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(Spikblad)

Tapasin

- The star of the show in HLA-I maturation

Camilla Thuring



LUND
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DOCTORAL DISSERTATION

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To be defended on Friday 20th March, 9.00 at
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Department of Immunology, University of Tübingen

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Abstract Human leukocyte antigen class I (HLA-I) molecules are present on the cell surface of all nucleated cells. They bind intracellular peptides and present them to the immune system thus providing a readout to cytotoxic T-cells about the status of a cell. Maturation of the HLA-I molecule includes peptide binding in the endoplasmic reticulum with the assistance of several other proteins collectively called the antigen processing machinery (APM). One of these proteins, tapasin, is of particular interest as it is dedicated to HLA-I and plays a key role in the selection and binding of peptides that induce stable HLA-I molecules. In this thesis I have studied HLA-I maturation, and tapasin in particular, both biochemically and in tumor material. The first paper is a study of HLA-I folding in the presence and absence of a part of recombinant tapasin (tpn1-87). Here we studied how different HLA-I allomorphs depend on tapasin and the influence of peptide length. This is the first study where a large number of HLA-I allomorphs have been simultaneously analyzed for tapasin facilitation. We found that the influence of peptide length for the different allomorphs increased with their tapasin dependence. In paper II and III we studied HLA-I, tapasin and other APM proteins in tumor sections and cell lines of glioblastoma multiforme (GBM). In tumors APM proteins are commonly downregulated as a strategy to evade the immune system. We also found the APM proteins in our tumor material to be highly dysregulated with strongly linked HLA-I and tapasin expression. In tumor tissue sections HLA-I and tapasin expression also correlated with survival of GBM patients. High resolution HLA-I typing allowed us to study the HLA-I allomorphs expressed in GBM patients and also each allomorph's tapasin dependence. We found that GBM patients display HLA-I allomorph profiles with mixed dependency of tapasin, similar as in a healthy cohort. In addition we show that tapasin deficient cells present suboptimally loaded HLA-I molecules on the cell surface. By exogenous addition of high affinity peptides we were able to increase the stability of presented HLA-A*02:01 molecules. The tapasin dependency of each allomorph as well as composition and proportions of HLA-I allomorphs presented on the cell surface is of importance not only for mechanistic understanding but also for immunotherapy settings in different diseases. GBM is an aggressive brain tumor with poor prognosis and there are high demands for new and more effective treatments. We propose individualized immunotherapy protocols where tapasin expression and tapasin dependency of allomorphs expressed in each patient are taken into consideration to improve the selection of peptide:HLA-I combinations for peptide vaccines.			
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Tapasin

- The star of the show in HLA-I maturation

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Papers included in the thesis

Paper I

Tapasin facilitation of natural HLA-A and -B allomorphs is strongly influenced by peptide length, depends on stability, and separates closely related allomorphs

Linda Geironson, Camilla Thuring, Mikkel Harndahl, Michael Rasmussen, Søren Buus, Gustav Røder and Kajsa Paulsson

Journal of Immunology, 2013, Volume 191, Issue 7, Pages 3939-47

Paper II

Tapasin and human leukocyte antigen class I dysregulation correlates with survival in glioblastoma multiforme

Camilla Thuring, Linda Geironson and Kajsa Paulsson

Anticancer Agents in Medicinal Chemistry, 2014, Volume 14, Issue 8, Pages 1101-9

Paper III

HLA class I is most tightly linked to levels of tapasin compared to other antigen processing proteins in glioblastoma

Camilla Thuring, Elna Follin, Linda Geironson, Eva Freyhult, Victoria Junghans, Mikkel Harndahl, Søren Buus and Kajsa Paulsson

Manuscript

Papers not included in the thesis

HLA-I antigen presentation and tapasin influence immune responses against malignant brain tumors - considerations for successful immunotherapy.

Anna Darabi, Camilla Thuring, Kajsa Paulsson

Anticancer Agents in Medicinal Chemistry. 2014, Volume 14, Issue 4, Pages 1094-1100

Histocompatibility, Chapter 10, MHC Class I Control

Gustav Røder, Linda Geironson, Elna Follin, Camilla Thuring, Kajsa Paulsson

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Abbreviations

ABC	ATP-binding cassette
APC	Antigen presenting cell
APM	Antigen processing machinery
ATP	Adenosine triphosphate
β_2m	β_2 -microglobulin
BAP31	B-cell receptor associated protein
BFA	Brefeldin A
BiP	Binding immunoglobulin protein
B-LCL	B-lymphoblastoid cell line
CNS	Central nervous system
COP	Coat protein complex
DC	Dendritic cell
DNA	deoxyribonucleic acid
DRiP	Defective ribosomal product
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERAP	Endoplasmic reticulum aminopeptidase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glioma cell line
HC	Heavy chain
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
ICC	Immunocytochemistry
IHC	Immunohistochemistry

IL	Interleukin
IFN	Interferon
LMP	Low molecular weight protein
LOCI	Luminescent oxygen channeling immunoassay
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PGE ₂	Prostaglandin E ₂
pHLA-I	peptide:HLA-I
pMHC-I	peptide:MHC-I
PLC	Peptide loading complex
RAG	Recombinase activating gene
STAT	Signal transducer and activator of transcription-4
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
Tapasin	TAP-associated glycoprotein
TAPBPR	Tapasin-related binding protein
TCR	T-cell receptor
TMZ	Temozolamide
TNF	Tumor necrosis factor
Tpn	Tapasin
TSA	Tumor specific antigen
UGGT	UDP-glucose:glycoprotein glucosyltransferase

Introduction to the immune system

Our immune system works to protect us from pathogens such as bacteria and virus. Immune responses are mediated by an array of different cells and mechanisms, which clears the body from organisms and substances that could potentially be harmful. The immune system can be divided into two major systems, the innate and the adaptive immune system.

The innate immune system is our first line of defense and includes the physical barriers such as skin and mucosa as well as several types of white blood cells, the complement system and the ability to recruit immune cells to a site of infection by the production of cytokines. It works in a non-specific manner and recognizes conserved molecular structures i.e. pathogen-associated molecular patterns (PAMPs) expressed by microorganisms such as viruses or bacteria. The effect of the innate immune system is immediate but does not provide any long-lasting protection to the host.

In contrast to the innate immune system, the adaptive immune system is highly specific and has the ability to recognize and selectively eliminate foreign microorganisms and molecules (i.e. foreign antigens). It is activated when the defenses of the innate system are breached and mainly made up of two types of lymphocytes, the B-lymphocytes and T-lymphocytes.

B-lymphocytes (B-cells) mature in the bone marrow and are responsible for the production of antibodies. The antibodies bind to antigen and thus prevent it from interacting with the host cells. Also, they make the pathogen recognizable to phagocytes, which can ingest and degrade the pathogen.

T-lymphocytes (T-cells) migrate from the bone marrow to mature in the thymus. These cells express T-cell receptors (TCRs) with which they can recognize antigen on the surface of an infected cell. For the antigen to be

recognized it has to be displayed in the form of a peptide presented by a major histocompatibility complex (MHC) molecule. T-cells are divided into two different subsets, which can be distinguished from each other by the presence of either CD4 or CD8 glycoproteins, also known as co-receptors, on the cell surface. CD4⁺ T-cells, or T-helper cells, recognize peptides presented by MHC class II (MHC-II) molecules on the surface of professional antigen presenting cells (APCs) such as macrophages, dendritic cells (DCs) and B-cells. Recognition and binding to a peptide:MHC-II complex result in activation of several mechanisms e.g. secretion of antibodies by activated B-cells, which aid the innate immune response in the elimination of extracellular antigens. The CD8⁺ T-cells, also known as cytotoxic T-cells, can recognize and kill an infected cell. The CD8⁺ T-cells recognize another class of the major histocompatibility complex, MHC class I (MHC-I), which is expressed on all nucleated cells.

To avoid autoimmunity it is important for the immune system to not react on self-antigens. Central tolerance is achieved during the maturation of B- and T-cells in the bone marrow and thymus. Immature B- and T-cells that recognize self-antigens are deleted or inactivated before they develop into fully immunocompetent cells. Newly formed B- and T-cells are sensitive to strong signals through their antigen receptor whereas the same signals would activate a mature B- or T-cell to initiate an immune response. This process is most active during fetal life but continues throughout life as new B- and T-cells mature. Tolerance can also be developed after the B- and T-cells have matured and entered the peripheral tissues. One example of this is anergy, which is a state of unresponsiveness where circulating T-lymphocytes, which failed to be removed during negative selection in the thymus, are made tolerant against proteins that are expressed in a high and constant concentration, like self-proteins.

The priming/activation of CD8⁺ T-cells most commonly takes place in the secondary lymphoid organs where they are activated by professional antigen

presenting DCs capable of cross presentation. The CD8⁺ T-cells then leave the site of activation to circulate the body and scan for infected cells. On the discovery of a cell presenting peptide:MHC-I (pMHC-I) complexes with altered self/non-self peptide matching the TCRs on the clonally expanded CD8⁺ T-cells results in a cytotoxic response killing the infected cell. Initiation of a cytotoxic response requires sufficient quantity and quality of the pMHC-I complexes. The affinity and number of TCR-pMHC-I interactions and the density per area of stable pMHC-I complexes, determine the duration of the interaction, and the outcome of the TCR-MHC-I interaction (Henrickson *et al.*, 2008).

The peptides presented on MHC-I are generated from intracellular self-proteins and, in a diseased state, also from viral or tumor proteins. Proteins are degraded into peptides by the proteasome and only a small fraction of all peptides produced binds to MHC-I. The peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) and then loaded onto MHC-I. MHC-I molecules are folded inside the ER supported by an array of different proteins included in the so-called antigen processing machinery (APM). The peptide is loaded while the MHC-I molecule is bound in the so-called peptide loading complex (PLC), which is of crucial importance for the formation of a stable pMHC-I complex. Once loaded with an optimal peptide, which induces a mature conformation of the pMHC-I complex, it is released from the PLC and transported to the cell surface.

A functional APM, including the PLC component tapasin, is crucial for the presentation of stable MHC-I molecules at the cell surface. Downregulation of APM components is commonly observed in tumors and serve as an immune evasion strategy to avoid recognition by T-cells. In our studies we have investigated the expression of APM components and MHC-I in a brain tumor called glioblastoma multiforme. We have focused particularly on tapasin and its role in the maturation of MHC-I molecules.

MHC class I and the antigen processing machinery

MHC gene organisation and polymorphism

Research in transplantation done in the mid-twentieth century provided insight in the rules that govern the acceptance or rejection of tissue when transplanted between members of the same species – literally histocompatibility. The experiments indicated that rapid rejection of a transplant was determined by a single gene, which was called major histocompatibility gene. Later studies indicated that this “gene” was a set of closely linked genes inherited linked as a unit and thus it became known as the major histocompatibility complex (MHC). The human MHC is known as human leukocyte antigen (HLA).

The HLA region is large, with over 200 genes spanning four mega bases, and located on the short arm of chromosome 6 and they are known to be the most polymorphic genes of the whole genome with over 9000 HLA-I alleles identified to this date (Feb 2015). However, not all of these alleles are translated into unique proteins since some differences in the deoxyribonucleic acid (DNA) sequences do not result in a difference at amino acid level and some alleles are null alleles. The 9000 different alleles give rise to about 6500 unique HLA-I protein products i.e. HLA-I allomorphs (HLA Informatics group, 2015).

The HLA-I molecules are divided into two groups, the classical and the non-classical HLA-I. The classical HLA-I genes are characterized by high sequence polymorphism and encoded by three genes, HLA-A, HLA-B and HLA-C. They are co-dominantly expressed meaning that each individual can express

between three and six different types of classical HLA-I molecules. The non-classical HLA-E, HLA-F and HLA-G constitute only a smaller fraction of the identified alleles. They have a structure and sequence similar to the classical HLA-I but are much less polymorphic. Also they are tissue specific, expressed at lower levels at the cell surface and bind a more limited repertoire of peptides. 98% of the HLA-I genes are made up by the classical HLA-A, -B and -C alleles and HLA-B is the most polymorphic (~40%) of the alleles followed by HLA-A (~32%) and HLA-C (~27%) (HLA Informatics group, 2015).

MHC-I structure

The MHC molecule is made up of two polypeptide chains, the polymorphic MHC-I heavy chain (HC) and the non-covalently bound invariant light chain called β_2 -microglobulin (β_2m) (figure 1a). During maturation the HC folds into three domains, α_1 , α_2 and α_3 . The α_3 domain contains a transmembrane region, which anchors the MHC-I molecule in the cell membrane and is also the main binding site for the CD8⁺ T-cell co-receptor, CD8. The α_1 and α_2 domains represent the most polymorphic part of the MHC-I molecule and they are bound together to make up the peptide binding cleft. After initial folding of the HC and association with β_2m , in the absence of a peptide in the peptide binding cleft, most MHC-I molecules require the association with ER chaperones to prevent unfolding and subsequent degradation. With no peptide, or alternatively a loosely bound peptide, the peptide binding cleft is in a more open state i.e. a peptide-receptive state, allowing peptides to be exchanged. When an optimal peptide binds the peptide binding cleft adopts a closed state. This structural change of the peptide binding cleft might also affect the structure of other parts of the molecule and the stability of the MHC-I increases to a level where it is not dependent on the association with ER chaperones any longer.

Peptide binding

Despite the high polymorphism in the peptide binding cleft some amino acids involved in the binding of a peptide via hydrogen bond formation are conserved and shared by all MHC molecules. These include a cluster of tyrosine residues on one side of the cleft where the N-terminal part of the peptide is bound and Thr-143, Lys-146 and Trp-147 in the α_2 -1 helix binding the C-terminal region of the peptide in the other end of the cleft (Praveen *et al.*, 2010). These interactions are independent on peptide sequence and have been shown to contribute the most to the binding energy (Bouvier & Wiley, 1994). The bottom of the MHC-I peptide binding cleft typically contains six pockets termed A-F (figure 1b). These pockets provide interaction sites for anchor residues in the peptide, which bind non-covalently in a sequence-dependent manner. The high polymorphism of the α_1/α_2 domains results in thousands of MHC-I allomorphs each with a distinct preference for certain amino acids at specific positions in the peptide, so called anchor positions, which can fit into the pockets (Falk *et al.*, 2006). Although the preferences for peptide anchor positions vary between different MHC-I allomorphs many allomorphs, among them A*02:01, define position two (binding in the B-pocket) and the C-terminal end (binding in the F-pocket) as anchor positions in the peptide (Lund *et al.*, 2004). The interactions between the amino acids in the anchor positions and the binding pockets in the bottom of the binding cleft are of crucial importance to obtain high affinity of the peptide binding and high stability of the MHC-I complex. The general idea has been that the optimal length for a peptide to bind in the peptide binding cleft is 8-10 amino acids (Rammensee *et al.*, 1995). However, work by our group and others have shown that the peptide length specificity for some HLA-I molecules also include longer peptides (Bell *et al.*, 2009; Geironsen *et al.*, 2013).

