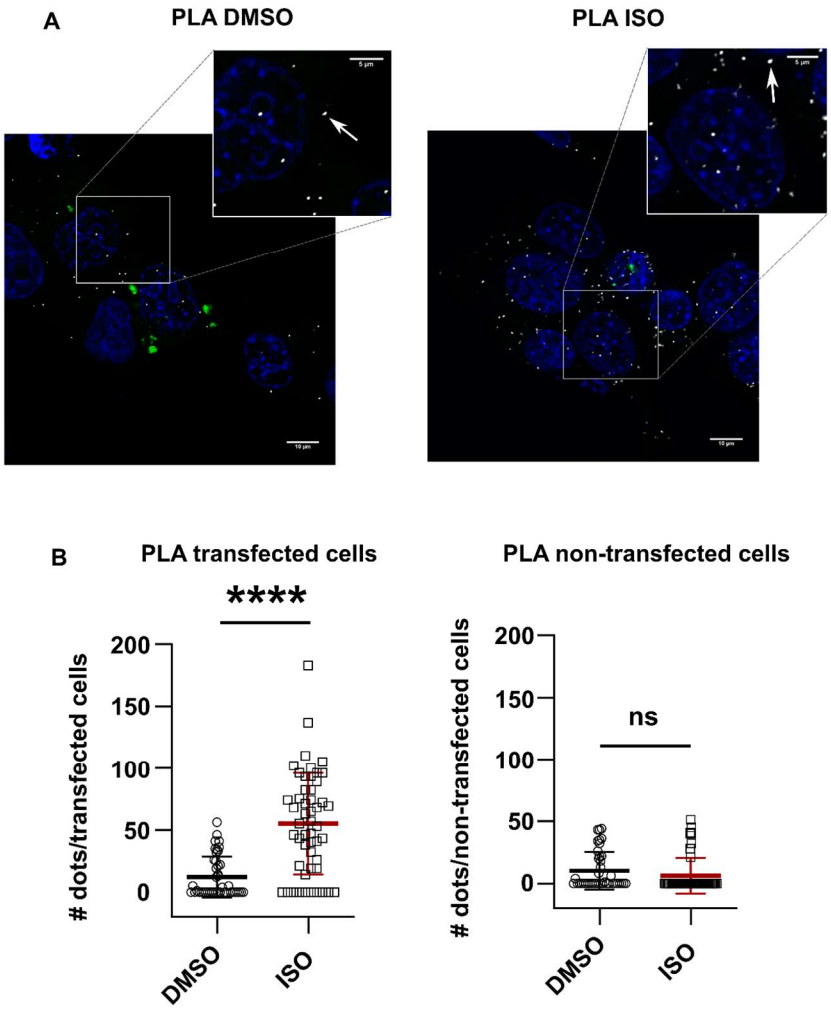


Figure 12. (A) PLA assay was performed with the use of customized antibodies (Fig. 11) to visualise the expression of the intron-derived SL8 substrate in H1299 cells expressing the β-globin-SL8 construct (enlarged squares, white dots). Immunohistochemistry was carried out in parallel using mouse anti-β-globin antibodies (green fluorescent signal). Treatment with 30M of ISO for 20 hours increased the expression of intron-derived SL8-carrying peptides and reduced the amount of β-globin protein, as compared to the DMSO control. (B) The number of PLA dots in (A) was calculated using custom-made automated script in FIJI (**** $p<0,0001$).

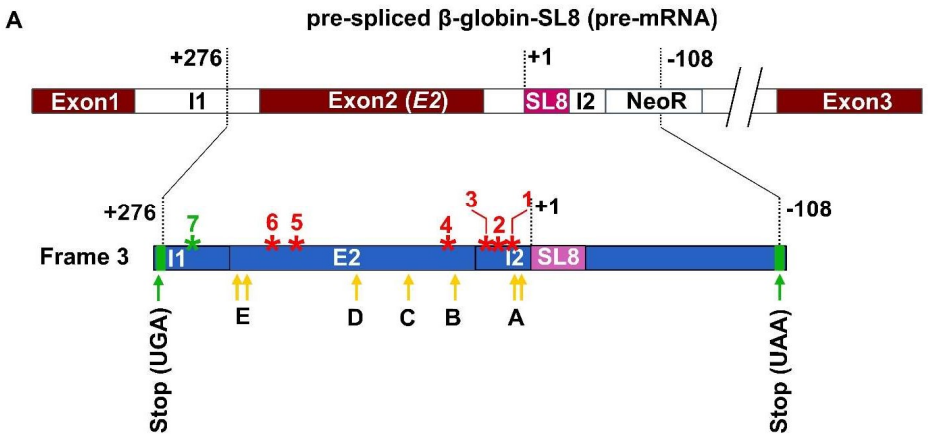
III. Establishment of model constructs for studying translation initiation of SIINFEKL peptide precursor

In order to identify the translation initiation site of the SIINFEKL precursor we have generated several constructs with substitutions of potential START codons into non-AUG codons or constructs carrying UAA stop codons.

A. Constructs

The SL8 epitope is in the third frame of the **β**-globin-SL8 construct. 276 nucleotides (nts) upstream of the SL8 epitope (+276) is an in frame stop codon (Fig. 13 A and B). Within this sequence there are two AUG codons in the same open reading frame +12, +171. In order to assess if these AUG codons are responsible for translation initiation of SIINFEKL precursor we substituted them with AUC codons by site-directed mutagenesis and tested by functional assays described in next chapters. Further on, we identified within this sequence potential non-AUG initiation codons that have been implicated in the past with translation initiation [140]. Leucine encoding CUG codons in positions +15, +48, +111 +240 and +243 were substituted by CUC codons and were subjected to further analysis described in next chapters.

We have also created constructs in which certain codons upstream SIINFEKL were substituted with UAA stop codons in order to identify the potential alternative translation initiation region (Fig. 13A and B). All above constructs were made by site-directed mutagenesis with the primers stated in Table 1.



B

5'3' Frame 3

G A P D Stop C Stop E G C C L W P V G K G E R R Stop S W W Stop G P G Q V G I Q V T R Q L T R S W V
L G D R G L L T S R H Stop L Stop V S P V Y V S L F R L L V V Y P W T Q R Y F D S F G D L S S A S A I Met
G N A K V K A H G K K V I T A F N D G L N H L D S L K G T F A S L S E L H C D K L H V D P E N F R V S L Met
G T S S I I N F E K L W V S F P W L F C S T F L S E G N S S S R K L A Y N F V Stop H T L Y E V I P I H
R Y L D L E G E A I L G S S L H D C V W S V D K S L D I L F S T Q N R C S P S L C S V L S F P L S L V S F
Stop F P V A F Y Stop I N Stop A G Y L L P I L I S A S P P P F L P V L L S L L S L S L I L S F P S V H F F F
F D L L L F V F L N I A L Stop L T Q R T R K I C P C F F S Stop L S R I V A Stop L A F Met P G Stop Q G K
N I F Y I Stop I L F D I G F L Stop Stop F V S S L R L Q T N V F I N K P A V S G I F A L Q L C Stop Stop F F
H L P T A P G Q Y D R D C A G P P P G Q G F H P R C T G C L P E G G G W S G R C P G S Q V P L

Figure 13. (A) The SL8 epitope is in the third reading frame (blue) of the β-globin-SL8 construct and flanked by stop codons at nucleotide positions +276 and -108 relative to the SIINFEKL. Stop codons (1-7) were introduced upstream of the SL8 epitope creating 7 individual constructs. Selected AUG and CUG codons upstream SL8 epitope were substituted by AUC and CUC codons, respectively (A-E), generating further 5 constructs. All constructs were cloned in order to investigate translation initiation site of SIINFEKL precursor. (B) Complete amino acids sequence of translated 3rd frame of the β-globin-SL8 construct with indicated mutation sites. Green and red – sites of substitution to UAA stop codons. Orange – sites of leucines (CUG) and methionines (AUG) substituted to CUC and AUC, respectively.

B. Analysis of RNA expression

To further investigate if mutations in **β**-globin-SL8 did not affect the RNA expression we performed the RT-qPCR analysis on transiently co-transfected H1299 cells with Kb and individual constructs. The results were normalized to actin, compared to cells transfected with empty vector (EV) and show high expression levels of mutated **β**-globin-SL8 pre-mRNA expression (Fig. 14 A and B) in all transfected cells.

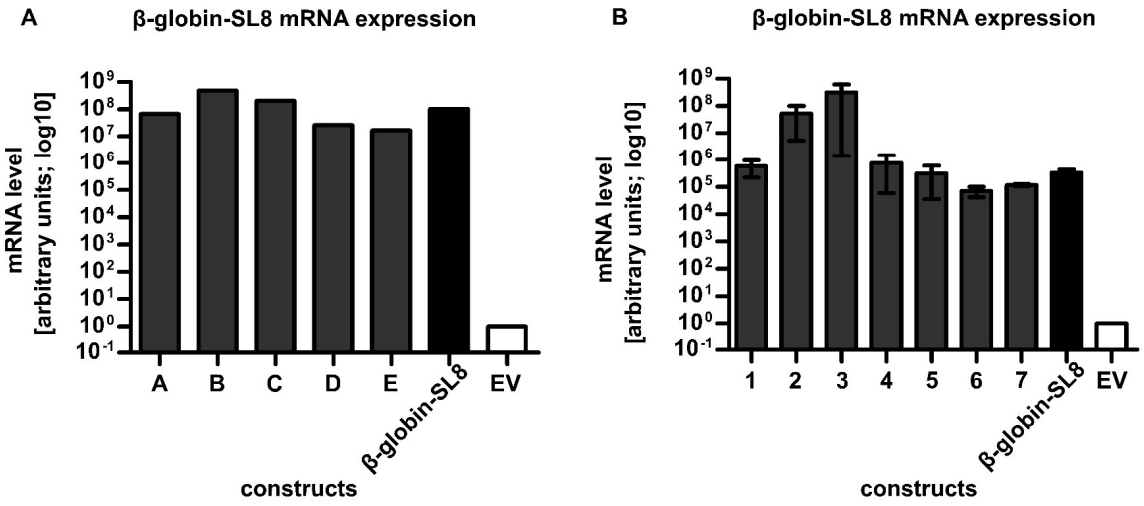


Figure 14. β-globin-SL8 pre-mRNA expression levels in H1299 cells co-transfected with Kb and individual mutated constructs. RT-qPCR with primers specific to exon 2 (Ex2; forward; Table 7) and SL8 (I2SL8; reverse; Table 7) confirms RNA expression of β-globin-SL8 gene constructs A-E (A) and 1-7 (B) in transiently transfected cells. The data were normalized against actin and represent fold increase towards cells transfected with empty vector (EV).

IV. Indirect analysis of translation initiation of SIINFEKL peptide precursor by a functional assay

To study the effect of codon substitutions on translation of SIINFEKL precursor and generation of antigenic peptides we evaluated described in previous chapter constructs by indirect functional assay - *in vitro* antigen presentation.

A. Analysis of antigen presentation from described constructs with substitutions of potential START codons

We performed *in vitro* antigen presentation as described in the previous chapter to indirectly assess the effect of mutations on the translation of SIINFEKL precursor and generation of antigenic peptide. We analysed the levels of IL-2 released into medium supernatants during co-culture of transiently transfected H1299 cells with OT-1 CD8+ T cells by ELISA. First, we analysed the constructs with substitutions of AUG and CUG codons into AUC and CUC, respectively. ELISA results show no significant changes in IL-2 secretion levels as compared to non-mutated **β**-globin-SL8 (Fig. 15).

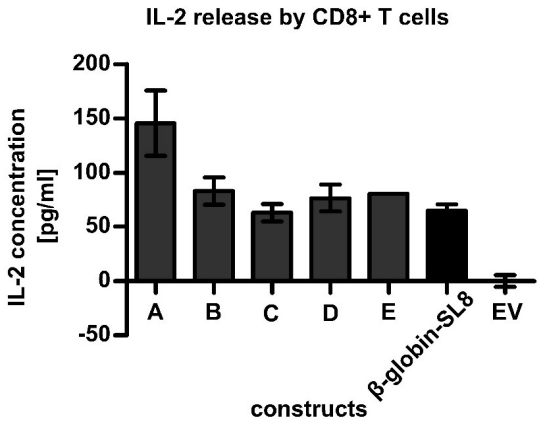


Figure 15. IL-2 release by OT-1 CD8+ T cells in response to transiently co-transfected H1299 cells with Kb and individual constructs A-E or **β**-globin-SL8 or empty vector (EV). IL-2 concentration in the medium from co-cultured cells was analysed by ELISA.

B. Analysis of antigen presentation from described constructs with UAA stop codons

Next, we assessed the effect of UAA stop codons in generated constructs on the translation of SIINFEKL precursor and generation of antigenic peptide by the same method as in A. As shown by ELISA results (Fig. 16) the IL-2 levels were significantly lower in supernatants collected from co-cultures with cells transfected with **β**-globin-SL8 mutants carrying stop codons up until +228 upstream SIINFEKL. The IL-2 levels achieved from co-cultures with cells transfected with mutants carrying stop codons at positions +255 and +273 remained unaffected as compared to non-mutated **β**-globin-SL8 construct.

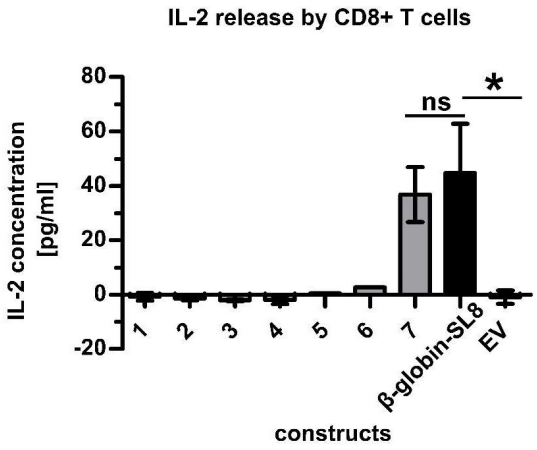


Figure 16. IL-2 release by OT-1 CD8+ T cells in response to transiently co-transfected H1299 cells with Kb and individual constructs 1-7 or **β**-globin-SL8 or empty vector (EV). IL-2 concentration in the medium from co-cultured cells was analysed by ELISA.

V. Analysis of ribosomes' presence on potential SIINFEKL precursor translation initiation site by adapted ribosome profiling

Gradually introducing stop codons upstream of SL8 sequence prevented the synthesis of the SL8 epitope up to the position +228 (Fig. 6 star no. 6). However, when a stop codon was introduced at position +255 (star no. 7) SL8 expression was unaffected (Fig. 13 and Fig. 14 B). The mutations did not affect the pre-mRNA expression of β-globin-SL8 (Fig. 14 B). This indicates that the synthesis of the SL8-carrying peptide substrates is initiated within 27 nts between +228 and +255. To verify this, we carried out a modified ribosome profiling protocol in which HEK293T cells expressing the β-globin-SL8 construct were treated with harringtonin and cycloheximide to maintain the ribosome in position of initiation in order to generate ribosome-protected RNA fragments (ribosome footprint) [197]. After RNAse treatment, the protected RNA fragments were isolated (Fig. 17 A) and linked with stemloop primers[198]in the 3' by reverse transcription and qPCR confirmed the presence of the +255 to +228 sequence in the pool of ribosome footprints (Fig. 17 B and Tables 2 and 3) . In this 27 nts sequence there are two adjacent leucine (CUG) codons in frame with the SL8. CUG codons have been implicated in translation initiation of antigenic peptide substrates [87], [140] but introducing synonymous mutations in the third position in both codons (CUG>CUC) did not affect expression of the SL8-carrying peptide substrate (Fig. 14 A construct E).

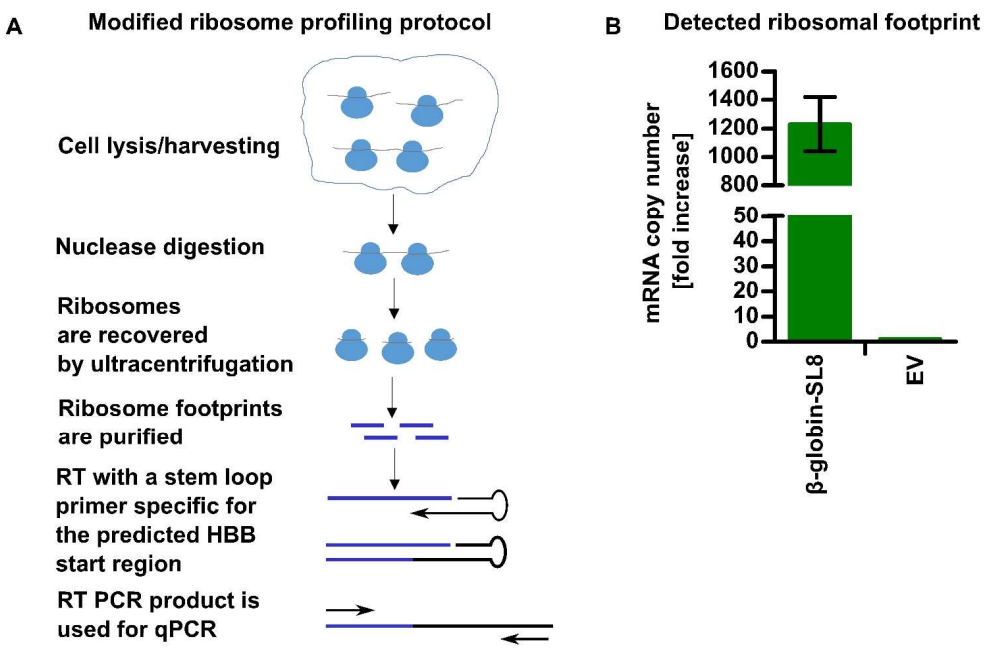


Figure 17. A modified ribosome profiling protocol was carried out to determine if the 27 nt sequence harbours the ribosome initiation site (A). Cells were lysed under conditions that maintain the ribosome in position using Harringtonin treatment followed by cycloheximide [197]. RNAse and DNAse were added to generate ribosome footprint and ribosomes were isolated following ultracentrifugation for 22 hours at 36000 rpm. The ribosome-protected RNA fragments were isolated and stem loop primers were fused to the 3' (Table 2). RT-qPCR was performed using primers corresponding to the 5' of the predicted 27 nts initiation sequence and the stem loop (Table 3). (B) The graph shows that the predicted 27 nt sequence was included in the ribosome footprint of the pre-spliced β-globin-SL8 RNA (EV: empty vector).

VI. Establishment of transgenic mice encoding SIINFEKL sequence in the intron2 of the mouse beta-globin gene (HBB)

In order to take forward knowledge from *in vitro* studies we have created a mouse model to investigate the physiological role of PTPs *in vivo*.

A. Construct

The fragment of 109 bp encoding SIINFEKL sequence and other critical regions for knock-in was integrated into the intron 2 of the β-globin gene which is placed on chromosome 7 in mice by homologous recombination in Ciphe's laboratories, Marseille (Fig. 18).

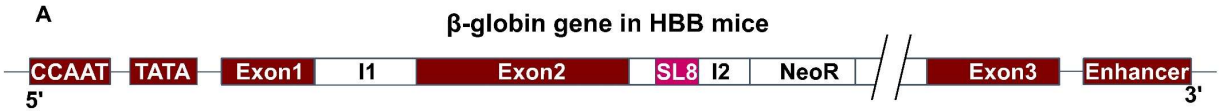


Figure 18. Cartoon represents a map of the HBB knock-in mice with the SIINFEKL encoding sequence (SL8) inserted in the second intron of the β-globin gene. I1: Intron1; I2: Intron2

B. Identification of transgenic mice

The PCR analysis of DNA isolated from murine tails' biopsies enabled us to identify the animals carrying the insertion of interest in their genome. Separation by agarose gel of PCR amplicons allowed to distinct the WT allele (742bp) from mutant HBB allele (851bp) as shown in Figure 19.

First 3 heterozygous animals delivered to our laboratories were used as founders of 3 lines. Subsequent analyses of numbers of positive animals per litter allowed us to select for one family as a founder of the HBB line. Heterozygous animal female mouse number 005 once backcrossed to C57BL/6 N (B6) mouse gave the highest number of pups with 44 % of transgene positive HET animals (Fig. 20; Table 8). Heterozygous males and females

were coupled in order to produce homozygous (HOM), heterozygous (HET) and wild (WT) type animals. Only HOM and WT animals were used for experiments.

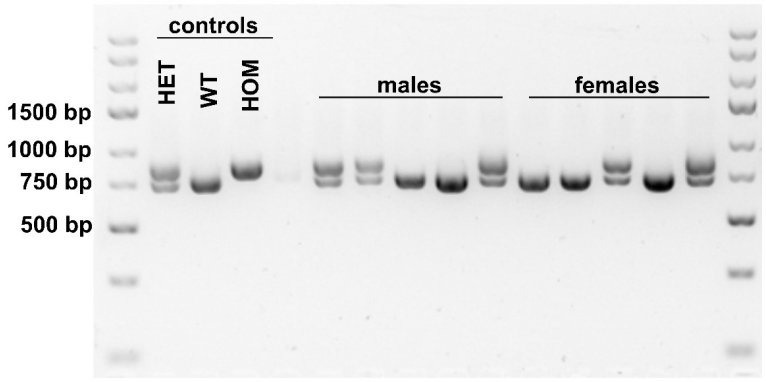


Figure 19. Transgenic mice characterisation. Distribution of PCR amplicons in agarose gel after mice genomic DNA analysis towards the presence of knock-in SIINFEKL sequence. Bands on above agarose gel represent frequency of heterozygous (HET) and WT mice in selected litter obtained from the 005 HET founder backcrossed to C57BL/6 N mouse. Out of 10 pups in litter, 50% had HET genotype. Simultaneously, there was an equal number of males and females within the litter.

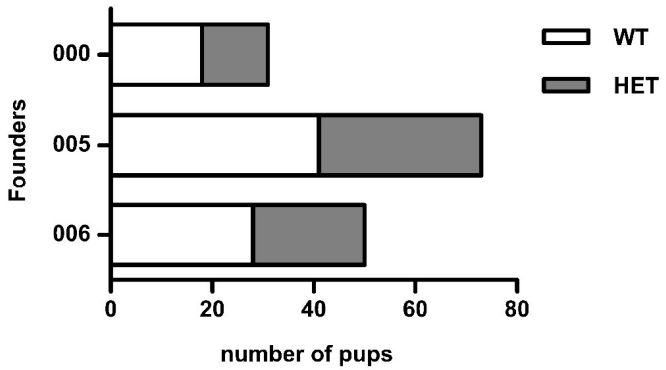


Figure 20. Frequency of transgene positive mice per litter. Graph represents the number of HET and WT mice obtained from 3 separate founders (number 000, 005 and 006) backcrossed to B6 mice. Line 005 gave the highest number of pups (73) with simultaneous high frequency of HET animals (44%) per litter (see Table 8).

Founders	number of pups			
	WT	HET	Total	% of HET
000	18	13	31	42
005	41	32	73	44
006	28	22	50	44

Table 8. Number of transgene positive and negative pups delivered in families number 000, 005 and 006.