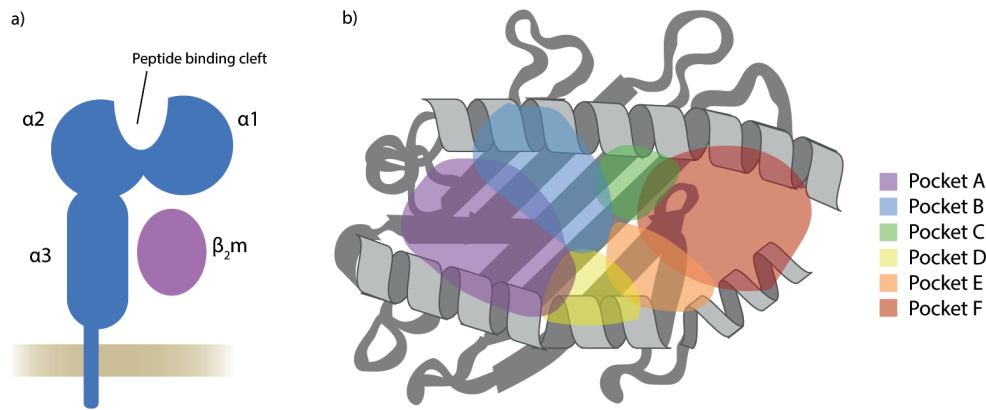


Figure 1. Structure of the MHC-I molecule. a) The MHC-I molecule folds into three different domains, α_1 , α_2 , and α_3 . The α_3 domain contains the transmembrane region and the α_1 and α_2 domains make up the polymorphic peptide binding cleft. b) The bottom of the peptide binding cleft contains six pockets (A-F), which provide interaction sites for anchor residues of the peptide.

MHC-I maturation

Degradation of proteins to peptides in the cytosol

Peptides that are destined to be loaded onto MHC-I are generated by the numerous amounts of proteasomes in the cytosol. The ATP-dependent ubiquitin-proteasome system is the major protein degradation system in cells and is essential to maintain cellular homeostasis and ensure that misfolded proteins are degraded (Rock & Goldberg, 1999). In addition, other cytosolic proteases such as puromycin-sensitive aminopeptidase, bleomycin hydrolase and also metallo-endopeptidases contribute to the generation of peptides inside the cell (Lopez *et al.*, 2000; Stoltze *et al.*, 2000).

The proteasome is made up of the regulatory 19S subunit and the 20S proteasome catalytic core. Together they form the 26S proteasome. Proteins targeted for degradation are tagged with polyubiquitin chains, which can be recognized by the 19S subunit of the proteasome. The regulatory 19S subunit is present on each side of the 20S subunit and allows entry to the proteolytic proteasome core only for those proteins that are targeted for degradation and

at the same time prevents entry for others. Also, the entrance is limited due to its narrow opening of 13 Ångström ensuring that only unfolded proteins can enter the catalytic chamber. This tight control is essential for cellular homeostasis since the proteolytic activity of the proteasome otherwise might damage proteins which are not destined for degradation. The 20S subunit is cylindrical and composed of four stacked rings with seven subunits in each ring. The two outer rings are identical and made up of α -subunits and the two inner rings is made up from seven β -subunits, three of which have catalytic activity, β 1, β 2 and β 5 (Fenteany *et al.*, 1995; Groll *et al.*, 1997; Lowe *et al.*, 1995).

In immune cells or in response to the inflammatory cytokines interferon- γ (IFN γ) or to some extent tumor necrosis factor- α (TNF α) the β 1, β 2 and β 5 are exchanged for alternative catalytic subunits named β 1i (LMP2), β 2i (MECL-I) and β 5i (LMP7) to form the immunoproteasome (Griffin *et al.*, 1998). Proteasomes can also be composed by a mixture of cytokine-inducible and non-inducible subunits (Joeris *et al.*, 2012). Due to differences in their catalytic activity the standard proteasome and the immunoproteasome produce distinct sets of peptides, which overlap only partially (Boes *et al.*, 1994; Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993; Toes *et al.*, 2001). The immunoproteasome have a higher tendency to cleave after basic or hydrophobic residues and have thus been predicted to be more efficient in producing peptides with high affinity for HLA-I molecules, as basic or hydrophobic residues are preferred at the C-termini for peptides binding to MHC-I (Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993). In conclusion, the immunoproteasome plays an important role in MHC-I antigen presentation. Although the immunoproteasome forms in response to inflammatory cytokines the exchange is not absolute and thus cells do not possess only one kind of proteasome but rather a mixture of proteasomes.

In 2007 another proteasome called the thymoproteasome was identified (Murata *et al.*, 2007). The structure of the thymoproteasome resembles the

26S proteasome but has a distinct $\beta 5$ subunit, the $\beta 5t$ subunit. In humans they are specifically found in the cortical epithelial cells in the thymus and in a fraction of dendritic cells in the thymic cortex (Tomaru *et al.*, 2009). The thymoproteasome has been proposed to play role in the positive selection of T-cells (Xing *et al.*, 2013).

It takes several hours for a virus to replicate in the cell and yet CD8⁺ T-cells are able to recognize in infected cell within an hour after viral penetration (Esquivel *et al.*, 1992; Yewdell *et al.*, 1996). This fast recognition is due to the production of defective ribosomal products (DRiPs), products with errors in the mRNA or protein synthesis e.g. misincorporation or deletion of amino acids, premature termination of replication etc. (Yewdell *et al.*, 1996). Peptides presented by MHC-I molecules at the cell surface are to a large extent fragments of what is currently being translated within the cell rather than already expressed proteins (Qian *et al.*, 2006) and thus DRiPs are most likely a main source of antigenic peptides.

Transport of peptides

Transport of peptides into the ER is mediated by the TAP complex, which belongs to the family of ATP-binding cassette (ABC) transporters. The heterodimeric TAP complex consists of TAP1 and TAP2 that form a pore in the ER membrane through which peptides can be actively transported. The transport of a peptide is a two-step process involving the binding of a peptide and subsequent ATP-dependent translocation (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993). Testing of various epitopes has revealed a preference for TAP to bind peptides with positively charged residues in the N-terminal position 1-3 and a strong preference for basic and hydrophobic residues at the C-terminal end (Momburg *et al.*, 1994; Parcej & Tampe, 2010). Notably, this C-terminal preference matches the requirements for peptide binding by MHC-I molecules. The most efficient transport occurs for peptides of 8-16 amino acids in length, however, peptides up to 40 amino acids in length have

also been shown to be transported but with lower efficiency (Koopmann *et al.*, 1996; van Endert *et al.*, 1994). A recent study suggested that both ends of a peptide are bound to TAP whereas the central part is mobile and unlikely to interact (Herget *et al.*, 2011). Thus TAP combines both specificity and variability as it recognizes its substrate peptides by N- and C-terminal anchor positions while the middle of the peptide can be diverse. TAP deficient cells show decreased quantity and quality of cell surface expressed MHC-I molecules (Salter & Cresswell, 1986; Spies *et al.*, 1992; Spies & DeMars, 1991).

Trimming of peptides in the ER

Following transport into the ER peptides are trimmed at the extended N-terminal end by the enzyme ER aminopeptidase associated with antigen processing (i.e. ERAAP in mice, and ERAP1 and ERAP2 in humans) to be of suitable length for binding to MHC-I (Chang *et al.*, 2005; Saric *et al.*, 2002; Serwold *et al.*, 2002). ERAP1 and ERAP2 work in concert as they cleave substrates with different preference for the N-terminal residues i.e. ERAP1 binds to peptides with large hydrophobic C-terminals whereas ERAP2 prefers basic residues at the C-terminal (Saveanu *et al.*, 2005).

Quality control of MHC-I

The MHC-I heavy chain (HC) is translocated into the ER in an unfolded state. During or shortly following translocation in to the ER lumen an oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) is attached to the unfolded HC, which also interacts with a chaperone called binding immunoglobulin protein (BiP). Subsequently, two of the glucoses are removed by glucosidase I and II to generate a monoglucosylated glycoprotein that interacts with the ER resident lectins calnexin and calreticulin. Calnexin and calreticulin also associates with ERp57, a disulfide isomerase, which facilitates the formation of disulfide bonds within the MHC-I molecule and thus assists the acquisition of a correct

conformation (Oliver *et al.*, 1999). Cleavage of the remaining glucose by glucosidase II results in release from calnexin/calreticulin. Upon release from calnexin/calreticulin the folding status of the MHC-I molecules is evaluated by the soluble enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT). Prematurely dissociated, unstable MHC-I molecules will be re-glucosylated by UGGT and rebound to calnexin (Helenius & Aebi, 2004). During the folding process the MHC-I HC may go through several rounds of binding, release and re-binding of calnexin and calreticulin in what is referred to as the glycoprotein quality control cycle. Permanently misfolded proteins will be recognized and translocated back across the ER membrane to the cytosol for degradation by the ubiquitin-proteasome system, a mechanism called endoplasmic reticulum-associated degradation (ERAD) (Helenius & Aebi, 2004) (figure 2).

The glycoprotein quality control cycle is of importance during the initial folding events of the MHC-I HC. However calreticulin is also involved in another quality control process, which further promotes the folding of the MHC-I and optimizes the peptide loading. Following association of the MHC-I HC with β_2m the MHC-I heterodimer is rapidly recruited into the PLC, escorted by calreticulin (Wearsch *et al.*, 2011). The PLC is a multi-protein complex that is made up of MHC-I, TAP, ERp57, calreticulin and a key protein called tapasin. Within the PLC calreticulin is involved in multiple interactions with MHC-I, ERp57 and tapasin, however, the individual contribution of each of these interactions is still controversial (Del Cid *et al.*, 2010; Ireland *et al.*, 2008; Rizvi *et al.*, 2011; Wearsch *et al.*, 2011; Zhang *et al.*, 2009).

Tapasin is a membrane bound glycoprotein that binds to MHC-I and also to several PLC components including TAP. Tapasin ensures close proximity between TAP and MHC-I and thus place the MHC-I molecule in a milieu where the density of TAP transported peptide is high (Hulpke *et al.*, 2012). In the PLC, tapasin also binds to the oxidoreductase ERp57 via a mixed disulfide bridge between Cys95 of tapasin and Cys57 of ERp57 (Dick *et al.*, 2002;

Peaper *et al.*, 2005). ERp57 is a member of the protein disulphide isomerase family and contains four thioredoxin-like domains called a, b, b' and a'. The a and a' domains each contains a CXXC motif with the ability to catalyze reduction, oxidation and isomerization of disulphide bonds. ERp57 forms transient disulphide bonds between the substrate and the N-terminal cysteine in the CXXC motif, which can subsequently be resolved by the C-terminal cysteine of the same motif in a process called the resolving escape mechanism (Walker & Gilbert, 1997). The 3D structure of the tapasin-ERp57 conjugate shows that the ER-luminal part of tapasin adopts an L-shaped conformation and interacts with both the catalytic domains a and a' of ERp57 (Dong *et al.*, 2009). However, tapasin association specifically inhibits the escape pathway through noncovalent interactions between tapasin and ERp57, which inhibits the reductase activity of the CXXC motif in the N-terminal domain of ERp57 (Peaper *et al.*, 2005). In a cell-free system, a conjugate of soluble tapasin and ERp57 was shown to stabilize empty MHC-I molecules and selectively facilitate the loading of high affinity peptides (i.e. peptide editing) more efficiently than soluble tapasin alone (Wearsch & Cresswell, 2007). Previous studies have suggested that tapasin solely was responsible for these tasks (Barber *et al.*, 2001; Barnden *et al.*, 2000; Chen & Bouvier, 2007; Garbi *et al.*, 2000; Williams *et al.*, 2002). However, as ERp57 has been shown to be necessary for PLC structural integrity (Stepensky *et al.*, 2007) whereas its redox activity is dispensable for PLC function (Peaper & Cresswell, 2008; Zhang *et al.*, 2009), it is possible that the structural role of ERp57 boosts the effect of tapasin by stabilizing the interactions in the PLC. ERp57 is undoubtedly relevant for antigen processing as deletion leads to reduced MHC-I surface expression in a mouse model (Garbi *et al.*, 2006) and mutation of cysteine 95, which prevents the association between tapasin and ERp57, results in sub optimally loaded MHC-I in human cells (Dick *et al.*, 2002).

To exit the ER the MHC-I must achieve a correct conformation and high stability to pass the glycoprotein quality control checkpoints including

glucosidase II trimming and UGGT-mediated reglucosylation. The loading of an optimal peptide induces a final state of high stability in the MHC-I molecule, which results in release from the PLC. It has been shown that calreticulin and MHC-I are still associated when the MHC-I is released from the PLC (Wearsch *et al.*, 2011). It has also been shown that N-glycans of free MHC-I HCs, but not PLC-associated HCs, are accessible to glucosidase II and thus the final trimming by glucosidase II has been suggested to occur after the release of MHC-I from the PLC (Wearsch *et al.*, 2011). A fully matured MHC-I, which has passed all the quality control checkpoints, can exit the ER with the assistance of the B-cell receptor-associated protein 31 (BAP31) and be transported to the cell surface.