C. Study of the transgene RNA expression

In order to validate our model we have analysed the expression levels of SIINFEKL specific pre-mRNAs by qPCRs in selected organs and tissues of HOM and WT mice. The results obtained showed the highest expression of the SIINFEKL specific pre-mRNAs in organs, namely spleen, bone marrow and blood (Fig. 21 A). However, using this method we were unable to detect expression in thymus, lymph nodes and liver. the high expression levels in organs with simultaneous lack of transgene pre-mRNA expression in the liver led us to investigate further the pre-mRNA expression in splenocytes separated from mature red blood cells (RBCs). The analysis showed the significant difference between the insert pre-mRNA expression in RBC depleted population as compared to total HBB splenocytes (Fig. 21 B).

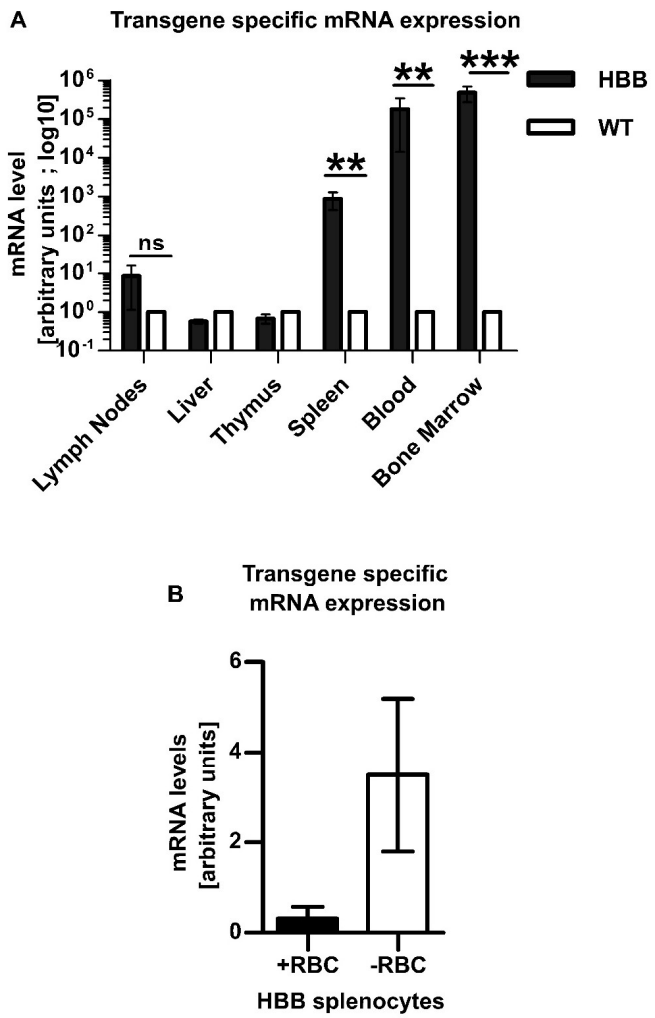


Figure 21. The analysis of transgene specific mRNA expression in selected tissues of HBB mice. (A) RT-qPCR confirms RNA expression of the HBB knock-in sequence in indicated tissues with the highest levels in Bone Marrow, Blood and Spleen. (B) Transgene specific mRNA levels analysed in HBB splenocytes depleted of red blood cells (-RBC) vs nondepleted controls (+RBC). All results were normalized to actin as a housekeeping gene. Data indicates over three fold increase in the expression after RBC depletion.

D. Western blot analysis of full-length beta-globin protein

In order to verify if the intronic insertion didn't affect beta-globin protein expression in HBB mice we performed Western blot analysis on protein lysates from splenic and bone marrow cells. There was no difference in size neither in level between HOM and WT mice in both tissues. The beta globin protein of correct size (14kDa) was detected in both HOM and

WT mice in both tissues and there was no difference in the protein levels between transgene positive and WT animals (Fig. 22).

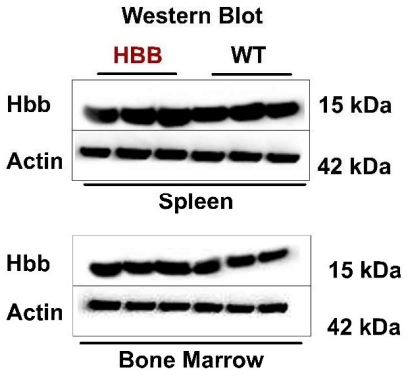


Figure 22. Western Blot analysis of β-globin protein (Hbb) in splenic and bone marrow cells from three HBB and WT animals.

E. Blood morphology analysis

To further evaluate whether the intronic insertion of what in HBB mice had influence on morphology of RBCs in HBB and WT mice we performed the blood test on samples collected from the submandibular veins. The results obtained (Fig. 23) have shown no difference between HBB and WT RBCs count. Simultaneously, no major differences between the two groups were detected while analysing other parameters describing red blood cells like hematocrit (Hct), mean corpuscular volume (MCV) and hemoglobin (Hgb).

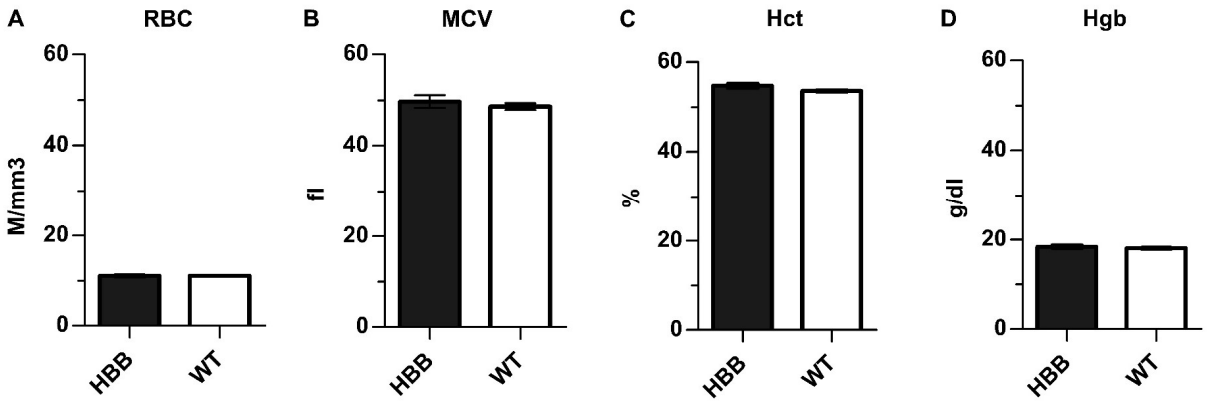


Figure 23. Blood morphology analysis of samples from HBB and WT mice.

F. Establishment of the HBB line on C57BL/6-Ly5.1 genetic background

In order to obtain the line which expresses CD45.1 allele on the surface of leukocytes and which would differentiate the endogenous leukocytes in HBB mice from OT-1 line, which has a transgenic CD8 T cells receptor specific to the SIINFEKL-Kb molecules and expresses CD45.2 receptor, the founder HBB mice were backcrossed to C57BL/6 - Ly5.1 congenic strain.

Taken together, two HBB lines were established which differ in the expression of CD45 alleles (CD45.1 or CD45.2) and both lines are important for experimental procedures described in following chapters.

VII. Evaluation of HBB mouse model by functional assays

In order to validate the HBB mouse model to study the physiological role of intron-derived antigenic peptides, we have performed *in vivo* analysis of SIINFEKL antigen presentation.

A. The analysis of OT-1 CD8+ T cells proliferation in HBB mice.

In order to evaluate whether intron derived SIINFEKL can be presented *in vivo* we have performed the antigen presentation assay. For that we have used OT-1 mice on RAG-1 knockout (KO) background as donors of CD8+ T cells expressing transgenic T cell receptors specific towards the SIINFEKL-Kb (SL8-Kb) complex (OT-1 cells). Enriched population of CD8+ T cells was labeled with CellTrace Violet dye which enables the assessment of cellular proliferation by Flow Cytometry. When OT-1 cells detect the SL8-Kb complex they proliferate and gradually lose the CellTrace label. Subsequently, 2x10^6 cells were injected intravenously (iv) to HBB and WT mice at day 0 (Fig. 24 A). The mice were sacrificed 3 days later and enriched CD8+ T cell proliferation was assessed by Flow Cytometry. The gating strategy was based on results obtained from positive and negative control mice and was used for the analysis during FACS acquisition (Fig. 24 B). For the positive control and assay validation WT mice have been injected intraperitoneally (ip) with transiently co-transfected cells with full-length OVA and Kb prior intravenous injection of labelled OT-1 cells. The FACS spleen cell analysis shows different proliferation of CD45.2+ CD8+ T cells after injection in CD45.1+ HBB and CD45.1+ WT mice, 2.74 % and 1.02 % of total OT-1 derived CD8+ cells, respectively (Fig. 24 C). The analysis of OT-1 cells in a positive control indicated 31.1% of proliferating cells. Collective data of 5 independent experiments show significant increase of OT-1 CD8+ T cells proliferation in HBB as compared to WT mice (Fig. 24 D; *** $p<0.001$).

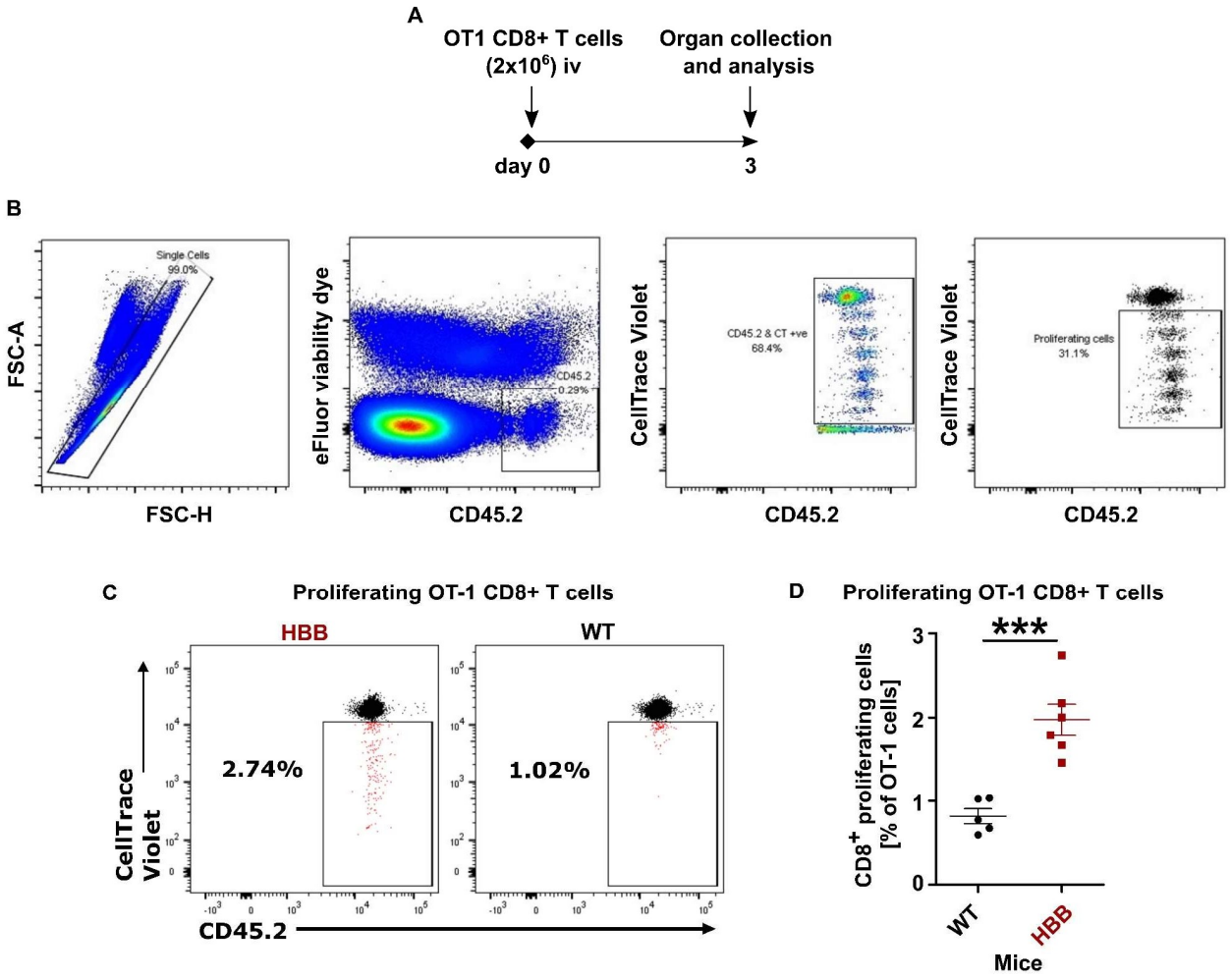


Figure 24. The analysis of OT-1 CD8+CD45.2+ T cells proliferation in HBB mice. Cartoon (A) represents the experimental outline. CellTrace labelled OT-1 CD8+T cells were injected intravenously (iv) to HBB and WT mice on day 0. FACS analysis of isolated CD8+ T cells was performed with the application of indicated gating strategy (B) to select for proliferating, CD45.2+ T cells. (C) FACS analysis of CellTrace violet labelled CD45.2+ CD8+ OT-1 cells specific for the SL8 epitope. When OT-1 cells detect the SL8-Kb complex they proliferate and gradually lose CellTrace violet label [199]. Spleen cell analysis shows different proliferation of CD45.2+ CD8+ T cells after injection in CD45.1+ HBB and CD45.1+ WT mice, 2.74% and 1.02% of total OT-1 derived CD8+ cells, respectively. (D) Graph shows data from 6 HBB mice analysed in 5 independent experiments (like in C) along with WT controls (*** $p<0.001$).

B. The analysis of OT-1 CD8+ T cells reactivity in HBB mice.

We assessed the effect of adoptive9 transfers of OT-1 cells on transgene expressing tissues in HBB mice. For this reason HBB and WT mice were injected intravenously with OT-1 CD8+ T cells as shown in Figure 25 A. First injection was performed with naive cells and mice were observed for the appearance of any signs of sickness. Due to the fact that no difference was observed between HBB and WT mice, animals were further injected 3 times with OT-1 cells that were activated ex vivo with synthetic SIINFEKL peptide for 24 h prior intravenous administration. After 24 h of OT-1 derived splenocyte stimulation with SIINFEKL peptide, CD8+T cell population was enriched and RT-qPCR analysis performed to determine the mRNA expression of key markers in cell proliferation and activation (Fig. 25 B). The analysis shows upregulated expression of proliferation marker Ki67 as compared to the levels in non-stimulated OT-1 CD8+ T cells controls. Also, we see lower levels of OT-1 transgenic T cell receptor (OT-1 TCR) and very low mRNA levels of IL-2 as compared to non-stimulated cells. Next, we also assessed the mRNA levels of activation markers. The analysis showed higher levels of Fas ligand (FasL), Perforin and GranzymeB in activated OT-1 CD8+ T cells as compared to non-stimulated cells. At day 45, the experiment was stopped and spleens collected for histopathology analysis. Organ microdissections and hematoxylin and eosin staining were used in order to analyse the anatomy. As shown in Figure 25 C there is an increase of the mantle zone and the marginal zone with similar stimulation in the germinal centers of lymphatic nodules, but in HBB mice the growth of the mantle zone and the marginal zone is much more irregular in shape. Using the same histopathology analysis method we also assessed the anatomy of noninjected HBB and WT mice (Fig. 25 D). The analysis shows no major differences in the shape nor size in mantle, marginal zones and germinal centers of lymphatic nodules between HBB and WT mice.

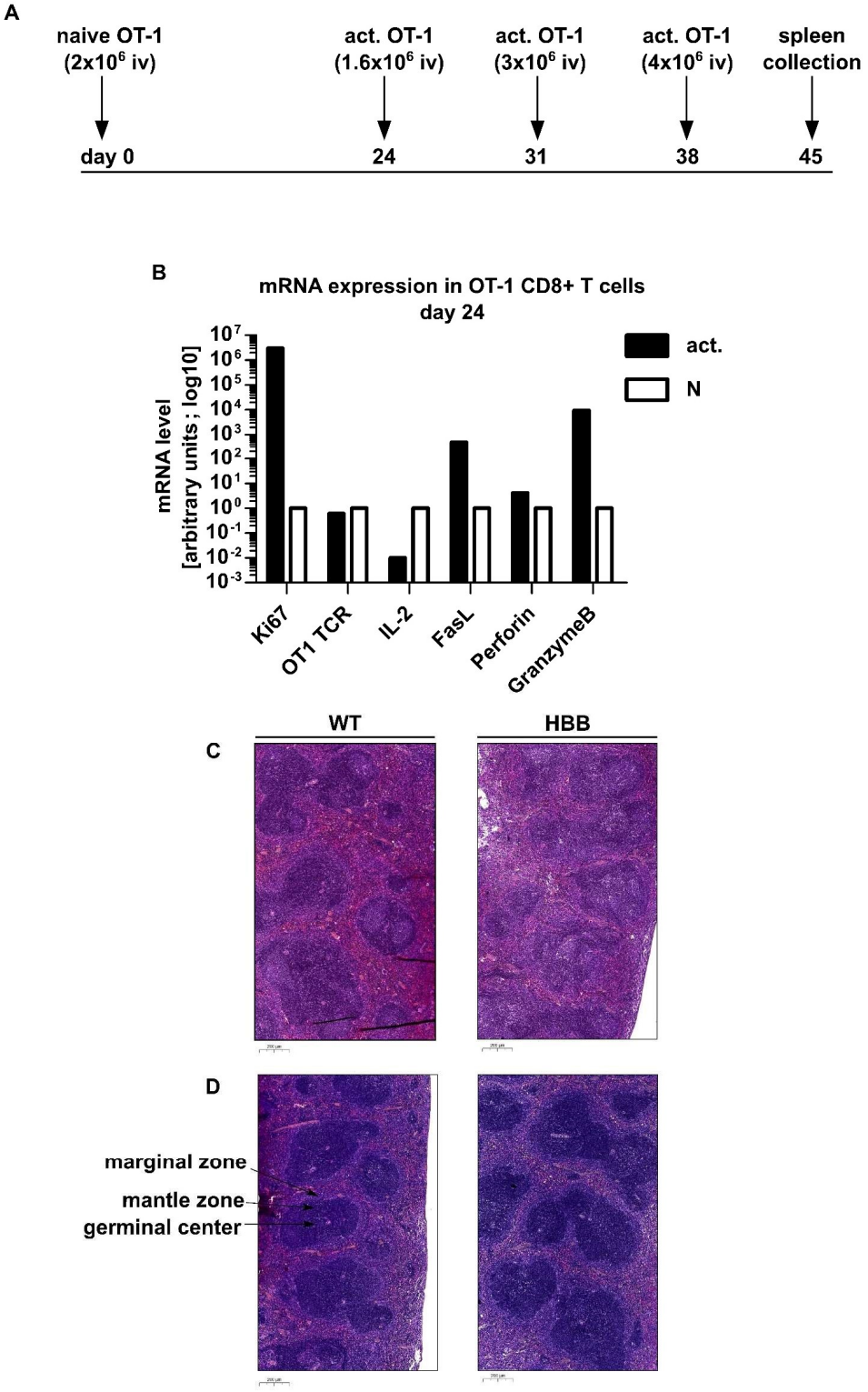


Figure 25. The analysis of OT-1 CD8+ T cells reactivity in HBB mice. Cartoon (A) represents experimental plan and the timing and number of adoptive transfers of OT-1 CD8+ T cells (OT-1). 24 h prior last 3 transfers OT-1 cells were activated *ex vivo*, by stimulation with

synthetic SL8 peptide. RT-qPCR analysis was performed to evaluate the mRNA levels of selected activation markers (FasL, Perforin, GranzymeB) and proliferation (Ki67, IL-2) markers as well as the level of OT-1 TCR in OT-1 cells (B). Histopathology analysis of spleens microdissections (C,D) was performed on HBB and WT mice with transferred OT-1 cells (C) as well as non-injected mice (D). (C) In all injected mice there is an increase of the mantle zone and the marginal zone with similar stimulation in the germinal centers of lymphatic nodules, but in HBB mice the growth of the mantle zone and the marginal zone is much more irregular in shape. (D) None of these changes were observed in noninjected steady state mice (both HBB and WT). Images represent HE stain of splenic microdissections from single experiment with 3 mice in each group. act. – cells activated ex vivo with SL8 peptide in co-culture with OT-1 splenocytes (24h) prior injection; N – nonactivated.

C. The analysis of endogenous SIINFEKL specific CD8+T cells responses in HBB mice

We assessed the response of endogenous SIINFEKL-specific CD8+ T cells in HBB and WT mice by Flow Cytometry with Kb-SIINFEKL (Kb-SL8) Tetramers after immunizing the animals with SL8-pulsed or not and lipopolysaccharide (LPS)-activated BMDCs. BMDCs were derived *ex vivo* from WT mice bone marrow and at day 8 the cells were harvested and used for antigen pulsing. In order to activate the cells *ex vivo* BMDCs were further pulsed with SL8 peptide or not in the presence of LPS for 2h at 37^{o}C. The 1x10^{6} cells were injected intravenously to HBB and WT mice twice on days 0 and 7 (Fig. 26 A). On day 10 we collected blood samples from all immunised animals in order to verify the impact of immunisation on the response of endogenous CD8+T cells towards SIINFEKL. FACS analysis with the use of specific Kb-SL8 Tetramers showed significantly lower increase of SL8 specific CD8+T cells in peripheral blood lymphocytes (PBL) in immunised HBB mice as opposed to WT controls (Fig. 26 C). Next, we also assessed the levels of SL8 specific CD8+ T cells in spleens, as it is the organ with high expression of SIINFEKL precursors mRNA in

HBB mice. After mice sacrifice at day 12 we performed FACS analysis with specific Kb-SL8 Tetramers which also showed that the numbers of SL8-specific CD8+T cells in spleens are significantly lower in HBB mice than in WT control group (Fig. 26 B and D).