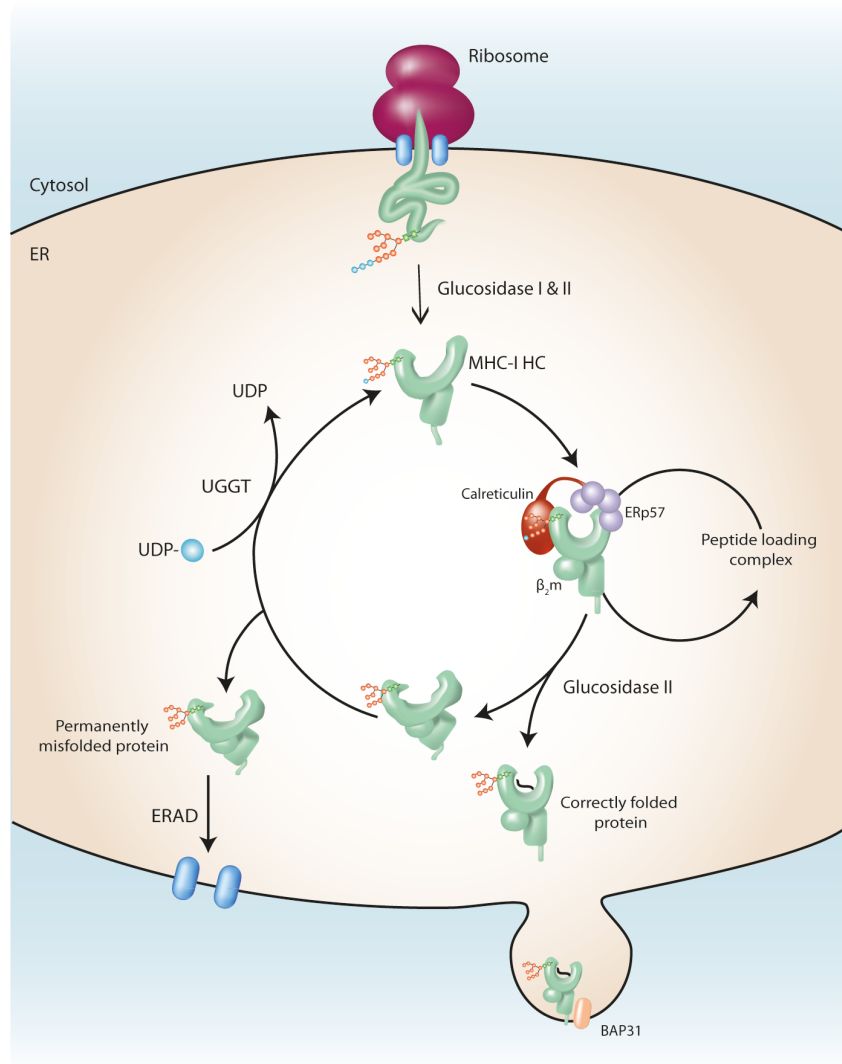


Figure 2. The glycoprotein quality control cycle. Shortly following translocation into the ER the unfolded MHC-I HC is provided with an oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, Glucose ●, Mannose ○, GlcNAc ■). Following removal of two glucoses by glucosylase I and II the MHC-I HC is bound to calnexin and recruited into the glycoprotein quality control cycle. Calnexin is also associated with ERp57, which promotes the formation of disulfide bonds within the MHC-I HC. After binding of β_2m calnexin is replaced by its soluble homologue calreticulin, which recruits the MHC-I heterodimer into the peptide loading complex for further maturation and quality control. Upon release from calnexin/calreticulin the state of the MHC-I is evaluated by UDP-glucose:glycoprotein glucosyltransferase (UGGT). Prematurely dissociated and unstable MHC-I molecules will be re-glucosylated and re-enter the glycoprotein quality control cycle. Permanently misfolded MHC-I will be recognized and translocated back into the cytosol for degradation by the proteasome, a mechanism called endoplasmic reticulum-associated degradation (ERAD).

Tapasin related protein

The tapasin-related protein (TAPBPR) is a newly discovered additional component in the MHC-I presentation pathway (Boyle *et al.*, 2013). It is similar to tapasin as it is also widely expressed, IFN γ -inducible and binds in a similar orientation to the MHC-I heterodimer in the ER. In contrast to tapasin, TAPBPR does not associate with ERp57 or calreticulin and is not an integral part of the PLC. Despite the similarities with tapasin the amino acid sequence of TAPBPR is only 22% identical to tapasin. Boyle *et al.* showed that over-expression of TAPBPR results in a phenotype resembling that of a tapasin deficient cell i.e. severely reduced surface levels of pMHC-I complexes and increased the expression of free MHC-I HC (Boyle *et al.*, 2013). They speculated that TAPBPR does not perform the function of peptide editing but rather acts as a chaperone monitoring the stability of the pMHC-I complex as it dissociates from the PLC (Boyle *et al.*, 2013; Hermann *et al.*, 2013).

Tapasin

The discovery of tapasin

Researchers in the middle 1990's were looking for additional molecules and/or mechanisms necessary for MHC-I maturation as experiments with the tapasin deficient B-lymphoblastoid cell line (B-LCL) 721.220 (.220) showed complete failure of MHC-I to associate with TAP (Grande *et al.*, 1995). Also transfection of *MHC-I* genes into .220 did not result in complete restoration of surface expressed MHC-I. Moreover, transfection of genes of certain MHC-I alleles resulted in levels of surface MHC-I expression comparable to control cells whereas the transfection of other alleles resulted in significantly reduced levels of surface expressed MHC-I. Fusion with B-LCL-721.174 cells, which lack TAP1 and TAP2, restored the MHC-I surface

expression and fusion with Daudi cells, which are missing β_2m , also restored the surface expression in a similar way (Greenwood *et al.*, 1994).

In 1994 Ortmann *et al.* found a 48 kDa glycoprotein co-precipitating with TAP and suggested it to act as a bridge between TAP and MHC-I (Ortmann *et al.*, 1994). It was later given the name TAP-associated glycoprotein, more commonly known as tapasin, and was shown to be able to restore the surface expression of MHC-I in .220 cells (Li *et al.*, 1997).

Interactions between MHC-I and tapasin

Tapasin is a 428 amino acid long transmembrane protein, which consists of three parts: the large ER luminal N-terminal part made up by two domains, a single transmembrane-spanning domain and a short cytosolic tail. All the interaction sites between MHC-I and tapasin are not fully unraveled. Several different regions or residues in the N-terminal and C-terminal part of the ER luminal domain of tapasin have been suggested to be involved in MHC-I binding (Dong *et al.*, 2009; Simone *et al.*, 2012; Turnquist *et al.*, 2001). Dong *et al.* reported in 2009 a conserved surface of tapasin suggested to be involved in binding to MHC-I. When residues central to this conserved patch were altered (E185K, R187E, Q189S and Q261S), it completely abrogated MHC-I binding. In other mutants, where residues located in or around this conserved patch were altered, the effect was less severe but still resulted in only trace amounts of MHC-I interacting with tapasin (Dong *et al.*, 2009).

A well-studied mutation of MHC-I is the T134K in A*02:01 (A*02:01-T134K). This mutation has been shown to prohibit the interaction with tapasin (Lewis *et al.*, 1996; Peace-Brewer *et al.*, 1996; Yu *et al.*, 1999). The interaction site in tapasin, for residue 134 in MHC-I, has been suggested to include R187 (Van Hateren *et al.*, 2010). However it is likely that tapasin holds another interaction site(s) within the first 87 N-terminal amino acids as the tapasin fragment tpn₁₋₈₇ efficiently facilitates peptide binding to A*02:01

and other allomorphs (Roder *et al.*, 2009; Roder *et al.*, 2011). Also, tapasin truncated at the N-terminal end does not co-precipitate with MHC-I, suggesting that the 50 most N-terminal residues of tapasin are indeed crucial for its interaction with MHC-I (Lehner *et al.*, 1998). In contrast the deletion of the transmembrane region of tapasin does not affect the interaction between tapasin and MHC-I (Lehner *et al.*, 1998).

Tapasin - a multifunctional protein

Several studies have demonstrated that tapasin increases the stability of cell surface expressed pMHC-I complexes (Barber *et al.*, 2001; Barnden *et al.*, 2000; Garbi *et al.*, 2000; Thirdborough *et al.*, 2008). Tapasin is a key component of the PLC and has multiple functions, which all aim to optimize the presentation of peptides by MHC-I at the cell surface. By simultaneous binding to MHC-I and TAP tapasin links the two major tasks of the PLC: peptide transport and peptide loading. This is important for the overall PLC function as soluble tapasin, which lacks the transmembrane domain has been shown to be unable to support peptide loading to its full extent (Everett & Edidin, 2007; Rizvi & Raghavan, 2010; Tan *et al.*, 2002; Vigneron *et al.*, 2009). Also, tapasin binding has been shown to stabilize the TAP complex and promote the binding and transport of peptides into the ER (Garbi *et al.*, 2003; Lehner *et al.*, 1998; Li *et al.*, 2000; Tan *et al.*, 2002). In the absence of tapasin TAP levels as well as thermal stability of TAP is significantly decreased (Bangia *et al.*, 1999; Garbi *et al.*, 2000; Lehner *et al.*, 1998; Raghuraman *et al.*, 2002). Another crucial function of tapasin is its ability to retain unstable MHC-I molecules in the ER until an optimal peptide has been loaded (figure 3). The cytosolic C-terminal end of tapasin contains a double lysine motif (KKXX), which is involved in retrograde transport of peptide-receptive MHC-I molecules in COP-I vesicles from the Golgi (Paulsson *et al.*, 2006; Paulsson *et al.*, 2002). An MHC-I allomorph known to interact with tapasin, A*02:01, takes almost three times longer to export to the cell surface compared to a non-tapasin interacting allomorph (Lewis & Elliott, 1998). In the absence of

tapasin, and thus its retention mechanism, peptide receptive MHC-I molecules have been shown to be transported to the surface at an increased rate but also to be less stable at the cell surface (Barber *et al.*, 2001; Barnden *et al.*, 2000; Paulsson *et al.*, 2006; Paulsson *et al.*, 2002; Schoenhals *et al.*, 1999). Maybe the most important function of tapasin is the ability to promote exchange of low affinity peptides for high affinity peptides in the peptide binding cleft of MHC-I, a process called peptide editing or peptide optimization (Barnden *et al.*, 2000; Chen & Bouvier, 2007; Garbi *et al.*, 2000; Howarth *et al.*, 2004; Praveen *et al.*, 2010; Wearsch & Cresswell, 2007; Williams *et al.*, 2002).

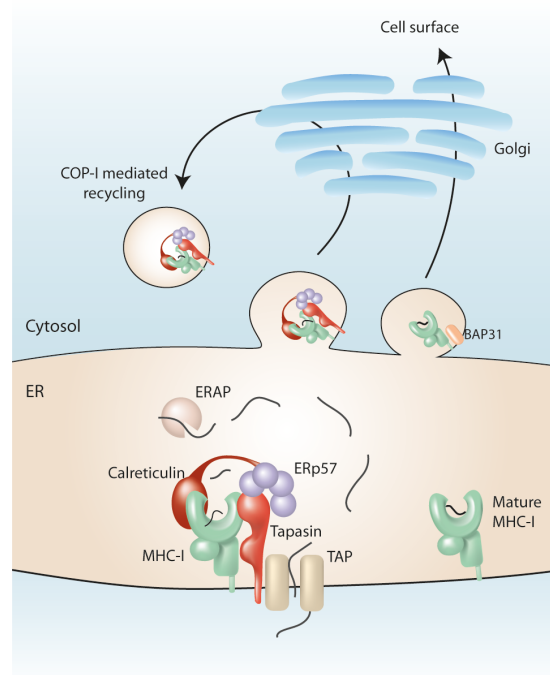


Figure 3. Tapasin is a key component of the PLC and retains unstable MHC-I in the ER. The peptide loading complex (PLC) is a multi-protein complex made up of MHC-I, tapasin, TAP, ERp57 and calreticulin. Tapasin is a multifunctional protein that binds to both MHC-I and TAP. Tapasin stabilizes TAP and also ensures close proximity between MHC-I and TAP, thereby placing MHC-I in a milieu where the density of peptide is high. Most importantly tapasin works to optimize peptides bound in the peptide binding cleft and also retains unstable MHC-I molecules in the ER by retrograde transport of peptide receptive MHC-I in COP-I vesicles from the Golgi.

Tapasin has been suggested to stabilize MHC-I molecules that bind suboptimal peptides (Barnden *et al.*, 2000; Garstka *et al.*, 2011; Ortmann *et al.*, 1997; Schoenhals *et al.*, 1999) and keep them in a peptide-receptive state (Chen & Bouvier, 2007). The peptide editing function of tapasin has been described in several studies (Barnden *et al.*, 2000; Chen & Bouvier, 2007; Garbi *et al.*, 2000; Howarth *et al.*, 2004; Praveen *et al.*, 2010; Wearsch & Cresswell, 2007; Williams *et al.*, 2002). Peptide editing, or peptide optimization, have been referred to as the process where tapasin promote exchange of low affinity peptides for peptides with higher affinity. However, “peptide editing” is not primarily based on the exchange for peptides with higher affinity but instead on the increase of MHC-I stability as studies have suggested that factors other than affinity are involved in peptide editing (Assarsson *et al.*, 2007; Harndahl *et al.*, 2012).

One of the earlier studies on peptide editing by tapasin was done on the B*44:02 and B*27:05 allomorphs by Williams *et al.* in 2002. They referred to the “known correlation” between the thermostability of peptide:HLA-I (pHLA-I) complexes and the affinity of their loaded peptide (previously shown by (Bouvier & Wiley, 1994; Fahnstock *et al.*, 1992)) and used thermostability of HLA-I as readout for the optimization. In the presence of tapasin they observed an optimization of peptides over time, which occurred much faster and to a greater extent for both allomorphs as compared to in the absence of tapasin. They also observed a qualitative improvement i.e. increased thermostability for both B*44:02 and B*27:05 (Williams *et al.*, 2002).

The correlation between peptide affinity and stability of pHLA-I complexes was questioned the year after in a study by Zarling *et al.* who observed no difference in the average affinity of peptides presented in the presence or absence of tapasin despite a significant difference in the half-life of cell surface presented B*08:01 and A*02:01. Their data was inconsistent with the role of

tapasin as a peptide editor and instead suggested tapasin to be a facilitator stabilizing peptide-receptive MHC-I molecules (Zarling *et al.*, 2003).

Mechanistic knowledge about what defines a mature stable MHC-I molecule comes from studies including the work by Praveen *et al.* who observed that in a competitive situation between high- and low-affinity peptides, tapasin mediates the binding of high-affinity peptides by accelerating the dissociation of the peptide from what they suggested to be an unstable intermediate form of MHC-I in the binding reaction (Praveen *et al.*, 2010). Tapasin was suggested to be an editor of this unstable intermediate complex, which converts into a stable complex at the binding of an optimal peptide (Praveen *et al.*, 2010). Binding of an optimal peptide results in dissociation from tapasin (Paulsson *et al.*, 2001; Rizvi & Raghavan, 2006).