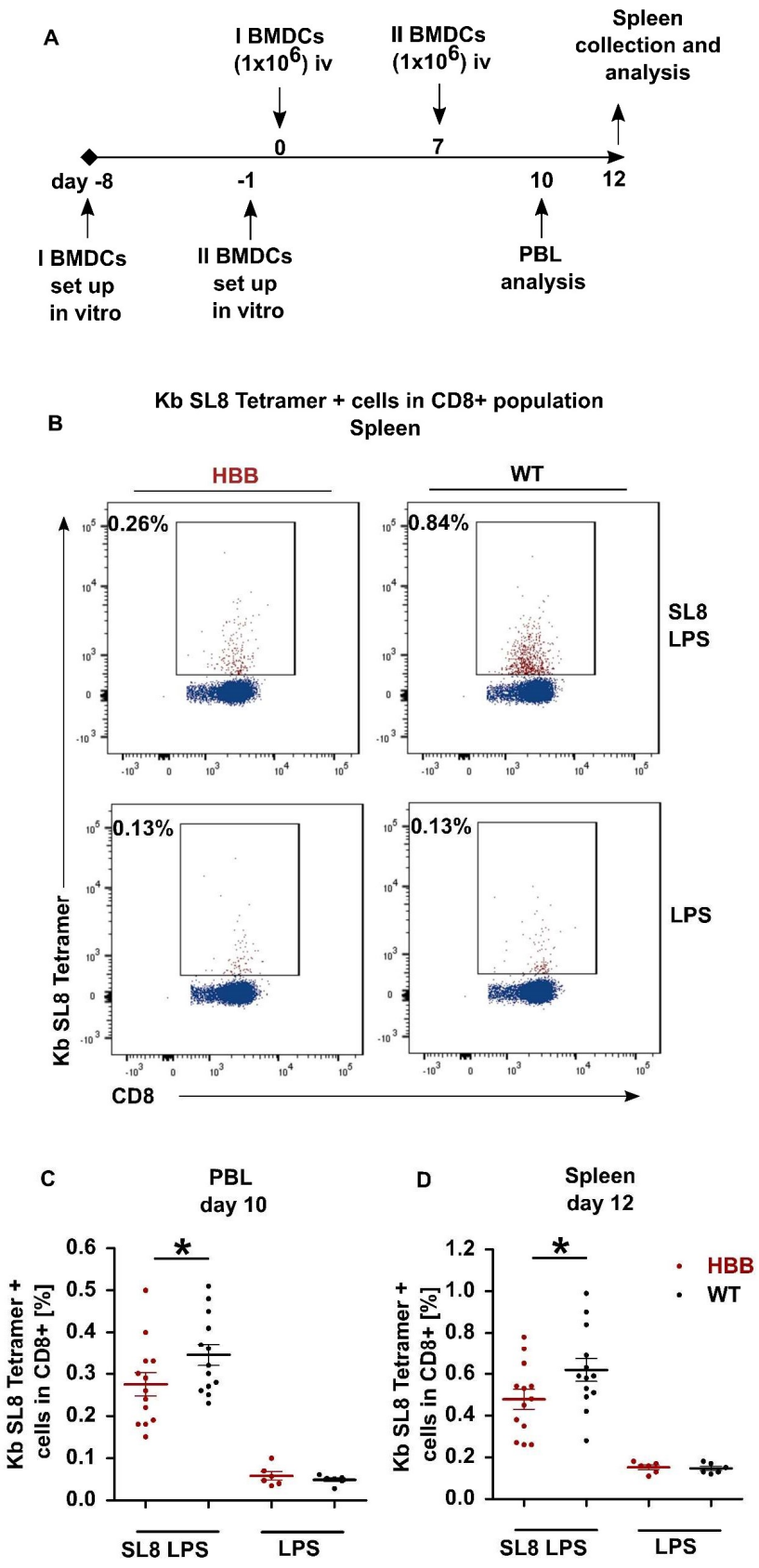


Figure 26. The analysis of endogenous SIINFEKL specific CD8+T cells responses in HBB mice. (A) 1x106 BMDCs pulsed with SL8 peptide, or not, and treated with LPS were injected intravenously (iv) in HBB and WT mice as indicated. (B) Following (A), FACS analysis was

performed with Kb-SL8 Tetramer and showed the percentage of CD8+ T cells specific for the Kb-SL8 epitope in splenic cells of HBB mice (0.26%), as compared to WT mice (0.84%). (C and D) show the percentage of Kb-SL8 Tetramer+ cells in peripheral blood lymphocytes (PBL) (C) or spleen (D) of HBB or WT animals following 10 or 12 days incubation, respectively (* $p<0.05$).

D. Tumor tolerance assay

To further address if intron-derived translation products generate tolerance and have impact on the endogenous responses against tumors expressing peptides from the same context, we have injected HBB mice with tumor cells stably expressing β-globin-SL8 construct and assessed its growth. To create the construct we used the β-globin-SL8 and cloned it into the pLVX-IRES-ZsGreen1 vector which also encodes for Green Fluorescence Protein under the IRES promoter. The vector expresses the two proteins from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection by flow cytometry. The construct was transduced into MCA205 mouse fibrosarcoma cells (MCA205) and the transduction efficiency was assessed by Flow Cytometry. Positive GFP signal shows over 99% transduction efficiency of β-globin-SL8 in MCA205 cells (Fig. 27).

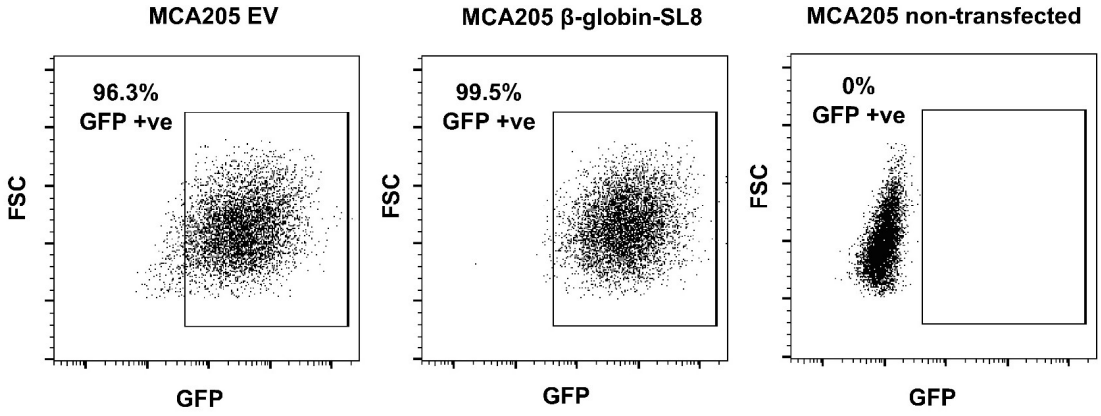


Figure 27. FACS analysis of GFP positive mouse fibrosarcoma MCA205 cells stably expressing empty vector (EV) or β-globin with SIINFEKL in intron2 (β-globin-SL8) in pLVX-IRES-ZsGreen1 vectors as compared to the non-transfected MCA205 cells. The vector expresses the two proteins from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection by flow cytometry. Positive GFP signal shows over 95% transduction efficiency.

MCA205 b-globin-SL8 cells (5 x 10^4) were injected subcutaneously in the flanks of HBB and WT animals (Fig. 28 A). Tumor growth from three independent experiments was estimated 9, 16 and 21 days following injection (Fig. 28 B) or until the ethical point was reached. On day 21, the volume of the tumors injected in HBB mice was estimated to an average of size of 300 mm^2, while the size of tumors injected in wild type animals were less than 100 mm^2. There was a significant increase in tumor size in HBB animals after 21 days, as compared to WT mice (Fig. 28 C).

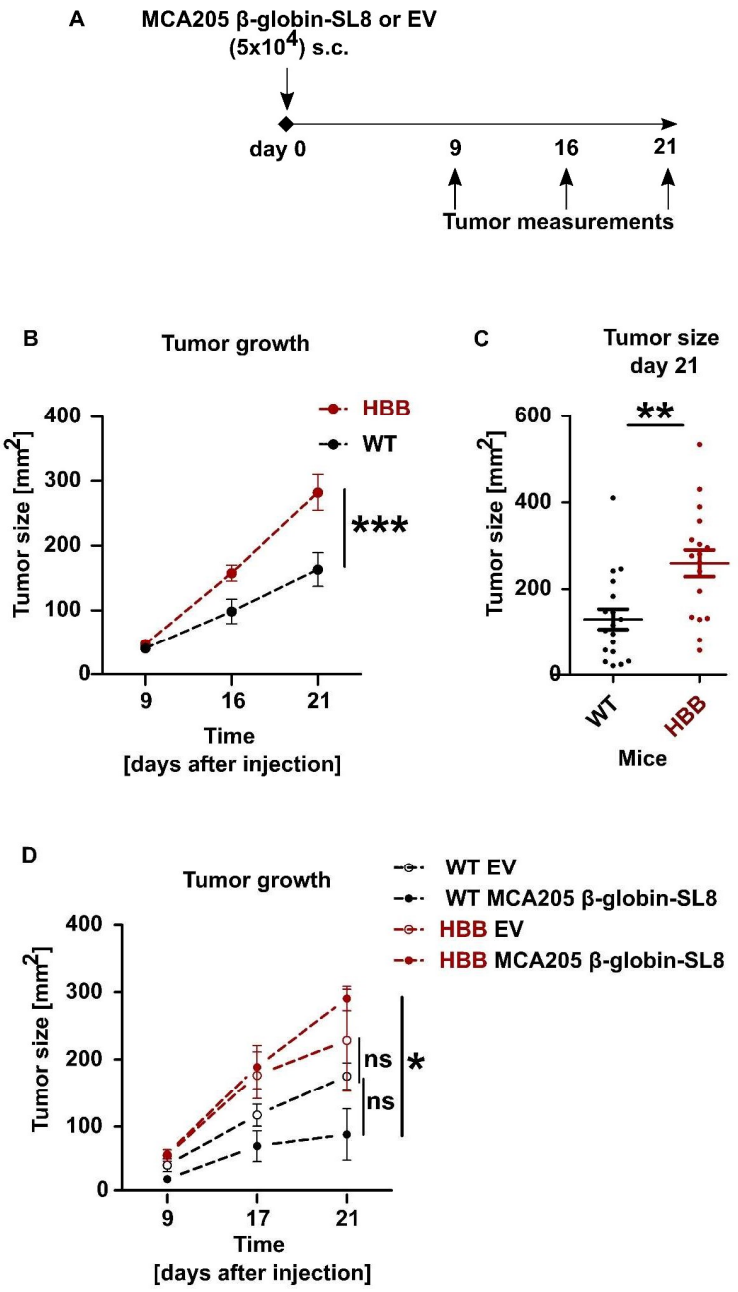


Figure 28. The scheme (A) of the experimental protocol in which 5x104 murine fibrosarcoma MCA205 cells stably expressing SIINFEKL in intron 2 of β-globin (β-globin-SL8) were injected subcutaneously (s.c.) in the flanks of HBB (red) and WT (black) mice. (B) Data represent tumor size (mm2) following injection of MCA205 cells expressing the β-globin-SL8 (Mean ± SEM) in HBB and WT mice over indicated time until the ethical endpoint is reached. (C) Post-mortem measurements of MCA205 tumor size (like in B) at day 21. Graph represents individual data of 3 independent experiments with a total of 18 WT and 17 HBB animals (*** p<0.0005; ** p<0.005). Graph (D) shows MCA205 EV (empty circles) and

MCA205 β-globin-SL8 (full circles) tumor growth over time in HBB mice or WT mice and indicates no significant difference in sizes of tumors carrying EV (ns $p>0.05$) in both mice strains as opposed to tumors expressing β-globin-SL8. (D) Data represent single experiment with 3 individual mice in each group (* $p<0.05$).

In order to assess HBB mice responses to tumors without the SIINFEKL precursor we created a construct expressing only GFP from the empty vector (EV). The transduction efficiency in MCA205 cells was also evaluated by FACS and shows over 95% of positive signal (Fig. 27). The assay was performed as described above and the evaluation of tumor size showed no significant difference between HBB and WT mice (Fig. 28D).

VIII. Establishment of double transgenic mice encoding SIINFEKL sequence in the intron2 of the mouse beta-globin gene (HBB), SIINFEKL specific T cell receptor (OT-1) on recombination activating gene 1 (RAG-1) knock-out (KO) or sufficient C57BL/6 N background

In order to prepare next models for further studies of the effect of intron-derived SIINFEKL on the mechanisms of immune tolerance we developed double and triple transgenic mouse models.

A. Establishment of line and identification of triple transgenic mice

HBB mice were crossed with OT-1 mice on RAG-1 KO or RAG-1 sufficient background. The first generation of pups (F1) inherited heterozygous β-globin gene with SIINFEKL in the intron 2, hemizygous copy number of transgenic T cell receptor specific to SIINFEKL-MHC I complex and hemizygous copy number of RAG-1 gene. We have further crossed these triple transgenic of hetero and hemizygous genetic backgrounds to generate population F2. Mice with complete RAG-1 KO background have no B cells no CD4+ T cells and no CD8+ T cells unless the transgenic T cell receptor is expressed. Hence, we have used this phenomenon to indirectly identify RAG-1 KO mice expressing transgenic T cell receptors specific to SIINFEKL-MHC I complex in F2 generation. Blood samples were collected by a punch of submandibular vein and prepared for FACS analysis as described in Materials and Methods. Samples were stained with a viability dye, anti-CD3 and anti-CD19 antibodies.

Out of 152 screened mice from F2 generation we identified 20 animals that were CD19 negative and CD3 positive. Within this group there were 3 mice (2 females and 1 male) that inherited homozygous HBB gene with the SIINFEKL in intron2, 13 mice (7 females and 6 males) with HET HBB gene and 4 WT (2 females and 2 males). That allowed us to establish breeding couples for next generation F3. However, this method did not allow us to distinguish between the mice with homo- or hemizygous status of OT-1 transgenic T cell receptor.

Therefore, we established and performed the analysis of a copy number variation (CNV) with the use of primers and probes listed in Tables number 5 and 6. Simultaneously, all the mice were genotyped for the presence of SIINFEKL knock-in in the β-globin gene using methods described in previous chapters.

B. Establishment of HBBxOT-1 line on RAG-1 sufficient background

In order to obtain the line which carries both SIINFEKL in the intron 2 of the β-globin gene, OT-1 TCR and RAG-1 the HBB mice were crossed with OT-1 mice of sufficient RAG-1 background. Mice were identified using the same methods as described in the previous chapter.

Around the time of thesis submission all homozygous mice strains successfully reproduced and delivered pups that will undergo FACS analysis of SIINFEKL specific CD8+ T cells with the use of Kb SL8 Tetramers.

DISCUSSION AND PERSPECTIVES

In order to have an experimental model to study the biological functions of intron derived antigenic peptides we created the β-globin-SL8 construct which encodes SIINFEKL sequence in the intron 2. Studying the context in which the SL8 epitope is generated *in vitro* helps us better understand the mechanism of translation of intron-derived peptide precursors and gives the possibility to translate this knowledge to animal studies. The concept of antigenic peptide precursors coming from so-called non-coding regions of genes e.g. introns, was thoroughly studied by our group and led to the introduction of Pioneer Translation Products. Here, we wanted to take forward the knowledge from *in vitro* studies to investigate the potential physiological role of intron-derived antigenic peptides in animal models. Therefore, based on previous studies described in the introduction we designed β-globin-SL8 construct of murine origin and studied it in order to verify its suitability to generate a mouse model with the same β-globin-SL8 sequence (Fig. 5A) [87], [88].

We first addressed the basic question regarding the influence of the SIINFEKL insertion in the intron 2 of the β-globin gene on splicing. The special design of primers made it possible to verify it by RT-qPCR. Hence, the forward primer annealed to the short nucleotides sequence at the 3' end of the exon1 and the 5' beginning of the exon 2. The reverse primer in turn was complementary to the 3' end of the exon 2 and the 5' end of the exon 3. Both primers could only anneal if the exons were correctly spliced (Fig. 5 A and Table 7). The analysis performed by RT-qPCR gave clear positive signal of correctly spliced β-globin-SL8 mRNA as compared to RNA from cells transfected with empty vector (EV).

Knowing that the SIINFEKL (SL8) insertion did not affect splicing we further investigated the levels of SL8 encoding pre-mRNAs using also RT-qPCR method but different primers pairs (Fig. 6 and Table 7). We were able to identify both intronic and exonic mRNA regions (Fig. 6). After normalisation we see similar levels of pre-spliced mRNA identified by both primer pairs specific to the modified region in the intron2 (NeoR/NeoR and Ex2/I2SL8). We also see higher expression levels of exon2 mRNA which comprises both the pre-spliced and spliced RNAs.

In order to investigate whether the SIINFEKL in the context of β-globin-SL8 construct can be used as a source for MHC-I pathway we performed a functional *in vitro* antigen presentation assay. The release of IL-2 by OT-1 CD8+ T cells is a specific and sensitive detection system to verify MHC-I antigen presentation. By co-culturing OT-1 cells with human cells co-transfected with β-globin-SL8 construct and Kb, we able to detect significantly higher levels of IL-2 as compared to cells transfected with empty vector (Fig. 7). This indicated prominent presentation of intron-derived SIINFEKL. The same assay helped us also understand better the capacity of generation of antigenic peptides from the intron as compared to full-length Ovalbumin (OVA). We see significant difference in the levels of antigen presentation between β-globin-SL8 and OVA, and those differences can trigger important questions regarding the origin of the SIINFEKL precursor in the OVA context. However, it is not possible to determine if SIINFEKL is produced during the degradation of full-length protein by the proteasome or rather during the pioneer round of translation via a temporarily different translation mechanism when encoded from an open reading frame. It should also be taken into account that when comparing the presentation of an AP derived from different context it is possible that the flanking sequences influence peptide processing. Regarding the source of MHC I peptide substrates a previous study showed in cells transfected with the *OVA* mRNA, that the synthesis of MHC I antigenic peptides stops well before that of the synthesis of full-length proteins [87]. The IκBα requires Ser32 and Ser36 in order to be ubiquitinated and degraded by 26S proteasome following treatment with TNFα [201]. This makes it an interesting model to test if ubiquitinated full length proteins are a source of MHC I peptides. When SIINFEKL was fused to IκBα and after treatment with TNFα there was no difference in antigen presentation from the wild type IκBα or an IκBα carrying mutations in codons 32 and 36 [87]. These data support the hypothesis that degradation of full length proteins via the ubiquitinated pathway is not a source of antigenic peptides for the MHC I pathway.

Hence, we next addressed the question whether indeed the SIINFEKL peptide precursor is indeed translated by a temporarily different mechanism from full-length proteins. To address this question we have used a polysome profiling method (Fig. 8) to evaluate the β-globin-SL8 mRNA distribution profile and translational status. Polysome profiling allows to separate by molecular weight the RNAs from total cell lysates that are attached to ribosomes. At the beginning of the fractionation profile the spectrophotometer detects free mRNAs that are not bound to ribosomes and it is indicated by the highest pick in the diagram. Next 3 peaks indicate forming ribosomal 40S, 60S subunits and then fully formed 80S monosomes. Further peaks represent LMW polysomes and the longer the RNA is and the more ribosomes are attached to it, the heavier it is. Hence, such mRNA can be further identified in the latest fraction collected during separation on HMW polysomes. It has been also described that polysome distribution indicates the robustness of mRNA translation [202]. The heavier the polysomes are detected on particular mRNAs the more robust expression is. The isolation and qPCR analysis of mRNAs from collected fractions allowed us to investigate which mRNAs are being translated. qPCR analysis on RNAs from pooled fractions 3-8 followed by gel electrophoresis (Fig. 9) revealed that both pre-spliced and spliced mRNAs are detected on forming ribosomes. indicating the possibility of translation from all reading frames.

The analysis of the relative mRNA levels in individual fractions revealed that the majority of the pre-spliced and SIINFEKL encoding mRNAs are present in fractions containing LMW ribosomes (Fig. 10 A). It indicates that the part of intron 2 which encodes SIINFEKL is present on the ribosomes and is actively translated. Taking into account that spliced mRNAs were detected in fractions containing heavy polysomes (Fig. 10 B) the above data suggests that these particular mRNAs can be translated at a different rate. Those differences may also become a basis to the hypothesis that different ribosomes can translate spliced or pre-spliced mRNAs. This would in fact go along with previous data published by our team, which indicated differences in the recruitment of translation initiation factors between antigenic peptide precursors and full-length proteins [88]. It would be very

interesting to further perform comparative proteomic analysis on the ribosomes from different fractions.

The polysome fractionation analysis performed on transfected cells treated with splicing inhibitor Isoginkgetin showed an increase in SIINFEKL encoding pre-spliced mRNAs as compared to DMSO controls in fractions with LMW polysomes. Interestingly, we see a shift in the levels of those mRNAs towards the fraction number 4 which contains fully formed translating LMW polysomes. As expected, the analysis also reveals the decrease of spliced mRNAs in ISO treated cells as compared to DMSO, which indicates splicing inhibition by the drug treatment. However, it is interesting to see higher levels of spliced mRNAs in ISO treated samples in fractions number 4 and 5, which also contain LMW polysomes. It may be due to the fact that the fraction of mRNAs detected by the primers specific to the exon 2 may in fact detect not only spliced but also pre-spliced mRNAs. Taken together, splicing inhibition by commercially available drug Isoginkgetin results in the upregulation of SIINFEKL peptide precursor translation and in a shift of overall β-globin-SL8 translation towards LMW polysomes (Fig. 10 C).

The data achieved from polysome profiling analysis is consistent with the results obtained from PLA assay which has many applications but most importantly for our study it enables sensitive detection of protein-protein interactions [203]. Since sensitive and direct peptide detection remains challenging we have decided to adapt this technique to detect SIINFEKL peptide precursors. In order to perform the assay *in vitro* special custom made antibodies were generated in rabbits and goat (Fig.11). Due to the fact that the length of peptide precursor was not known prior commencing animals' immunisation, it was challenging to predict the length of peptide immunogens that would trigger effective B cells response in animals and in the same time generate specific antibodies towards the SIINFEKL target. Hence, we have limited ourselves to generate peptides of 13 amino acids long and each containing 4 SL8 residues and its flanking amino acids residues respective to sequences from the intron 2 of β-globin-SL8 construct. To enhance the immunogenicity of these peptides the KLM immunogen was coupled to cystein at the C terminus of peptide II.

However, in case of peptide I additional cysteine residue was adjoined to the N terminus to enable KLM coupling. Animals were immunised with the mix of the peptides and antibodies levels in sera were monitored by ELISAs performed by Eurogentec. After the termination of immunisations program sera were further purified on the resins loaded with biotynylated peptides without KLM, which allowed to enrich the pool of antibodies recognizing peptides epitopes. The specificity of achieved polyclonal antibodies was further tested by ELISA. Such generated antibodies are a valuable tool in studies of intron-derived SIINFEKL peptides and can be applied to a wide range of immunoassays besides PLA, e.g. radioactive detection of peptides precursors expression levels. Here, we have focused on the detection of intron-derived SIINFEKL precursors in transiently transfected cells in order to verify its translation. The results from this study showed that treatment of transiently transfected cells with Isoginkgetin resulted in a significant increase in the amount of PLA signal which translates into the increase of SL8-carrying peptide substrate. Importantly, immunohistochemistry analysis with antibodies detecting full-length β-globin protein revealed decrease in GFP signal, meaning that splicing was effectively inhibited in those cells and resulted in downregulation of β-globin translation (Fig. 12 A and B). Since the SL8 peptide precursor and β-globin full-length proteins are encoded in different reading frames in the β-globin-SL8 construct it is worth taking into account the possibilities of different translation mechanisms of each product as well as the role of splicing inhibition in modulating these mechanisms. Data presented here go along with previous studies in which splicing inhibition increases MHC I antigen presentation as verified *in vitro* [88]. Other studies have also shown the impact of Isoginkgetin derivatives on shaping immunosurveillance by enhancing tumor associated antigen presentation [137].