Tapasin dependence

Different HLA-I allomorphs depend to different degrees on tapasin for maturation, peptide loading and subsequent peptide presentation at the cell surface. Several mutational studies and also studies of natural allomorphs have been done to elucidate which residues are responsible for tapasin dependence. Two allomorphs, B*44:02 and B*44:05 have been well studied in this regard since they are closely related, differing in only one residue at position 116, but yet totally different in terms of tapasin dependency. B*44:02 stands out as highly dependent on tapasin whereas B*44:05 is in the very opposite end of the spectra being largely independent of tapasin (Peh *et al.*, 1998; Sieker *et al.*, 2007).

Park *et al.* reported in 2003 that tapasin dependence of HLA-I correlated with the nature of the amino acids present at position 114 (Park *et al.*, 2003). The tapasin dependency increased in the order of acidity of the residue at position 114 with high tapasin dependence for acidic amino acids, moderate tapasin dependence for neutral amino acids and low tapasin dependence for basic amino acids. Also, substituting the glutamic acid to a histidine at position 114

allowed the otherwise highly tapasin dependent allomorph B*44:02 to load high affinity peptides independently on tapasin and the surface expression level was comparable to the level seen in the presence of tapasin. The opposite substitution, histidine to glutamic acid at position 114, rendered the otherwise tapasin independent allomorph B*27:05 more tapasin dependent (Park *et al.*, 2003). However, B*44:02 and B*44:05, which are in opposite ends of the tapasin dependency spectrum, both have aspartic acid at position 114 and thus position 114 does not alone dictate the tapasin dependence of an HLA-I allomorph.

A study by Gartska *et al.* investigated the effect of substitutions in position 116 in HLA*44:02 and B*44:05. The residue at position 116 is located at the floor of the F-pocket (which binds the C-terminal residue of the peptide) in the peptide binding cleft and is an aspartic acid in B*44:02 and a tyrosine in B*44:05. When substituting the aspartic acid in B*44:02 for a histidine (which has a shape resembling tyrosine), the molecule became less dependent of tapasin and had a stable conformation of the F-pocket. They hypothesized that it is the combination of two aspartic acids in position 114 and 116 of B*44:02 that results in a conformational disruption of the F-pocket due to excessive hydration (Garstka *et al.*, 2011). Along with this suggestion others have also proposed that the ionic/hydrophobic environment at the bottom of the F-pocket affect the conformational flexibility of peptide free HLA-I molecules (Garstka *et al.*, 2011; Sieker *et al.*, 2008; Zernich *et al.*, 2004). Sieker *et al.* reported that the residue at position 116 influences the tendency of the peptide binding cleft to open up in the absence of a peptide (Sieker *et al.*, 2008). Also, in a recent study by Ostermeir *et al.* molecular dynamics simulations indicated a significantly higher conformational flexibility of the F-pocket in the absence of peptide for B*44:02 compared to B*44:05 (Ostermeir *et al.*, 2015). Free energy simulations on B*44:02 indicated a molecular side chain switch mechanism, involving both the aspartic acid residues at position 114 and 116, to be responsible for the opening motion of the F-pocket (Ostermeir *et al.*, 2015). For B*44:05 the free energy barrier for opening of the

F-pocket was significantly higher compared to B*44:02 and no side chain switch was observed (Ostermeir *et al.*, 2015). All together this suggests a model where an open, peptide-receptive state of the binding cleft is required for peptide binding and that the opening can be supported by the residues in the peptide binding cleft or by tapasin.

Another recent study by Saini *et al.* reported that dipeptides binding in the F-pocket of A*02:01 and B*27:05 accelerate the dissociation of prebound peptides (Saini *et al.*, 2015). By binding into the F-pocket of the HLA-I the dipeptides interfered with the binding of the peptide C-terminus and thus prevented rebinding of the peptide in a partially dissociated pHLA-complex. As tapasin appears to bind close to the F-pocket (Dong *et al.*, 2009) they suggested that tapasin might interfere with the binding of the peptide C-terminal in a similar way as the dipeptides, possibly be the insertion of an amino acid chain into the F-pocket. They also found that the C-terminal amino acid of the dipeptide was different for those dipeptides inhibiting peptide binding in B*27:05 and those inhibiting peptide binding in A*02:01. The mechanism of allomorph specific dipeptides might be similar to tapasin dependency and hence used to study differences of HLA-I allomorphs regarding the need of quality control.

The current view is that tapasin acts as a chaperone protecting the HLA-I and inducing a more open conformation of the peptide binding cleft, which results in the accelerated dissociation of low-affinity peptides and binding of high-affinity peptides. (Chen & Bouvier, 2007; Praveen *et al.*, 2010; Wearsch & Cresswell, 2007). Once loaded with an optimal peptide, the HLA-I complex can overcome the energy barrier necessary to convert to a closed conformation, dissociate from tapasin and leave the PLC (Chen & Bouvier, 2007).

The tapasin dependency may to some extent be explained by the influence of specific amino acids in defined positions of the HLA-I molecule (Roder *et al.*, 2011). However, the allomorph is not the only regulating factor as the identity of the bound peptide also regulates the interaction between HLA-I and tapasin

(Geironson *et al.*, 2013; Roder *et al.*, 2009; Roder *et al.*, 2011; Zernich *et al.*, 2004). The B*44:02 and B*44:05 allomorphs have been shown to have different preferences for the amino acid residue in position 9 of a bound peptide, both aiming to optimize the environment in the F-pocket in the peptide binding cleft (Zernich *et al.*, 2004). Also, a recent study by Hawse *et al.* reported that the flexibility of the peptide binding cleft of A*02:01 varies significantly with different peptides (Hawse *et al.*, 2013). Thus peptide identity influences the conformational flexibility of the HLA-I peptide binding cleft and thereby also the extent of the interaction between HLA-I and tapasin.

Another rather well studied HLA-I allomorph in terms of tapasin dependency is B*27:05. In contrast to B*44:02, the B*27:05 allomorph has been shown to be relatively independent on tapasin with respect to its surface expression (Park *et al.*, 2003; Peh *et al.*, 1998; Purcell *et al.*, 2001; Williams *et al.*, 2002; Zernich *et al.*, 2004). However, the peptide repertoire presented at the cell surface by B*27:05 has been shown to differ significantly in the presence and absence of tapasin (Peh *et al.*, 1998; Purcell *et al.*, 2001). Also, in an assay where cytotoxicity (i.e. cell lysis) was measured on .220*B27:05 cells four hours post infection with recombinant vaccinia virus, the cells displayed only half the cytotoxicity compared to tapasin positive cells (Peh *et al.*, 1998).

Tapasin dependence could be defined as the dependence of tapasin to increase the stability and thus the surface expression of HLA-I allomorphs. However, even though an allomorph is sometimes referred to as being "independent" of tapasin, tapasin still exerts a very important role in terms of optimizing the loading of peptides with high immunogenicity. Thus allomorphs should be denoted as more or less tapasin dependent and not "tapasin independent".

Studies on tapasin dependence have usually been executed by using the .220 cell line in which different HLA-I alleles have been used for transfection and for subsequent analysis of steady state surface expression in the presence and absence of co-transfected tapasin (Park *et al.*, 2003; Peh *et al.*, 1998; Zernich *et al.*, 2004). In recent studies by our group ((Geironson *et al.*, 2013), III) we

used a biochemical approach with recombinant proteins to determine tapasin dependency of a large set of HLA-I allomorphs. The biochemical assay, in which we studied the ability of the recombinant HLA-I proteins to form pHLA-I complexes in the presence and absence of tapasin, we observed highly variable levels of tapasin facilitation for the allomorphs studied ((Geironson *et al.*, 2013), III). The hierarchy of tapasin facilitation closely resembled the order established for the small number of allomorphs previously studied in cellular models (Park *et al.*, 2003; Peh *et al.*, 1998).

HLA-I and APM in tumors

Tumor cells produce some antigens that are relatively restricted to tumor cells (tumor-associated antigens (TAA)) or even antigens that are unique to tumor cells (tumor-specific antigens (TSA)). The discovery of these antigens, which are presented by HLA-I and recognized by T-cells, opened up new ways to understand the concept of immune surveillance against tumors (Boon & van der Bruggen, 1996). These findings urged many laboratories to develop novel vaccination therapies that would boost the T-cell response and induce tumor regression in cancer patients. However, despite many years of research the results from clinical trials have been disappointing and there is demands for improvement. Despite general immune activation against tumor antigens and presence of specific cytotoxic T-cells, tumors have still been observed to progress (Rosenberg *et al.*, 2005). This is likely partly due to the immune evasion strategies that tumors develop to avoid recognition and clearance by the immune system. Such evasion strategies include downregulation of HLA-I and APM proteins as part of immunoediting as well as immunosuppression, which will be discussed in the chapter about tumor immunology.

Loss or downregulation of HLA-I in tumor cells represent an important cancer immune escape mechanism (Drake *et al.*, 2006; Garrido *et al.*, 1997; Marincola *et al.*, 2000). Results from immunohistochemical stainings during the past 30 years have revealed that loss of HLA-I is a frequent event in cancer. For most cancer types, various alterations of HLA-I expression can be found in 60-90% of tumors, depending of the histological type of cancer (Blades *et al.*, 1995; Cabrera *et al.*, 1996; Cabrera *et al.*, 1998; Cabrera *et al.*, 2000; Kageshita *et al.*, 2005; Koopman *et al.*, 2000). The alterations of HLA-I expression varies from total loss or downregulation of all HLA-I molecules to selective losses of HLA-I haplotypes or alleles. The defects can occur at genetic, epigenetic, transcriptional and post-transcriptional level and are the result of either regulatory abnormalities (reversible), which can be restored with e.g. immunostimulatory cytokine treatment, or more severe structural defects (irreversible) (Garrido *et al.*, 2010).

The reversible HLA-I deficiencies, which involve the APM proteins at transcriptional level, include repression of gene transcription by different oncogenes such as Her2/Neu and HPV E7 (Georgopoulos *et al.*, 2000; Mimura *et al.*, 2011). The activation of these oncogenes most often leads to histone modifications, which decrease the expression of HLA-I and APM proteins. Such modifications can however be reversed by DNA de-methylating agents and de-acetylase inhibitors (Garrido *et al.*, 2010; Khan *et al.*, 2008; Rodriguez *et al.*, 2007). Also, reversible HLA-I and APM deficiencies can be restored by treatment with immunostimulatory cytokines such as interferons since most genes involved in the HLA-I antigen presentation encode an interferon stimulated responsive element (ISRE) in their promoter regions (Seliger, 2008). IFN-mediated upregulation of APM components normally leads to enhanced HLA-I surface expression and improves anti-tumor cytotoxic T-cell responses (Seliger, 2008). This suggests that T-cell based therapy can still be successfully applied on tumors with reversible HLA-I and APM deficiencies. Lack of IFN γ -mediated upregulation of HLA-I, caused by

defects in the Jak-STAT components of IFN-mediated signaling pathway, can also be a mechanism to producing tumor escape variants (Rodriguez *et al.*, 2007; Seliger, 2008) and calls for non-IFN based treatments.

The irreversible, structural HLA-I defects may have more profound effects on T-cell mediated rejection of tumor cells and on the outcome of cancer immunotherapy. The irreversible defects are caused by various mutations and chromosomal defects involving the genes encoding for HLA-I, β_2m and other antigen presentation related genes (Lampen & van Hall, 2011). Structural defects in various APM components have been shown for some cancer types although dysregulations rather than structural alterations have been suggested to cause the HLA-I and APM deficiencies in most tumors (Garrido *et al.*, 2010; Koopman *et al.*, 2000).

HLA-I and APM dysregulations in tumors

Downregulation of HLA-I and APM components are common in tumors of different origin. Defects in the expression of APM components occur individually or in combination and the frequency and nature of the defects vary substantially between tumor types (Leone *et al.*, 2013). These abnormalities are of particular clinical interest since they are strongly linked to aggressiveness of the disease as well as outcome for the patient. Beside the downregulation of HLA-I many tumors show downregulation of APM proteins e.g. the TAP and LMP proteins (Leone *et al.*, 2013) but deficient expression of tapasin has also been reported to be linked to metastasis and poor outcome in a number of different tumors (Dissemond *et al.*, 2003; Facoetti *et al.*, 2005; Jiang *et al.*, 2010).

The downregulation of APM components results in a dysfunctional process of HLA-I maturation, which in turn exerts a negative effect on immune recognition of tumor cells (figure 4). Firstly, the downregulation of the proteasome subunits can inhibit the processing of antigens and thus decrease the generation of epitopes. In addition, variations in proteasome subunit ratios

may alter the repertoire of antigenic peptides in the cell and thus modify the characteristics of the presented peptides (resulting in changes in the immunodominance hierarchies). Defects in TAP expression may reduce the transport of peptides into the ER with decreased formation of pHLA-I complexes as a result. Also, downregulation of chaperone proteins may interfere with the quality control cycles during HLA-I maturation. Tapasin deficiencies in particular may have severe consequences for HLA-I allomorphs that are highly dependent on tapasin for maturation. Even allomorphs with lower tapasin dependence will also be affected, as tapasin's function of peptide optimization is lost. All these events can have profound consequences for immune recognition as CD8⁺ T-cells recognize mature and presumably stable HLA-I molecules with a peptide in their binding cleft. Several studies have shown the correlation between the extent of CD8⁺ T-cells infiltration and the expression of APM components (Han *et al.*, 2008; Kasajima *et al.*, 2010; Liu *et al.*, 2012; Ogino *et al.*, 2006). In some cases the downregulation of specific APM components has been correlated with lack of cytotoxic CD8⁺ T-cell recognition (Lopez-Albaitero *et al.*, 2006; Racanelli *et al.*, 2010).

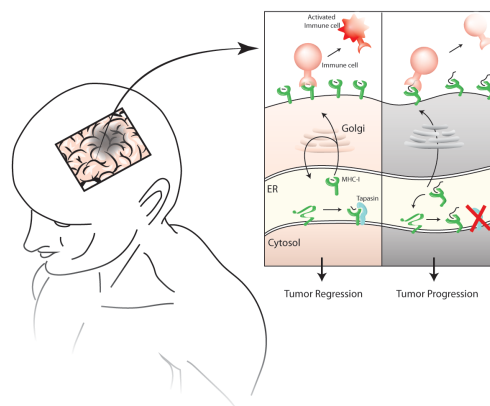


Figure 4. Tumors downregulate HLA-I and APM proteins as a strategy to evade the immune system. HLA-I and APM deficiencies are of clinical interest as they are strongly linked to aggressiveness of the tumor. HLA-I, TAP and LMP are commonly downregulated but also deficient expression of tapasin is linked to worse prognosis. Absence of tapasin results in poor quality of tapasin dependent HLA-I allomorphs and a general loss of quality control and peptide optimization for all HLA-I allomorphs.

In addition to their immunological role several APM components also participate in activities that are essential for the cell to survive, including control of newly synthesized proteins in the ER and degradation of proteins tagged by ubiquitin. Thus there are two opposing forces that shape the APM phenotype of tumors cells. Some parts of the APM activities e.g. protein degradation and ER chaperone function must be active for all cells to survive but at the same time these processes sustain the generation of pHLA-I complexes recognized by CD8⁺ T-cells, thus exposing the tumors to negative immune selection for APM deficiencies. As a result only tumors cells with certain APM defects (not essential for cell survival) will survive whereas cells with widespread defects in most APM components are eliminated, a process called immunoediting.

Glioblastoma multiforme

Glioma

Glioma is the collective term for all brain tumors originating from glial cells or glial precursor cells. Together they represent approximately 30% of tumors occurring in the central nervous system (CNS) and 80% of all malignant brain tumors. Depending on what cell types they originate from or share histological phenotype with, gliomas are further divided into subcategories. The majority of all gliomas (75%) originate from astrocytes (astrocytomas) but there are also oligodendral tumors deriving from oligodendrocytes (6%), mixed gliomas, e.g. oligoastrocytic tumors of mixed glial cell origin (3%) and ependymal tumors (7%) (Dolecek *et al.*, 2012). The world health organization (WHO) has further classified all CNS tumors in grades I-IV according to their malignancy with grade I being the least aggressive and grade IV the most aggressive tumors.

Grade IV tumors, such as glioblastoma multiforme (GBM), most commonly develop as a primary *de novo* tumors but can also occur as secondary tumors from lower-grade astrocytomas. They are malignant tumors with nuclear atypia, high mitotic activity, anaplasia and microvascular proliferation (multi-layering of endothelium) and/or necrosis. Typically, grade IV tumors infiltrate the surrounding healthy brain parenchyma and are associated with rapid disease progression with fatal outcome (Louis *et al.*, 2007).

Glioblastoma multiforme

GBM is the most common and also most aggressive primary brain tumor in adults. It typically occurs in patients older than 50 years and

represents 16% of all primary brain tumors and 54% of all gliomas (Dolecek *et al.*, 2012). GBM is classified by WHO as a grade IV tumor and the median survival for patients after diagnosis is less than 15 months despite currently available treatments (Wen & Kesari, 2008). The symptoms include headaches, seizures, nausea, focal neurologic signs (impairment of nerve, spinal cord or brain function that affects a specific region of the body) depending on where the tumor is located and alteration in mental status. The clinical onset of symptoms usually occurs very abrupt and is caused by increased intracranial pressure due to tumor growth, tumor infiltration into the brain parenchyma or tissue destruction (Buckner *et al.*, 2007; Wen & Kesari, 2008) (figure 5).

GBM is histologically characterized by morphology and display all the hallmarks of a grade IV tumor including e.g. nuclear atypia, mitotic activity, anaplasia and necrosis within the core of the tumor (Collins, 2004). The tumor is preferentially located in the cerebral hemispheres and rarely metastasizes outside the brain. However GBM tumors have diffuse borders and aggressive growth pattern with extensions of tumors cells migrating into the brain parenchyma and forming distal tumor microsatellites. This infiltrative nature makes GBMs almost impossible to remove completely by surgical resection and thus the tumor eventually reappears in nearly all patients.

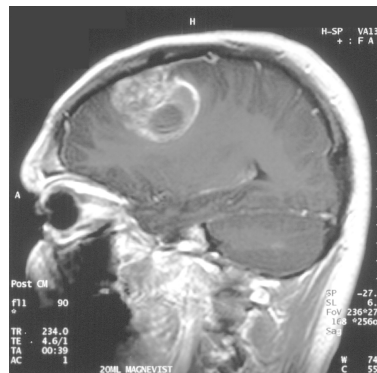


Figure 5. Glioblastoma multiforme. Sagittal magnetic resonance imaging (MRI) of a GBM in a 15-year-old boy. Used under the Creative Commons attribution Share Alike 3.0

Current standard of care for GBM includes maximal safe surgical resection followed by radiotherapy and concurrent treatment with Temozolomide (TMZ) followed by adjuvant TMZ. Surgical resection relieves the patients from symptoms but also allows for analysis of the tumor tissue and confirmation of diagnosis.

TMZ was included in the standard treatment of care after studies by Stupp *et al.*. In a phase III-trial they reported that surgical resection followed by radiotherapy with concurrent TMZ followed by adjuvant TMZ prolonged survival compared to patients receiving surgical resection and radiotherapy only (Stupp *et al.*, 2005). The follow-up study also showed an increase in 5-year survival to 9.8% versus 1.9% for the patients receiving radiotherapy only (Stupp *et al.*, 2009). TMZ is an alkylating agent that attaches an alkyl group to the guanidine base of DNA, which stops the DNA strand from uncoiling and separation and thus interferes with DNA replication and proliferation. Despite the promising results from Stupp *et al.* an epidemiologic study with approximately 20.000 cases, published in 2012, showed that survival in GBM patients in the TMZ era is only modestly improved (Darefsky *et al.*, 2012).

Due to the modest improvements in survival emerging from the traditional treatment of surgical resection, radio- and chemotherapy there have been intensified efforts in research to find new and more effective treatments. Immunotherapy aims to induce a potent tumor-specific immune response that is sufficiently long lasting to allow for a durable tumor regression and/or eradication. It is less toxic for the normal brain parenchyma than chemotherapeutic agents and also has the ability to induce an immunological memory that controls recurrent tumors. Immunotherapy such as cancer vaccines have shown encouraging results and provides a promising approach to complement and enhance the current treatment. However, despite progresses in the preclinical and clinical work, the outcome of most immunotherapeutic

clinical trials have been unsatisfactory and there are still great demands for improvements.

Tumor immunology

The concept that the immune system can recognize and eliminate developing tumors has been known for over a century. However, under certain conditions the immune system may also promote tumor progression by the secretion of proangiogenic factors, cytokines and growth factors. The protection against premalignant lesions and early tumors by the immune system is termed immunosurveillance. However, the dual role of the immune system has lead to a refinement of this concept, the immunoediting hypothesis.

Immunosurveillance

Already in 1909 Paul Erlich first proposed the idea that the immune system could protect against the occurrence of neoplastic disease. In 1959 and 1970 Thomas and Burnet proposed that lymphocytes were responsible for the recognition and elimination of continuously arising transformed cells and thus formulated the hypothesis of cancer immunosurveillance (Burnet, 1970). Despite the development in the field of immunology since the first idea of immunosurveillance was introduced in 1909, Thomas and Burnet failed to provide evidence for the process as they were using mice with spontaneous mutations rendering them immunocompromised but not completely immunodeficient. Only much later, with the development of gene targeting and transgenic mice, could the cancer immunosurveillance hypothesis be tested in molecularly defined murine models of immunodeficiency. The definite work demonstrating the existence of an IFN γ - and lymphocyte-dependent cancer immunosurveillance process

was performed with recombinaase activating gene (RAG)-2 knockout mice (lacking lymphocytes and NKT-cells) as well as STAT1^{-/-} knockout mice (lacking interferon-mediated pathways) (Shankaran *et al.*, 2001). This study showed a higher incidence of tumors induced by chemical carcinogens and spontaneous arising tumors compared to wild type mice (Shankaran *et al.*, 2001).

In a human study, data from a transplant registry on Nordic recipients of renal transplants was linked with the national cancer registries and analyzed. The transplant patients, who received immunosuppressive treatment or had primary immunodeficiencies, showed a higher risk of cancer development. The transplant patients had higher incidences of cancers in multiple different tissues (Birkeland *et al.*, 1995).

Immunoediting

Despite the evidence that support the existence of a cancer immunosurveillance process, immunocompetent individuals still develop cancer. This is due to a selection process where tumors of high immunogenicity are eradicated whereas tumors with low immunogenicity, which have a better chance of surviving in an immunocompetent host, are not. Several studies have shown that tumors are imprinted by the immunologic environment they are formed in e.g. tumors formed in the absence of an intact immune system are more immunogenic than those formed in an immunocompetent host (Engel *et al.*, 1997; Shankaran *et al.*, 2001; Street *et al.*, 2002; Svane *et al.*, 1996; Takeda *et al.*, 2002). This imprinting process selects for tumor cell variants with low immunogenicity and thus favors the formation of tumors that are either poorly recognized by the immune system and/or have developed mechanisms that suppress immune effector functions. The shaping of a tumor's immunogenicity most likely continues during tumor development but the major events probably occur early before the tumor is clinically detectable.

Based on this knowledge the term “cancer immunosurveillance” no longer sufficed to describe the complex interactions between a developing tumor and the immune system of the host. The original idea of cancer immunosurveillance referred to the protective function of the adaptive immune system only at the earliest stages of cellular transformation. Now, because of the recognition of the dual role of the immune system where not only protection from tumor development but also shaping of the tumor immunogenicity takes place, the concept of immunosurveillance has been proposed to be a part of a much broader term – immunoediting (Dunn *et al.*, 2002).

The dynamic process of immunoediting is defined with three different phases: elimination, equilibrium and escape (figure 6).

Elimination

The elimination phase corresponds to the concept of cancer immunosurveillance. In this phase transformed cells begin to grow invasively causing local tissue disruption in the surrounding tissue. The initiation of an immune response occurs when stressed cells start to produce endogenous danger signals, which initiate an inflammatory response. These danger signals recruit cells of the innate immune system such as DCs, macrophages, natural killer (NK) cells, NKT-cells and $\gamma\delta$ T-cells to the tumor site where these cells produce cytokines such as $\text{IFN}\gamma$, $\text{TNF}\alpha$ and nitric oxide (NO). The cytokine and NO production can have a direct toxic effect on the tumor cells but NK-cells can also directly eliminate tumors cells with decreased expression of classical HLA-I. As a result of these processes tumor antigens from dead tumor cells becomes available and cells of the adaptive immune system are recruited. Cell debris from dying tumor cells are phagocytized by professional APCs, which present the antigenic material to tumor specific naïve CD4^+ and CD8^+ T-cells in the draining lymph nodes. Activated CD4^+ T-cells start to produce cytokines such as $\text{IFN}\gamma$ and IL-2 that

facilitate the development of tumor specific cytotoxic CD8⁺ T-cells, but APCs can also directly activate the CD8⁺ T-cells via cross-priming. Activated CD8⁺ T-cells leave the lymph node and home to the tumor site where they can attack the remaining tumor cells (Dunn *et al.*, 2002).

Equilibrium

In the equilibrium phase the tumor cell variants that survived the elimination phase enter a dynamic equilibrium with the cells of the immune system of the host. In this phase the lymphocytes exert pressure on the tumor cells, which is enough to contain, but not fully eradicate them. During this phase many of the original tumor cells are destroyed but mutations in the tumor cell population give rise to new tumor cell variants with increased resistance to immune attack. Thus the immune system sculpts the heterogenous parental population and promotes the formation of a new population of tumor clones with lower immunogenicity (Dunn *et al.*, 2002).

Escape

The breach of the host's immune defense occurs when surviving tumor cells have acquired resistance to immune detection and/or elimination through genetic and epigenetic changes. In the escape phase the tumor cell variants selected in the equilibrium phase are allowed to expand (Dunn *et al.*, 2002).

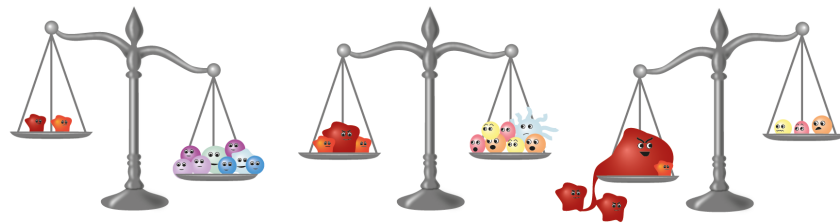


Figure 6. The three phases of immunoediting: elimination, equilibrium and escape.

Immunosuppression

As previously mentioned tumor cells can down regulate the expression of HLA-I to avoid recognition by the immune system. But tumors also have several other ways of evading the immune system. Immunosuppression is an important process to avoid over-activation of the immune system and autoimmune diseases. However, the down side of immunosuppression is that in a tumor setting it allows the tumor to avoid recognition and elimination. Mechanisms of immunosuppression, in addition to downregulation of HLA-I, include:

- Secretion of immunosuppressive factors such as TGF β (Maxwell *et al.*, 1992), interleukin-10 (IL-10) (Huettnner *et al.*, 1997) and prostaglandin E₂ (PGE₂) (Kokoglu *et al.*, 1998) or expression of apoptosis-inducing molecules such as FasL (Saas *et al.*, 1997).
- Regulatory T-cells (Tregs), which are recruited to the tumor site through soluble factors secreted by the tumor cells and promote the survival and expansion of the tumor (Crane *et al.*, 2012).
- Myeloid derived suppressor cells (MDSCs) (Bronte *et al.*, 2001) and tumor-associated macrophages (Van Ginderachter *et al.*, 2006), which in addition to Tregs also accumulate at the tumor site and have immunosuppressive capacity.
- Infection of viruses e.g. human cytomegalovirus (HCMV), which further enhance the tumor immune evasion and escape e.g. by inducing expression of cyclooxygenase-2 (COX-2) (Maussang *et al.*, 2009) or by the production of viral proteins targeting the APM (Hewitt *et al.*, 2001; Park *et al.*, 2004).