Noteworthy is also another aspect of intron-derived peptide translation, namely the intracellular space where it can happen. The PLA analysis presented in these studies visualised SIINFEKL precursor both in cytoplasmic and nuclear compartments. However, in order to draw conclusions from these results it is necessary to discuss a few important aspects as well as answer more questions. Namely, as it's been already pointed out in the

results sections, the difference between signal detected in DMSO treated β-globin-SL8 non-transfected and transfected cells is statistically non-significant. It indicates a high signal background detected in all cells and hence should trigger cautiousness in drawing conclusions regarding compartmentalisation of translation mechanisms. Further optimization on antibodies dilutions should be carried out to reduce the detection of background nonspecific signals. Especially that it may clear out the differences between the current means between β-globin-SL8 negative and positive DMSO treated cells (Fig. 12 A and B). The automated quantification of these samples revealed that the mean number of PLA signals per transfected cell is 12 whereas the mean in non-transfected cells is 10. Reducing the background may help to precise those differences as well as indicate localisation of peptide precursors. In the future, high accuracy of the method would be helpful in determining co-localization of peptide precursors with other factors not only associated with translation but also with antigen processing for the MHC I pathway. Nevertheless, several groups including ours have been reporting on the possibility of nuclear translation [88], [106], [139], [204] and besides the fact that the topic remains underappreciated, with the emergence of new technologies it periodically returns to laboratory benches to undergo scientific scrutiny. It has been shown before that blocking mRNA export from nucleus also resulted in the increase of generation of MHC I antigenic peptides from introns as shown by their enhanced antigen presentation. Interestingly this study also showed that antigen presentation from the exonic context was inhibited along with the arrest of mRNA nuclear export [106]. The data presented here by no means prove this but they clearly state enhanced translation of antigenic peptide precursors on pre-spliced mRNAs and therefore add to what's been already echoing in this area for a long time and is worth further investigation.

Above data indicate that SIINFEKL encoding pre-mRNA is actively translated, however it doesn’t show how long the peptide precursor is, or which codon is used for translation initiation. In order to address these questions we have further investigated the 3rd frame of β-globin-SL8 construct in which SIINFEKL amino acid sequence is encoded (Fig.

13). Substitution of AUG codons to AUC upstream SIINFEKL did not block the antigen presentation meaning that other codon than methionine is used for translation initiation (Fig. 15). It's been shown previously that translation can initiate at CUG codons and that single synonymous point mutations from CUG to CUC in positions 114 and 124 in ORF of ovalbumin significantly decreases antigen presentation [205], [206]. Silent mutations of codon 39 within ORF of Myelin Basic Protein (MBP) altered antigen presentation of MHC I epitope and in parallel with proteasome inhibition revealed alternative translation initiation of shorter polypeptide [87]. Substitution of CUG codons upstream SIINFEKL to CUC in the β-globin-SL8 context also did not stop antigen presentation (Fig. 13 and 15). These results indicate that neither AUG nor CUG codons are responsible for translation initiation of the SIINFEKL precursor and that potentially other codons may play a role. It has been shown in the past that other leucine or valine codons can initiate translation and that such translated products are a source for MHC I pathway [140]. On the other side, results presented here highlight also the importance of RNA structure in the regulation of the translation process. Simultaneous substitution of AUG and CUG codons in positions +12 and +15 nts upstream SIINFEKL significantly increased the level of antigen presentation (Fig. 15 construct A) as compared to β-globin-SL8, while keeping similar levels of mRNA expression (Fig. 14 A).

It has been shown before that mRNA structure can regulate the protein expression process by changes in 5' UTR regions [207], [208]. The data presented here may open for the new concept that RNA structure may also regulate translation mechanisms of mRNA regions that are so far perceived as 'non-coding'. However, knowing that changes in both AUG and CUG codons did not stop antigen presentation we undertook a different experimental approach to investigate the translation initiation regions upstream SIINFEKL. By substituting individual codons upstream SIINFEKL to UAA stop codon (Fig. 13 and 16) and subjecting those constructs to analysis by *in vitro* antigen presentation assay we were able to identify the region responsible for translation initiation of SIINFEKL precursor. Substitutions in positions +12 to +228 nts upstream SIINFEKL (Fig. 13 and Fig. 16) effectively stopped antigen presentation without affecting mRNA expression, meaning that

the translation initiates upstream of the codon +228 nts (construct no. 6). However, substitution in position +255 (construct no. 7) did not affect antigen presentation. This indicates that the synthesis of the SL8-carrying peptide substrates is initiated within 27 nts between +228 (no. 6) and +255 (no. 7). In this 27 nts sequence there are two adjacent leucine (CUG) codons in frame with the SL8 but introducing synonymous mutations in the third position in both codons (CUG>CUC) did not affect expression of the SL8-carrying peptide substrate (Fig. 15 construct E). Knowing that it may not be up to a single codon to initiate the translation process, we carried out a modified ribosome profiling protocol in which HEK293T cells expressing the β-globin-SL8 construct were treated with Harringtonin and Cycloheximide to maintain the ribosome in position of initiation in order to generate ribosome-protected RNA fragments (ribosome footprint) [197]. After RNAse treatment, the protected RNA fragments were isolated (Fig. 17 A) and linked with stemloop primers [198] in the 3' by reverse transcription. The qPCR analysis confirmed the presence of the +255 to +228 sequence in the pool of ribosome footprints (Fig. 17 B and Tables 2 and 3). Taking advantage of emerging techniques for studying the mRNA secondary structures performing SHAPE-MaP analysis on β-globin-SL8 construct could help in further understanding of noncanonical translation mechanisms [209].

Several reports have implicated alternative mRNA translation events, including nuclear translation, co-transcriptional translation and initiation at non-AUG codons but the physiological role of these events, or the functions of the encoded peptides, is not known [87], [88], [139], [202], [210], [211]. Therefore, in order to study the physiological role of intron-derived antigenic peptides we created a mouse model encoding SIINFEKL in the intron 2 of the β-globin gene (HBB mice) in the same position as in the β-globin-SL8 construct described so far. As described in the results section we see high pre-mRNA expression levels of knock-in specific sequence in blood, spleen and bone marrow in HBB mice (Fig. 21 A) The insertion in the intron 2 did not affect the splicing nor expression level of β-globin protein as confirmed by WB (Fig. 22). Since β-globin chain is a subunit of hemoglobin it was important to investigate whether SIINFEKL knock-in in the intron2 did not

affect the morphology and function of red blood cells (RBCs). It is known that certain mutations within the β-globin sequence may alter the transcription and translation and result in different forms of β-thalassemia or sickle cell anemia [131]–[133]. Blood analysis of HBB mice showed normal levels of RBCs count and hematocrit (Hct) which were of no difference as compared to WT animals (Fig. 23). The analysis has also indicated equal hemoglobin levels in both groups. There was also no difference in mean corpuscular volume (MCV) which describes the average volume of RBCs and if deviated from the norm indirectly indicates hemoglobin alterations or its abnormal distribution within the cells [212]. Taking together, these results show that SIINFEKL knock-in in the intron 2 of the β-globin gene did not affect the protein expression and function in HBB mice. It also did not affect the hemoglobin formation and function.

Knowing that SIINFEKL pre-mRNA is expressed in HBB mice we wanted to know whether it can be translated and presented *in vivo*. We addressed this question by performing an *in vivo* antigen presentation assay (Fig. 24) with the use of SIINFEKL specific CD8+T cells from OT-1 mice that are CD45.2+ (OT-1 cells). OT-1 cells' response to Kb-restricted SIINFEKL peptide has been well characterized in the literature and here they have been used to evaluate whether intron-derived antigenic peptide can be presented in HBB mice [213]. Adoptive transfer of 2 x 10^6 OT-1 CD8+ T cells labelled with CellTrace Violet to HBB animals (CD45.1+) followed by CD45.2+CD8+ T cell isolation revealed an average 2,5-fold increase in OT-1 cell proliferation, as compared to OT-1 cells injected in wild type (WT) mice (Fig. 24 B and C). This data indicates the recognition of SIINFEKL-MHC I complex by OT-1 cells and means that SIINFEKL antigen is presented *in vivo*. Despite the efforts undertaken we were unable to show precisely which cell population presents the antigen. However, taking into account that the knock-in specific pre-mRNA is expressed in splenic mononuclear cells depleted of RBCs (Fig. 21 B), we can speculate that the SL8 peptide can be presented by erythrocyte precursors prior enucleation or by pAPCs via cross-presentation. SIINFEKL precursor potentially could be subjected to both direct MHC I presentation pathway or cross-presentation. It has been previously shown that peptide

precursors originating from introns can be substrate for both pathways, however in this context, the exact mechanism remains to be understood [156].

Taking into account that presented intron-derived antigenic peptide induces OT-1 CD8+ T cells proliferation we have further investigated whether repetitive injections of SIINFEKL-specific CD8+T cells (OT-1) to HBB mice would have an effect on mice health (Fig. 25). After the injection of naive OT-1 T cells we did not see any change in the animals' behaviour that would otherwise indicate illness. Therefore, next injections were performed with the OT-1 cells preactivated *ex vivo* and the expression of proliferation and activation markers were assessed by RT-qPCR (Fig. 26). Preactivated OT-1 cells were highly proliferative as indicated by Ki67 upregulation compared to naive cells. The downregulation of IL-2 and slight decrease of OT-1 TCR expression are also common indicators of T cells intensive proliferation and response to over 24h peptide stimulation [200], [214]. The inhibition of IL-2 expression has been also correlated with T cells exhaustion, hence to verify the T cells cytotoxicity we analysed the expression levels of FasL, GranzymeB and Perforin [215]–[219]. The results show upregulation of all 3 markers in preactivated OT-1 cells and indicate their cytotoxic capacity to respond to SL8 peptide presented on Kb molecules. The continuous injections of activated cytotoxic OT-1 cells also did not cause any severe pathology in both groups during the experimental time course. However, taking into account high expression levels of SIINFEKL encoding mRNA in spleen the organs were subjected to further histopathology analysis. The analysis revealed anatomical changes in structures of splenic mantle and marginal zones between HBB and WT mice, which indicates immunological reaction within the white pulp of HBB mice. However, the nature of that reaction would need to be further investigated. There are few plausible scenarios which may be worth evaluating in the future. Knowing that injected OT-1 CD8+ T cells had cytotoxic capacities it would be interesting to investigate potential cytotoxic (apoptotic or necrotic) markers in the spleens of HBB mice and look for colocalization of dying cells with OT-1 cells which would determine if the antigen was presented directly or by cross-presentation. Direct

antigen presentation from β-globin *in vivo* may seem controversial due to the fact that mature RBCs are enucleated and hence do not express MHC I molecules. However recent studies on mice infected with *Plasmodium yoelii* revealed MHC I expression on immature RBCs – reticulocytes, both in spleen and bone marrow [220]. Spleen in mice are known to participate in erythropoiesis which distinguish them from humans where the development of RBCs is restricted to bone marrow [221]. Nevertheless, described phenomenon makes HBB mouse a suitable model for further investigation if intron-derived antigenic peptides can be processed and presented in reticulocytes via direct pathway.

Taking into account that spleens main function is filtering blood and removing non-functional erythrocytes via specialized macrophages it would be also interesting to investigate their role in mediating MHC I antigen cross-presentation. Recent studies have shown that murine $CD11c^{int}F4/80^{high}$ splenic red pulp macrophages are able to process MHC I OVA antigen and cross-present it to CD8+ T cells via cytosolic pathway both *in vivo* and *in vitro* [222]. Taking into account that macrophages have been mostly associated so far with processing and presentation of exogenous antigens it would be very interesting to investigate their potential contribution into MHC I pathway and mediating immune tolerance responses.
On the other hand, it is also possible that splenic cells from HBB mice were in fact triggering responses against OT-1 cells to prevent their reactivity against self peptide-MHC I complexes. In that case, it would be interesting to investigate the role of tolDCs and Tregs in regulating CD8+ T cells toxicity. Histopathology analysis from this experimental model provides another proof that intron-derived antigenic peptides play an important role in shaping immune responses *in vivo*. Interestingly, there is no difference in spleens' anatomy between WT and HBB mice in steady state. Knowing that the SIINFEKL peptide is presented in HBB mice, it led to think that those mice in fact tolerate intron-derived SL8 antigen.