Summary and discussion of the project

The aim of my PhD project was to elucidate the role of tapasin and other APM proteins in the maturation of HLA-I. Tapasin is of high interest as it is a key protein that affects both quality and quantity of surface expressed HLA-I through multiple mechanisms. Some of these mechanisms have been unraveled whereas others, e.g. tapasin dependence of different HLA-I allomorphs, remain to be studied further. Although it is a key protein, tapasin is only one of the players in the quality control process that ensures correctly folded HLA-I molecules on the cell surface. Downregulation of APM proteins is an immune evasion strategy commonly seen in tumors. In my project we studied GBM, a tumor with poor prognosis for the patient, to investigate the expression of HLA-I, tapasin and other APM proteins and to study how the dysregulations of APM proteins affect the HLA-I molecules at the cell surface.

Paper I

In this paper we studied 16 different HLA-I allomorphs and investigated how tapasin facilitation depends on the HLA-I allomorph as well as the length of the bound peptide.

We used recombinant proteins and random peptide libraries (7-13 amino acids in length) to fold pHLA-I complexes and measured the amount formed with a luminescent oxygen channeling immunoassay (LOCI, commercialized as AlphaScreen™) (figure 7). Previously our group has been able to demonstrate that the first 87 N-terminal amino acids of tapasin (tpn₁₋₈₇) increases the amount of W6/32 recognized A*02:01 complexes (Roder *et al.*, 2009). Also, with this same method our group demonstrated that tpn₁₋₈₇

facilitated folding of A*02:01, B*08:01, B*44:02 and B*27:05 to different degrees, which was perfectly consistent with other studies of full-length tapasin and HLA-I allomorphs in cellular models (Park *et al.*, 2003; Peh *et al.*, 1998; Roder *et al.*, 2011).

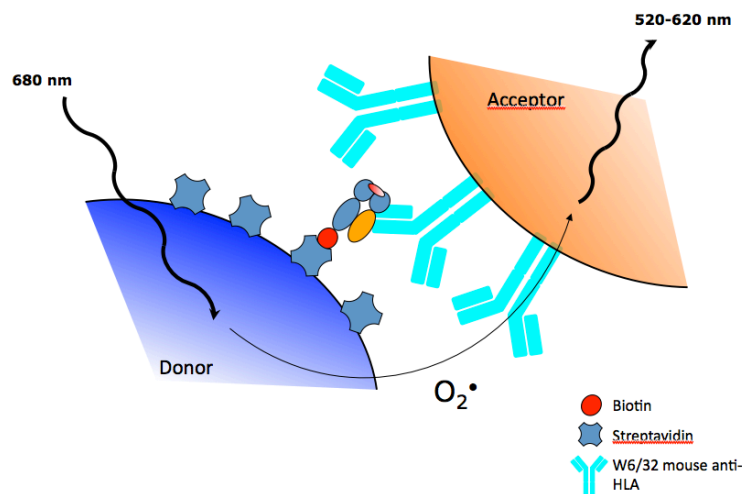


Figure 7. The principle of the AlphaScreen assay. The Alpha screen assay is a luminescent oxygen channeling immunoassay where the proximity of donor and acceptor beads are measured by light emission. The donor beads were coated with streptavidin and thus bind to the here used biotinylated recombinant HLA-I molecules. The acceptor beads are coated with W6/32, an HLA-I antibody that only recognizes the mature form of HLA-I i.e. HLA-I associated with β_2m . Binding of the W6/32 antibody to the HLA-I molecules brings the donor and the acceptor beads in close proximity. Upon illumination energy is transferred from the donor beads to the acceptor beads, which generates a luminescent signal.

The pHLA-I folding assay allows the simultaneous study of several (in our case 16) HLA-I allomorphs in the presence and absence of specific proteins e.g. tpn₁₋₈₇. This is the first study where such a high number of HLA-I allomorphs have been tested for tapasin facilitation in one single experimental set up. The 16 allomorphs showed a spectrum of tapasin facilitation ranging from very high to almost absent. The extremes i.e. the highly tapasin dependent B*44:02 and the tapasin independent mutant of A*02:01, HLA-A*02:01T134K, were

as expected placed in each end of the spectrum. However, the allomorph B*27:05, which has previously been suggested to have low dependency of tapasin (Park *et al.*, 2003), unexpectedly ended up further towards the middle in the tapasin dependency spectrum. Another member of the B*27 family, B*27:03, showed in our study to have the second highest dependence on tapasin following B*44:02. This is intriguing since B*27:03 and B*27:05 are in each end of the tapasin dependency spectrum and still differ in only a single amino acid in position 59. The same phenomenon has been observed for the more well studied B*44:02 and B*44:05, which also differ in a single amino acid but are placed in each end of the tapasin dependency spectrum (Peh *et al.*, 1998; Sieker *et al.*, 2007).

The T134K mutation of HLA-A*02:01 is commonly used as a negative control for tapasin facilitation as it has been shown to prohibit the interaction with tapasin (Lewis *et al.*, 1996; Peace-Brewer *et al.*, 1996; Yu *et al.*, 1999). However, some initial results from our group have indicated that HLA-A*02:01-T134K might not be completely independent on tapasin. For a set of peptides with a specific amino acid at a specific position, which I will refer to as “ghost peptides”, we have with the AlphaScreen assay been able to observe an effect of tapasin on the amount HLA-A*02:01-T134K complexes formed (figure 8). This has never been shown and is contradictory to previous studies on HLA-A*02:01-T134K. It is possible that the combination of residue and position in these ghost peptides affects the structure and the stability of the HLA-I molecule to a large extent and thus renders it dependent on tapasin to complete the maturation process.

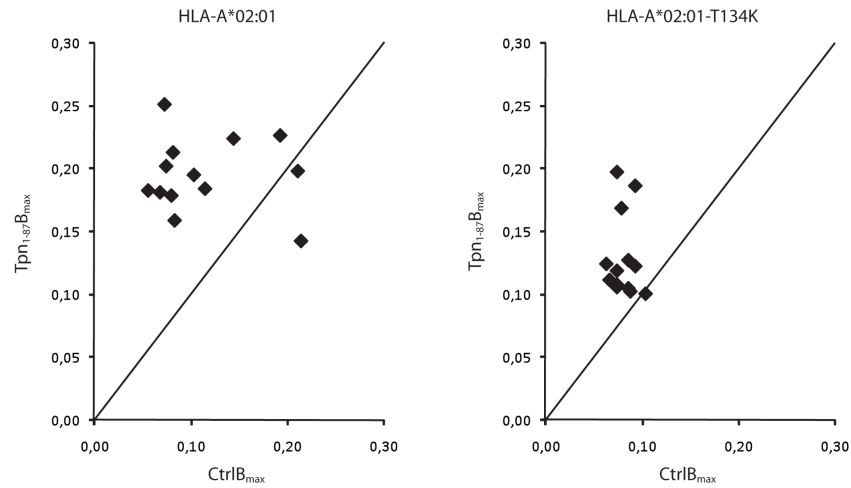


Figure 8. "Ghost peptides" make the HLA-A02:01-T134K tapasin dependent. The T134K mutation of A*02:01 is commonly used as a negative control for tapasin dependency. However, initial results from our group have indicated that tapasin facilitates the folding of HLA-A*02:01-T134K in the presence of peptides with certain amino acids at certain positions ("ghost peptides"). Shown here is the result from a representative experiment studying HLA-I folding with the previously described Alpha screen assay. As expected A*02:01 displayed a significant degree of tapasin facilitation with $Tpn_{1-87}B_{max}/CtrlB_{max} > 1.5$. Unexpectedly, A*02:01-T134K also showed some degree of tapasin facilitation for "ghost peptides" as $Tpn_{1-87}B_{max}/CtrlB_{max} > 1$. This was not seen for any other peptides tested for binding to HLA-A*02:01-T134K.

The AlphaScreen assay allowed us to determine tapasin facilitation based on the amounts of pHLA-I complexes formed in the absence and presence of tapasin. Interestingly the allomorphs with low tapasin dependency formed more pHLA-I complexes compared to the highly tapasin dependent allomorphs. Also the HLA-B allomorphs in this study showed to have a stronger preference (i.e. formed more complex) for the classical lengths 8- to 10-mers compared to the HLA-A allomorphs, which were less length sensitive and formed most complex with slightly longer peptides (10- to 11-mers).

Due to the co-dominant expression of three *HLA* genes each individual is expected to express three to six different HLA-I allomorphs at the surface of every cell. However, the proportion of allomorphs presented at the cell surface has not been studied. Studies of allomorph proportions are difficult partly due

to the lack of antibodies specific enough to distinguish between closely related HLA-I allomorphs. Other non-antibody dependent methods could possibly also be used to elucidate the proportions, at least to some degree. For example the proportions of HLA-A and -B allomorphs could be studied by labeling the HLA-I molecules with radioactivity and then pull them down using HLA-A and -B specific antibodies such as HCA2 and HC10. In our study of recombinant proteins we have shown that less tapasin dependent allomorphs form more complex than more tapasin dependent allomorphs both in the presence and absence of tpn₁₋₈₇. From this one may speculate that allomorphs of low tapasin dependence are expressed in higher proportions in cells. In tumor cells the proportion of allomorphs with low tapasin dependence would be even higher since the allomorphs of high tapasin dependence may not make it to the cell surface at all due to downregulations in tapasin (e.g. HLA-B*44:02). An increase in the proportion of allomorphs with low tapasin dependency might not be due to an increase in the quantity *per se* but rather a shift in the over all proportions due to the inability of more tapasin dependent allomorphs to survive on, or even make it to, the cell surface. With a general understanding of tapasin dependency and the proportions of cell surface expressed HLA-I allomorphs one would be able to predict what allomorphs that are presented on the cell surface and thus specifically select peptides with high immunogenicity. This would be highly relevant in the development of peptide vaccines. If it would be the case that less tapasin dependent HLA-I allomorphs are expressed at the cell surface in a higher proportion compared to more tapasin dependent allomorphs one could hypothesize that this is a result from evolutionary pressure from viruses and other intracellular organisms interfering with tapasin and other components of the PLC.

In addition to investigating the general tapasin facilitation of allomorphs in the presence of a mixture of peptide lengths we also evaluated the tapasin facilitation each specific peptide length. This was another question not addressed before that we could study with our system of recombinant proteins. We found that tapasin facilitation was more pronounced for complexes

formed with 7- to 8-mers and 12- to 13-mers, whereas facilitation of 10- to 11-mers was consistently low. The highly tapasin dependent allomorphs had a strong preference for specific lengths and formed the most complexes with 8- to 9-mers (and 10-mers for B*27:03). In contrast the allomorphs with intermediate and low dependency on tapasin were more promiscuous and formed higher amounts of complexes even with peptides ≥ 11 amino acids in length.

Previous studies by our group have shown, for a limited number of specified peptides, that low tapasin dependence correlate with high stability of the pHLA-I complex (Geironsen *et al.*, 2012; Roder *et al.*, 2011). In paper I we investigated, independently on peptide sequence, if two HLA-I molecules with distinct separation in tapasin facilitation (A*02:01 and A*02:01-T134K) also had different stabilities. After folding with random peptide libraries of 8-10 amino acids in length the dissociation was monitored over 24 hours at 37°C. Consistent with A*02:01 being more tapasin facilitated it also formed less stable pHLA-I complexes. When analyzing the dissociation curves for pHLA-I complexes of A*02:01 formed with peptides of 7-13 amino acids we found that 7- and 8-mers formed complexes with poorest quality and 13-mers formed the most stable complexes. The low stability of 7-mers was expected since the peptide binding requires a minimum length for the essential N- and C-terminal bonds to form. The high stability of 13-mers was more surprising since this length is usually considered to be too long for optimal binding. However, long peptides, including potential tumor epitopes, have also previously been identified for binding to A*02:01 (Chauvin *et al.*, 2012; Hu *et al.*, 2014).

The correlation between low tapasin dependence and stability of the pHLA complex may be due to a higher intrinsic stability of the HLA-I allomorph. Some allomorphs could have the capability to reach a fairly stable conformation already during the initial folding events and thus would not rely on tapasin to chaperone them. Studies have shown that tapasin dependency is connected to the flexibility of the peptide binding cleft (Ostermeir *et al.*, 2015;

Sieker *et al.*, 2007). Depending on the composition of amino acids in different HLA-I allomorphs, in the absence of a bound peptide, the molecule might be more or less prone to reach a conformation resulting in low flexibility of the peptide binding cleft. More tapasin dependent allomorphs become dependent on tapasin to stabilize the HLA molecule and to keep the flexible peptide binding cleft in an open state and allow peptides to bind. Once loaded with an optimal peptide the tapasin dependent HLA molecule can overcome the energy barrier necessary to convert to a closed conformation and thus be released from tapasin. Although an allomorph with low tapasin dependence does not need tapasin to reach a stable confirmation, tapasin likely still exerts an important role to allow loading of optimal peptides of high immunogenicity (Peh *et al.*, 1998; Purcell *et al.*, 2001).

Paper II

In paper II we turned to tumor material where we did immunohistological analysis of the expression of HLA-I and tapasin as well as the infiltration of CD8⁺ T-cells in 12 GBM tumor specimens. Initially we stained the tissue sections also for β_2m , LMP2 and TAP1 but struggled with high background in these stainings. Also, preliminary results from WB on other tumor material were indicating highly variable expression levels of tapasin and thus we chose to focus on optimizing the staining protocols for tapasin and HLA-I only.

Generally, evaluation of immunohistochemical (IHC) stainings includes more than one scientist who judge and score a tissue section by looking at it in a microscope. Such an evaluation could be biased by several things such as scientific background of the scientist, which parts of the tissue you look at and also the perception of color strength. To overcome the limitations of existing methods I developed a novel method of digital analysis that allows an unbiased analysis of whole tumor sections. Apart from these obvious advantages this method is also much faster and can be done by one single scientist.

Following the staining of the tissues the sections were scanned with the ScanScope CS system (Aperio) to create digital slides. These were subsequently analyzed in ImageScope 7.01 (Aperio) using the Positive Pixel Algorithm (figure 9). The Positive Pixel Algorithm evaluates and scores the intensity of the color that represents your target protein in each pixel of the digital slide. Each pixel is then placed in different groups depending on staining intensity. The groups of “strong positive”, “positive”, “weakly positive” and “negative” have certain colors assigned to them (red, orange, yellow and blue respectively) and thus after analysis a colored “mark up image” appears, which provide an overview of the analysis of your staining. The rules for what color to search for as well as the range of color intensity is determined by the user and thus you can set the program to not recognize e.g. background staining. Importantly you can also exclude parts of the tissue from the analysis e.g. necrotic tissue, which are frequently present in GBM tumors.

To be able to compare the tissues from different tumors we assigned the pixels of each group a score. Strong positive (red) pixels got a score of 3, positive pixels (orange) a score of 2, weakly positive (yellow) pixels a score of 1 and negative (blue) pixels a score of 0. In this way we could calculate a Positive Pixel Score and to be able to compare the tissues each Positive Pixel Score was divided by the total number of pixels analyzed, i.e. $(\text{Blue} \times 0 + \text{Yellow} \times 1 + \text{Orange} \times 2 + \text{Red} \times 3) / \text{Total number of pixels analyzed}$).

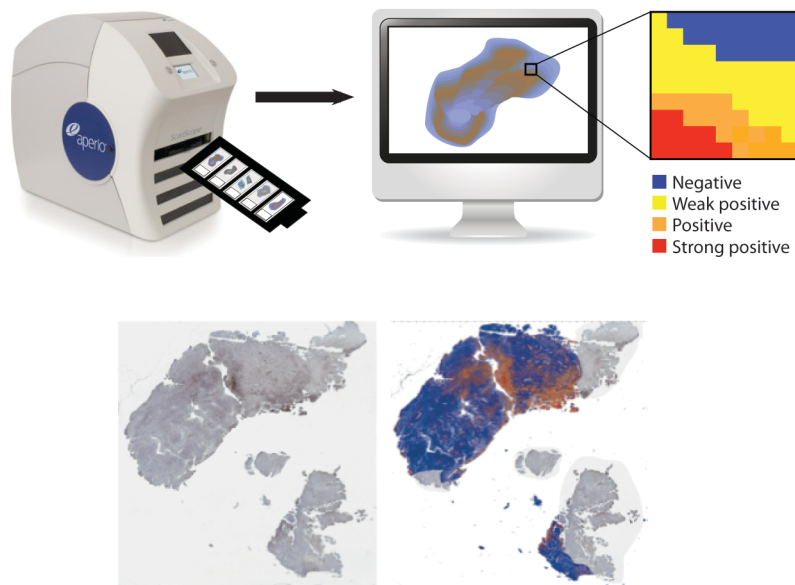


Figure 9. Principle of digital analysis of GBM specimens. Stained sections were scanned to create digital slides, which were subsequently analyzed in ImageScope 7.01 (Aperio) using the Positive Pixel Algorithm. The algorithm evaluates the staining intensity in each pixel of the digital image and divides each pixel into a group of “strong positive”, “positive”, “weakly positive” or “negative” (red, orange, yellow and blue respectively). The lower panel shows one example of GBM tissue analyzed with the Positive Pixel Algorithm. Lower left panel shows the scanned original GBM tissue stained for HLA-I HC and the lower right panel shows the same tissue after analysis with the Positive Pixel Algorithm. The areas positive for HLA-I are colored yellow-red and negative areas blue. Necrotic tissue and tumor of lower grade have been excluded from the analysis.

IHC stainings of HLA-I and tapasin revealed highly variable expression of both proteins between GBMs. However the expression patterns of HLA-I and tapasin in each tumor were strongly correlated. To assure that we were staining tumor cells and not other types of cells such as immune cells (which would express higher levels of HLA-I as they do not suffer from the genetic alterations occurring in tumor cells) we performed one double staining on a HLA-I high tumor with antibodies against HLA-I and CD45. CD45 is a surface glycoprotein expressed on all nucleated hematopoietic cells and was chosen to distinguish infiltrating immune cells in the tissues. In addition we

also made single CD45 stainings on all the GBMs. A number of HLA-I positive cells were also positive for CD45, however, most of the HLA-I positive cells were CD45⁻. The HLA-I and CD45 double staining showed that the HLA-I⁺ with highest intensity were also CD45⁺ indicating that HLA-I expression in these cells did indeed not suffer from downregulation. Despite this knowledge we chose to include all positive pixels in the analysis. The number of double positive CD45⁺ and HLA-I⁺ pixels constituted a very small part of the total number of analyzed pixels and restraining the analysis to not include pixels with high intensity staining might also then exclude staining from the tumor cells.

The extent of CD8⁺ lymphocyte infiltration has been shown to improve patient prognosis in gliomas (Kmiecik *et al.*, 2013; Lohr *et al.*, 2011) and thus we evaluated the extent of infiltrating CD8⁺ lymphocytes. CD8⁺ cells were distinct with strong intensity staining and thus for CD8, to exclude the background staining that occurred, we considered only the strong positive pixels as true positives. Infiltration of CD8⁺ cells was generally very low. The tissues with the highest amount of CD8⁺ cells displayed a range of HLA-I expression from high to low. A high expression of HLA-I together with a high infiltration of CD8⁺ cells could indicate the presence of cytotoxic CD8⁺ T-cells active against the tumor. A high infiltration of CD8⁺ cells together with intermediate/low amount of HLA-I is somewhat harder to explain. One possible reason could be that CD8 is not exclusively expressed on cytotoxic T-cells but also exists on other types of immune cells such as DCs (Winkel *et al.*, 1994), macrophages (Hirji *et al.*, 1997), NK-cells (Perussia *et al.*, 1983) and regulatory T cells (Gershon & Kondo, 1971).

We could not find a correlation between the amount of infiltrating CD8⁺ cells and HLA-I or tapasin expression. Nor could we find a correlation between CD8⁺ cells and survival. However three out of the four patients with highest infiltration of CD8⁺ cells also had a survival time above the median survival of

420 days. In contrast, patient survival did correlate with both HLA-I and tapasin expression.

Paper III

In Paper III we extended the set of GBMs and added another 11 tumors to the IHC analysis of HLA-I and tapasin. For these 11 tumors we also had corresponding cell lines, which we analyzed for the expression of not only HLA-I and tapasin but also the expression of other APM proteins using western blot. We also analyzed HLA-I surface expression on the cells with immunocytochemistry and flow cytometry and determined what HLA-I allomorphs were expressed and how they depend on tapasin for maturation. In addition we used .221 cells (tapasin proficient) and .220 cells (tapasin deficient) and a small set of exogenously loaded peptides to investigate how lack of tapasin affected the properties of HLA-I molecules on the cell surface.

The IHC stainings were performed and analyzed as previously described. The addition of these 11 tumor sections to the previously analyzed 12 tumor sections strengthened the correlation with survival for both HLA-I and tapasin.

In addition to HLA-I and tapasin we decided to analyze the expression of several other APM proteins in the corresponding glioma cell lines (GCLs). Protein expression was evaluated with western blot and we used a somewhat unconventional approach to correct for equal loading of lysates. Typically a house keeping protein such as β -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are used as a loading control since they are often stably and constitutively expressed at high levels in most tissues. However, tumor cells display unstable expression of many proteins, including housekeeping proteins (Ferguson *et al.*, 2005; Waxman & Wurmbach, 2007), and thus they should only be used as loading controls after careful evaluation that their expression indeed do not vary between samples. In the GCLs the

expression levels of both β -actin and GAPDH were highly variable and thus we used a total protein stain to correct for equal loading of protein lysates.

As we analyzed the expression of proteins in tumor cells one would have wished for a control to be able to compare expression levels with a “normal level”. However, normal brain cells are hard to obtain. Also, a normal brain cell generally express very low levels of HLA-I, a consequence of the brain being an immune privileged site and one may speculate that brain tumor cells initially up regulate HLA-I and subsequently down regulate it. This is in line with the elimination phase of immunoediting in which the invasive growth of the tumor initiates an inflammatory response. This in turn results in the production of cytokines such as IFN γ , which increases the expression of HLA-I and APM components. When the tumor cells then move into the equilibrium phase they have accumulated mutations that result in downregulation of HLA-I and APM proteins, which increases the tumor cells’ resistance to immune attack. In the search of a control we tried to use total brain lysates, which however were of too poor quality and produced only large smears in immunoblotting. We also considered to use a “non-brain cell”, B-LCL cell line .221 as a control but decided that it would not be representative for neither normal brain cells nor IFN γ stimulated brain cells. Hence, in the absence of an appropriate control we decided to compare the protein expression levels between GCLs and not elaborate on how the expression levels relate to a normal state.

HLA-I and several of the APM proteins showed highly variable expression among GCLs and some proteins were expressed in a correlated manner. HLA-I clustered with tapasin, LMP2 and LMP7, β_2m , TAP1 and TAP2 whereas calnexin and calreticulin formed another small cluster. Not included in the paper are the stimulation experiments with exogenous IFN γ , which increased the expression of tapasin, HLA-I HC (figure 10a), tapasin, TAP1 and TAP2, LMP2 and LMP7 whereas expression of ERAP1, BAP31 and calnexin was not affected. ERp57 was not, with one exception GCL2, upregulated by IFN γ .

Also, we found an increased expression of W6/32 reactive HLA-I molecules on the cell surface after treatment with IFN γ (analyzed by immunocytochemistry (ICC) and flow cytometry) (figure 10b). The upregulated expression in the presence of IFN γ indicated that the dysregulations in the GCLs are due to reversible defects at transcriptional level rather than irreversible structural defects.

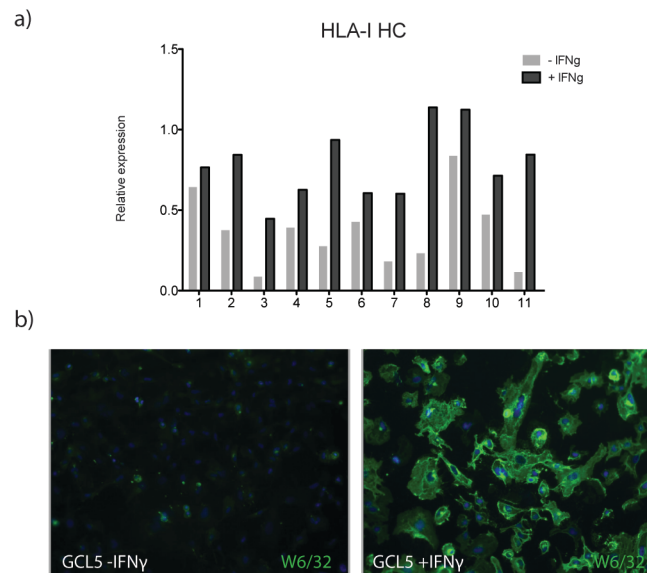


Figure 10. HLA-I is upregulated by IFN γ . a) Representative western blot of HLA-I HC levels in the presence and absence of exogenously added IFN γ . b) ICC stainings of one representative GCL in the presence and absence of IFN γ .

Together with the intracellular analysis of HLA-I HC and APM proteins we also wanted to assess the expression of mature HLA-I molecules (recognized by W6/32) on the cell surface of the GCLs. Highly variable expression of surface expressed HLA-I was initially observed with ICC and subsequently verified with flow cytometry.

As tapasin expression was highly dysregulated among GCLs and also strongly connected to HLA-I expression we wanted to investigate which HLA-I allomorphs were expressed in the GCLs and how they depend on tapasin for maturation. High resolution HLA-I typing revealed the allomorphs present in the GCLs. We were able to obtain most of recombinant proteins for the HLA-I allomorphs present in the GCLs, which we then studied with regards to tapasin facilitation with the previously described AlphaScreen assay. The analysis of tapasin facilitation revealed a broad spectrum where the HLA-I allomorphs ranged from very high tapasin dependency to very low tapasin dependency, which we have also observed before for the set of 16 allomorphs analyzed in paper I. We hypothesized that some HLA-I profiles, if they were expressing e.g. several HLA-I allomorphs with high tapasin dependency, could be more sensitive to downregulation of tapasin and thus more prone to develop a progressing tumor. However, determination of the tapasin facilitation of the allomorphs showed that each GCL expressed HLA-I allomorphs with variable tapasin dependency. Also, when comparing to a healthy cohort we could conclude that HLA-I profiles of glioma patients did not differ in either the dispersion of each HLA-I allomorph's tapasin facilitation or the average level of tapasin facilitation.

Due to the lack of allomorph specific antibodies it was not possible to determine what HLA-I allomorphs that were actually present on the cell surface or in what proportions they were expressed. Tapasin dependence has only been analyzed in cellular systems where one single allomorph at the time has been transfected into tapasin deficient or tapasin proficient cells and then compared to another transfectant. However, the question of how the presence or absence of tapasin might affect the proportions of different allomorphs at the cell surface remains unanswered. Although we cannot tell about the proportions yet this study is the first published paper with an analysis of tapasin dependence for a complete HLA-A and -B profile.

In an attempt to evaluate the quality of the HLA-I molecules expressed at the cell surface of the GCLs we tried to study the stability of these molecules. We

used brefeldin A (BFA), which blocks the intracellular transport of newly synthesized HLA-I, and assessed the remaining surface expression after 2-26 hours. Unfortunately, for the GCLs we were not able to reach a reliable and reproducible experimental set up and thus we took one step back and turned to the simpler cellular model of the tapasin deficient .220 cell lines, here transfected with only one allomorph, A*02:01. The advantages of this model is that we could study the effect on the quality of a particular allomorph resulting from a single APM deficiency i.e. tapasin. We hypothesized that in the absence of tapasin a tapasin dependent allomorph would not receive the assistance required in the maturation and peptide loading process and thus end up suboptimally loaded and unstable at the cell surface. By loading of exogenously supplied peptides we aimed to increase the stability of the cell surface expressed HLA-I molecules on .220 cells through the exchange to a more optimal peptide. The tapasin proficient .221 cell line transfected with A*02:01 was used as control. Three peptides, predicted to bind to A*02:01 with high affinity, were used in an extracellular peptide loading assay. For the tapasin proficient .221 cells we were not able to increase the stability of the A*02:01 molecules by the addition of any of the exogenous peptides. In contrast, for the tapasin deficient .220 cells, we were able to increase the stability of A*02:01 by adding exogenous peptide. Stabilization occurred for only one of the tested peptides, however, the peptide predictions were based on affinity, which is not the only factor that dictates the rules of an optimal peptide (Assarsson *et al.*, 2007; Harndahl *et al.*, 2012). Also the here studied peptides are exchanged on the cell surface, which is not identical to the peptide editing process in the ER. Surface expressed HLA-I molecules most likely have another, perhaps more rigid, conformation that may not allow peptides to be exchanged to the same extent. It is possible that different allomorphs, in the absence of tapasin, could be more or less rigid when presented at the cell surface and thus also be more or less prone to exchange their peptides. However, to survive on the cell surface the HLA-I molecules must reach some level of folding and stability and thus they will presumably always have a more

rigid conformation compared to the HLA-I molecules undergoing initial peptide loading in the ER.