In next experimental approaches we wanted to answer a more complex question, namely whether an intron derived antigenic peptide can shape endogenous CD8+T cells repertoire. By performing immunisation with LPS treated and SIINFEKL pulsed DCs (Fig. 26)

we were able to show that HBB mice have reduced numbers of SIINFEKL-specific CD8+T cells in peripheral blood lymphocyte population as compared to WT mice (Fig. 26 A and C). Knowing that SIINFEKL encoding pre-mRNA is expressed in spleen we further evaluated for possible accumulation of self SIINFEKL specific CD8+T cells in this organ. Interestingly, we also see significantly reduced numbers of those cells in the spleen of HBB mice as compared to WT animals (Fig. 26 B and D). For a long time it wasn't known why such an independent translation mechanism of so called 'non-coding' RNAs would be needed physiologically, however we show that antigenic peptides originated from pre-spliced mRNA could be used *in vivo* for generation of immune tolerance towards self-immunopeptidome. Main pathways responsible for shaping T cells repertoire take place in the thymus. In this organ developing thymocytes undergo positive and negative selection, once exposed to self-peptide:MHC complexes on cTECs and mTECs as well as professional antigen presenting cells (e.g. macrophages and dendritic cells) [223]–[225]. As a result of this process only T cells with particular moderate avidity towards self antigens exit thymus to the periphery ready to respond to pathogens or cancerous cells. All other T cells that bind to the antigens too strongly are destroyed, however, in some circumstances they escape to the periphery where the mechanisms involved in the peripheral tolerance perform a second round of control in order to prevent any autoimmune diseases. The fact that self-peptides expressed in thymus play a role in such important mechanisms and protecting us from immune malfunctions highlights even more the importance of knowing the real source of antigenic peptides for MHC pathways. Thymus contains only a low percentage of mTECs or cTECs and pAPCs compared to the number of maturing thymocytes [226]. It would be rather impossible to express all the tissue-restricted proteins suitable for the 26S proteasome degradation pathway in order to generate all necessary MHC I ligands in a single organ. Also one should think of particular biological and toxic consequences caused by potential expression of all functional proteins in one place. Therefore, expression of self-antigens from pre-spliced mRNA by a noncanonical translation pathway in the thymus, would explain how the immune tolerance is being generated towards all different splice variants of tissue-restricted proteins.

AIRE has been implicated in the mediation of PGE expression in thymus and it's deficiency has been associated with several CD8+ T cell mediated autoimmune disorders [183], [186]. HBB mouse model gives the opportunity for future studies of AIRE involvement in regulation of tolerance mechanisms towards intron-derived MHC I epitope. For example *in vivo* silencing of AIRE could indicate any changes in levels of SIINFEKL specific CD8+ T cells in periphery as well as their potential cytotoxicity. Importantly, our data support the notion by indicating reduced numbers of SIINFEKL-specific CD8+T cells after mice immunizations. We do not know with what avidity the remaining CD8+ T cells bind to SL8-MHC tetramers, however we can only speculate that those which are still in the periphery have escaped the central mechanisms in the thymus. Alternatively, they may be of mild to moderate binding capacity and hence could be relatively easy controlled under steady state conditions by tolerogenic DCs from causing any autoimmune reaction. However, due to the fact that raised quantities of SIINFEKL-specific CD8+T cells in HBB mice are present as compared to levels detected in mice immunised with LPS+ DCs only, it would be interesting to investigate further T cells phenotype. This would give a better understanding about the nature of interaction between SL8 pulsed DCs and endogenous T cells and maintaining or break of peripheral tolerance mechanisms [227]. Another important aspect of this experimental approach relates to the concentration of synthetic SL8 peptide used for DCs pulsing prior injection. In our experiments we have used 1µg/ml/mln DCs which could potentially contribute to breaking safeguarding peripheral mechanisms and resulting in increasing the numbers of self-SIINFEKL specific CD8+T cells in HBB mice. Taken together this data and data from repetitive adoptive transfers they highlight the importance of identifying well sources of self immunopeptidome prior designing treatment strategies for cancer patients. The mechanisms described above are commonly discussed in the area of adoptive T cell therapies. As in any other treatment it unfortunately is also a source of adverse events due to off-target toxicities [228]–[231]. Patients undergoing such therapies should also undergo lymphodepletion to shut off the regulating mechanisms that would inhibit the reactivity of transferred cytotoxic CD8+ T cells. These procedures can generate potential off-target autoimmune reactions

triggered by other self reacting T cells which would otherwise be kept in guard, unresponsive [232]. On the other side, poorly known sources of antigenic peptides are a reason for low specificity of those treatment methods towards tumors and also lead to off-target reactivity in other tissues [228]–[230]. These adverse events highlight the gap in fundamental knowledge regarding sources of antigenic peptides for the MHC I pathway and the results presented here give explanation to key questions posed during the prediction of TAAs. On the other hand, the difference in levels of endogenous CD8+T cells specific to SIINFEKL between HBB and WT mice may add to explanation of low efficacy of DCs based vaccines in the treatment of certain cancers with poorly identified TAAs. Anti-cancer vaccination with DCs is a promising approach to cancer treatments, especially after FDA approval of Sipuleucel-T for late-stage prostate cancer [233]. However, the treatment raised multiple concerns including failure in translating CD8+T cells recognition of chimeric PA2024 antigen to physiologic PAP (prostatic acid phosphatase) antigen in tumors [234]. Currently there are many other clinical trials investigating the potential of DCs as immunotherapies for multiple malignancies – AML/MDS, melanoma, glioma and glioblastoma, lung and prostate cancers as well as lymphomas [235]. Our data gives better understanding of the role of intron-derived antigenic peptides in response to vaccination stimuli. It would be very interesting in the future to investigate other vaccination methods that are currently undergoing clinical trials, e.g. peptides vaccination and the effect of intron-derived self antigens on the treatment efficacy. However, in the long term the data presented here highlight the need for improvement of bioinformatics tools and the inclusion of non-coding genomic regions in predictions of self-immunopeptidome and identification of antigens specific to tumors or viruses as it may have a great impact on overall patients safety and treatments efficacies.

Interestingly, the data from tumor growth experiments have shown that HBB mice are not able to respond to cancer cells expressing peptide precursors from a known genomic context (Fig. 28). It was clear that overall immune responses in HBB mice are intact and do not differ from WT mice as shown by the growth of tumors expressing empty vector (Fig. 28 D). Hence, the lack of response towards SIINFEKL expressing tumors was due to tolerance

mechanisms. However, we cannot omit here the intricacy of the tumor microenvironment and its complex interactions with the host. The immune-suppressive pathways on the tumor's side play an important role in hiding from the host's detection as it's been shown by many examples [236], [237]. Our data imply the necessity for inclusion of whole genome sequencing (WGS) analysis and particularly the intronic regions in predictions of MHC I antigenic peptides in order to improve the preventive and therapeutic cancer vaccine design and overall prognosis of patients' survival.

Noteworthy is the fact that all experimental results presented here were performed in as much physiological conditions as possible. Several mice models expressing full-length Ovalbumin have been utilized to study immune tolerance mechanisms [238]–[240]. However these models are transgenic, expressing OVA mRNA and full-length protein under the control of different tissue restricted promoters (e.g. keratin or rat insulin promoter). Therefore, the levels of generated antigens do not mimic normal physiological conditions under which the central and peripheral tolerance mechanisms occur. Also, studying immune tolerance on these models do not answer fundamental questions regarding the real source of MHC I antigenic peptides as it is impossible to prove that an antigen encoded within exon is indeed translated by a non-canonical mechanism acting on pre-spliced mRNA. In those cases there will always be a doubt that the class I ligand was generated by the proteasomal degradation of full-length proteins translated by canonical mechanism rather than to look at protein synthesis as a real source. Hence, the HBB mice are the first model to study intron-derived MHC class I antigenic peptides and their role in shaping immune tolerance.

To take the studies from HBB mice forward, we have crossed them with OT-1 mice on RAG deficient or sufficient backgrounds. This led us to establish another two models to study the effect of intron-derived antigenic peptides not only on immune tolerance but also on induction of autoimmunity. Due to the fact that the mice are on triple or double transgenic background identification of mice has been established *de novo* during the course of the PhD program. At first we didn't have the established method for performing CNV analysis of the RAG gene the mice were identified indirectly by FACS phenotyping. For that we utilized the

phenomenon that RAG KO mice do not have any B cells nor CD4 and CD8 T cells unless the transgenic receptor is expressed. It allowed us to use only anti-CD19 and anti-CD3 antibodies to indirectly verify the genetic status of RAG and OT-1 TCR genes. Still, it did not allow us to distinguish between hemizygous and homozygous mice. Therefore, multiplex qPCR analysis has been utilized for the assessment of copy number variation between hemizygous and homozygous mice carrying transgenic OT-1 TCR. Since the breeding process of those mice required a long time, few preliminary experiments have been performed on mice heterozygous for HBB gene and hemizygous for RAG and OT-1 TCR backgrounds. The preliminary FACS analysis of OT-1 CD8+ T cells in those mice revealed that the cells were not deleted by central tolerance mechanisms and also that there was no difference between mice carrying HBB knock-in gene and WT animals. The interpretation of these results should be very careful due to the number of variables involved in these experimental models. However, they may also give insight into the role of intron-derived antigenic peptides in shaping immune tolerance. We do not see any signs of pathology in those mice and their life span is similar in both genetic backgrounds. Knowing from the studies performed on HBB mice that OT-1 cells proliferate after recognition of SL8-Kb complex *in vivo*, it will be important to investigate further the immune phenotype of those T cells. However, for clear understanding of achieved experimental data the analysis should be performed on animals of homozygous backgrounds of all three genes. On the other hand, once the OT-1 cells functional status is confirmed then the generated models will serve as great tools for studying the molecular mechanisms underlying immune tolerance to MHC I immunopeptidome.

CONCLUSIONS

The MHC class I presentation pathway plays a crucial role in the immune system and distinction between self and non-self. More studies report the presence of peptides originating from what's been previously thought non-coding DNA sequences. Also, we and others showed already that class I antigenic peptides originate during the early stages of ribosomal scanning during the pioneer round of translation. Here, we show that indeed the MHC class I peptide substrates can be translated from pre-spliced mRNA, without affecting the main open reading frame and the rate of translation of full-length proteins.

In these studies we have shown that intron-derived antigenic peptides are presented *in vivo* by MHC class I molecules and induce proliferation of specific CD8+T cells. We have also shown that adoptive transfer of specific and activated CD8+T cells causes some immunological reactions in spleens of HBB mice, where the SIINFEKL is expressed. However the nature of this interaction should be further investigated. On the other side, we described here the effect of immunization against self antigens which shows reduced endogenous levels of SIINFEKL specific CD8+T cells in HBB as compared to WT mice. Finally, we show that intron-derived antigenic peptides expressed by tumors from the known to host genomic context reduce overall capacities to anti-cancer response. The fact that hosts' immune responses towards known antigenic precursors expressed by cancer cells from pre-spliced mRNA can be diminished, influences also a drug discovery field. Our polysome profiling analysis showed here that pre-spliced mRNA is actively translated from an alternative open reading frame. Since the translation of full-length proteins and antigenic precursors are independent from one another, they potentially involve different ribosomal factors. Identifying the differences could bring a great improvement in the efficacy of drug design by targeting selective translation machinery to boost expression of antigens which have the highest probability of being identified as dangerous by the host's CD8+T cells. With the availability of bioinformatics tools and big data analytics it is more and more possible to compare the WGS data with cancer samples and hence decide which method of treatment would have the best treatment impact. Therefore, it is very important to expand research on

pre-mRNA translation and to better identify the exact mechanisms and cellular factors involved.

Taken together all the above studies highlight the importance of pursuing fundamental research in the area of Molecular and Cellular Biology and Immunology as they have the power to answer most intriguing questions regarding the safety and efficacy of certain immunotherapies of viral infections, cancer as well as autoimmunity. With the emergence of new technologies and expansion of the antigen processing and presentation field it is more and more possible to collectively answer all these basic questions. From a more personal perspective I hope the work presented here will be taken forward as well as be a source of scientific inspirations and thought provoking discussions.

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