In an attempt to find peptides that could potentially be considered for immunotherapy we investigated a set of HCMV peptides with our Alpha screen method. HCMV peptides are of high interest as they obstruct the function of tapasin and thus also the quality control of tapasin dependent HLA-I allomorphs. Previous work by our group has shown that the tapasin fragment tpn₁₋₈₇ facilitates the folding of HLA-I molecules with non-SYFPEITHI peptides but does not facilitate folding with SYFPEITHI peptides (Roder *et al.*, 2011). The SYFPEITHI database comprises peptide sequences that are known to bind to HLA-I in cells. Non-SYFPEITHI peptides may be predicted to bind to HLA-I or even bind in biochemical but have not been reported as natural ligands. We investigated a set of HCMV peptides, both SYFPEITHI and non-SYFPEITHI, to see if we with tpn₁₋₈₇ could distinguish any “new” SYFPEITHI peptides, which could have potential in immunotherapy. To ease the workload of pipetting we used a Janus Automated workstation from Perkin Elmer. The Janus was a new investment in our group and thus had to be programmed before it could be used for our particular experimental set up. As I was the first to use the Janus I also became responsible for the programming of it, which I spent a great effort on. With the Janus we were able to run larger experimental set ups with less room for human error. However, in this set of investigated HCMV peptides we could not reveal any new SYFPEITHI peptides with the use of tpn₁₋₈₇.

This paper is another piece of the puzzle towards improved understanding of immune evasion strategies and tumor immunology. New and more effective treatments for GBM are highly desirable and immunotherapeutic strategies such as peptide vaccination could be efficient although the outcome from clinical trials have so far been poor. Commonly peptides are chosen based on high affinity, however, to be able to chose which allomorph to target we propose that individualized protocols involving HLA-I binding antigenic peptides should also include consideration of the HLA-I allomorph profile and

its tapasin dependence. Including high resolution HLA-I typing along with determination of tapasin expression and dependency of allomorphs in each patient would allow better selection of peptides of higher immunogenicity as one could estimate which HLA-I allomorphs that are likely to be presented at the cell surface.

Conclusions

In conclusion my work has contributed to increased understanding of antigen presentation, and the function of tapasin in particular, on a molecular and mechanistic level. It has also provided a unique insight in tapasin dependence and folding capacity of different HLA-I allomorphs. We have shown that tapasin dependence of HLA-I allomorphs range from high to low with the mutant HLA*02:01-T134K in the extreme end considered not affected by tapasin. However, using a set of peptides with defined features we could observe an effect of tapasin also on the mutant HLA-A*02:01-T134K. This suggests that HLA-A*02:01-T134K is not completely independent on tapasin and that it is indeed the combination of peptide and HLA-I allomorph that dictates tapasin dependence. A large number of APM components were studied in glioma cell lines and in addition tapasin and HLA-I were studied in tumor sections. This study clearly pointed out that tapasin stands out among the APM components and that its expression level is strongly linked to HLA-I expression and patient survival. Moreover this is the first study contributing knowledge of the complete HLA-I profiles in terms of tapasin dependency of individual allomorphs as well as the average tapasin dependency of the HLA-I profile in both glioma patients and healthy individuals. In addition I have shown that certain high affinity peptides can be loaded on surface expressed A*02:01 in tapasin deficient cells while other high affinity peptides are not loaded. This suggests that the selection of appropriate high affinity peptides is highly relevant for the success of peptide-based immunotherapy. The other factors of importance are the tapasin expression level in combination with the choice of HLA-I allomorph for which the selected peptide is dedicated. The latter must be based on the knowledge of the entire HLA-I profile as well as the tapasin dependence of each HLA-I allomorph.

Populärvetenskaplig sammanfattning

Vårt immunförsvar består av en mängd olika celler och mekanismer som rensar kroppen från organismer, såsom bakterier och virus, som skulle kunna vara skadliga för oss. Det är uppdelat i två olika delar: det nativa (eller medfödda) och det adaptiva försvaret.

Det medfödda försvaret består av yttre fysiska barriärer såsom hud och slemhinnor men också av ett inre försvar som är uppbyggt av en rad olika celler. Det inre medfödda försvaret reagerar omedelbart men är inte särskilt specifikt och ger inte heller något långvarigt skydd.

Det adaptiva immunförsvaret tar längre tid att aktivera men är däremot mycket specifikt och kan känna igen och selektivt ta bort specifika inkräktare. Detta försvar aktiveras när det medfödda försvaret inte längre kan hålla emot och det byggs upp av två olika typer av celler B-celler och T-celler.

B-celler producerar antikroppar som kan binda till inkräktare och göra dem synliga för fagocyter som kan äta upp och förstöra dem. T-celler har en molekyll på ytan, en T-cell receptor, som kan scanna av cellerna i kroppen. På ytan av varje cell i vår kropp visas små fragment av alla proteiner som finns inuti cellen. Dessa kallas för peptider och kan läsas och utvärderas av T-cellernas receptor. Om man är helt frisk är det bara kropps-egna peptider på cellytan och T-cellerna kan scanna av peptiderna och sen gå vidare utan att initiera någon attack. Men om en cell är infekterad av t.ex. ett virus så kommer virus-proteiner att tillverkas inuti cellen och då även virus-peptider finnas på cellytan. T-cellerna kan då se att cellen har blivit infekterad och förstöra den.

För att T-celler ska kunna känna igen peptiderna på cellytan måste de vara bundna till en så kallad HLA-molekyl. HLA-molekylerna sitter på cellytan av nästan alla celler och har en klyfta högst upp där de binder in peptiden och

visar upp den för T-cellen. Varje människa har mellan tre och sex olika varianter av HLA-molekyler och dessa har alla olika preferenser för vilka peptider de kan binda in och sen presentera på cellytan. Innan HLA-molekylen kan binda en peptid måste den först gå igenom en matureringsprocess där den veckas på rätt sätt. Detta sker inuti cellens endoplasmiska reticulum med hjälp av flera andra proteiner som tillsammans på engelska kallas ”antigen processing machinery” (APM). Särskilda proteiner i en del av detta maskineri är ansvariga för själva laddningen av peptid på HLA-molekylen när den har veckats så att bindingsklyftan bildats. Kompositionen av proteiner som assisterar vid peptidladdningen kallas specifikt för peptidladdningskomplexet och består förutom HLA-molekylen av proteinerna TAP, tapasin, ERp57 och calreticulin. Speciellt ett av dessa proteiner, tapasin, är särskilt betydelsefullt eftersom det hjälper till att hålla upp peptidbindningsklyftan så att olika peptider kan prova att binda in. Det är viktigt att den peptid som ska presenteras på cellytan sitter fast ordentligt men också att den har bra passform till klyftan så att hela peptid:HLA-komplexet kan anta en stabil konformation. När en peptid som passar på dessa kriterier (d.v.s. en optimal peptid) har bundit in i klyftan kan tapasin känna av att peptid:HLA komplexet är stabilt och släppa iväg komplexet så att det kan presentera den laddade peptiden på cellytan.

Tumörer kan undvika att upptäckas av immunförsvaret genom genetiska mutationer som påverkar hur mycket HLA eller APM proteiner det finns i tumörcellen. Mutationerna kan resultera i nedreglering av uttrycket av själva HLA-molekylen så att T-cellerna inte kan läsa av tumörcellen och se att den är defekt. Eller så kan de resultera i nedreglering av någon eller flera av APM proteinerna så att dessa inte kan hjälpa HLA-molekylen att veckas och ladda peptid på rätt sätt. Detta leder till att HLA-molekylen inte blir stabil nog för att sitta kvar på cellytan tillräckligt länge för att en T-cell ska hinna läsa av den eller så är den så ostabil att den inte ens klarar resan ut till cellytan.

I mitt projekt har jag dels studerat tapasins effekt och tittat på hur mycket olika varianter av HLA beror av tapasins hjälp för att ladda en optimal peptid

samt hur peptidens längd påverkar effekten från tapasin (delarbete I). I den andra delen har jag dels studerat uttrycket av HLA och tapasin i vävnad från hjärntumören glioblastoma multiforme men också studerat de andra APM proteinerna i tumörceller från samma vävnader. Där har jag tittat på hur mycket av varje APM protein det finns i tumörcellerna och även hur HLA-molekylerna på cellytan av tumörcellerna påverkas av ett nedreglerat uttryck av tapasin och andra APM komponenter.

HLA-generna är de mest variabla generna vi har och i skrivande stund har ca 6500 olika HLA-varianter identifierats (HLA Informatics group, 2015). I delarbete I jämförde vi 16 olika varianter av HLA-molekyler med avseende på hur mycket de beror av tapasins hjälp för att bilda stabila peptid:HLA komplex. I den här studien använde vi oss av konstgjorda proteiner till skillnad från tidigare studier där man använt hela celler och bara kunnat titta på en HLA-variant i taget. Detta är den första studien där man tittar på tapasins effekt för så många HLA varianter i ett och samma experiment, en stor fördel om man vill kunna jämföra dem. HLA-varianterna i vår studie visade sig ha väldigt olika tapasin-beroende, från högt beroende till väldigt lågt beroende av tapasin. De mest extrema varianterna på skalan behöll sin plats i hierarkin jämfört med tidigare cell-baserade studier medan andra placerade sig något annorlunda. Vi kunde också se att de minst tapasin-beroende HLA-varianterna bildade mer peptid:HLA komplex är de som är mer tapasin-beroende. Detta resultat skulle möjligtvis kunna avspegla proportionerna av olika HLA-varianter på cellytan, något som hittills inte kunnat studeras pga. av experimentella begränsningar. Vi tittade även på hur beroendet av tapasin påverkades av längden på den inbundna peptiden och fann att peptidlängd har större betydelse för mer tapasin-beroende HLA varianter.

I delarbete II och III ville vi undersöka uttrycket av HLA och APM proteiner i vävnad och cellinjer från hjärntumören glioblastoma multiforme. Glioblastom är en mycket aggressiv hjärntumör med dyster prognos. Trots nuvarande behandling som inkluderar operation, strålning och kemoterapi är medellängden för överlevnad ca 15 månader, en siffra som inte förbättrats

under de senaste decennierna. Eftersom traditionella behandlingsmetoder inte har resulterat i förlängd överlevnad för glioblastompatienter pågår det intensiv forskning för att försöka hitta nya och mer effektiva behandlingsmetoder. En typ av behandling som skulle kunna vara effektiv är immunoterapi, en metod som går ut på att rikta och stimulera kroppens egna immunförsvar mot tumörcellerna.

Delarbete II innehåller en analys av tumörvävnader från 12 olika patienter med glioblastoma multiforme. Här analyserade vi uttrycket av HLA och tapasin och fann att uttrycket för båda HLA och tapasin var högst variabelt mellan olika vävnader men starkt korrelerade. Vi tittade också på infiltrationen av en viss typ av T-celler, eftersom det finns studier som har visat att detta kan förbättra prognosen för glioblastompatienter. Generellt var infiltrationen av dessa T-celler låg och vi kunde inte hitta någon korrelation med varken HLA, tapasin eller överlevnad. Däremot så hade tre av de fyra patienter med mest T-cell infiltration en överlevnad som var längre än medellängden på 420 dagar. Vårt viktigaste fynd var att överlevnad korrelerade med både HLA och tapasin.

I delarbete III utökade vi studien och analyserade uttrycket av HLA och tapasin i ytterligare 11 tumörvävnader. Även här observerade vi den starka korrelationen mellan HLA och tapasin. Genom att lägga till dessa 11 vävnader till de tidigare analyserade 12 vävnaderna kunde vi också stärka korrelationen med överlevnad för både HLA och tapasin. För dessa 11 vävnadssnitt hade vi också korresponderande cellinjer där vi analyserade uttrycket av HLA och tapasin men även flertalet andra APM proteiner samt relationen mellan dem. Flertalet av APM proteinerna samt HLA hade mycket variabelt uttryck i de olika cellinjerna och vi fann även vissa kluster av APM proteiner som korrelerade med varandra. Eftersom uttrycket av APM proteiner var så ojämnt i tumörcellerna ville vi undersöka hur mycket HLA molekyler som fanns på ytan av tumörcellerna. Även här fann vi stor variation mellan cellinjerna.

Eftersom tapasin uttrycktes i väldigt olika mängd i de olika cellinjerna och också var starkt kopplat till uttrycket av HLA ville vi undersöka vilka varianter

av HLA som fanns i våra celler och även hur mycket de berodde av tapasin för veckning och peptidladdning. Därför tog vi reda på vilka varianter som uttrycktes och använde sedan samma metod som i delarbete I för att bestämma tapasinberoendet för varje variant. Vi hade en hypotes om att vissa HLA-profiler, t.ex. om flera av HLA varianterna hos en patient är väldigt tapasinberoende, skulle kunna vara mer känsliga för nedreglering av tapasin och därmed mer benägna att utveckla tumörer. Men analys av tapasinberoendet av HLA varianterna som fanns i våra cellinjer visade att varje cellinje hade HLA varianter som var både mer och mindre tapasinberoende. Det visade sig också, när vi gjorde samma analys för en frisk grupp, att glioblastompatienternas HLA-profiler inte skiljer sig från de friska individernas profiler.

Vi var också intresserade av att studera hur tapasin påverkar kvaliteten av HLA molekyler som presenteras på cellytan. I våra tumör-cellinjer har många av APM proteinerna ett onormalt uttryck och därför är det svårt att studera effekten av endast tapasin. Därför använde vi istället en annan cellinje som har normalt uttryck av APM proteiner samt en liknande cellinje som totalt saknar tapasin. Här hade vi en hypotes om att avsaknaden av tapasin skulle resultera i laddning av dåligt passande peptider. Det skulle i sin tur resultera i HLA-molekyler på cellytan med peptider som skulle kunna bytas ut mot mer optimala peptider, vilket också skulle resultera i en mer stabil HLA-molekyl. Mycket riktigt kunde vi genom att tillsätta peptider vid cellytan öka stabiliteten för HLA-molekylerna på ytan av de celler som saknade tapasin (och som i och med denna egenskap liknar tumörceller), men inte på de celler som hade tapasin.

Det här projektet är ytterligare en pusselbit i arbetet för att öka förståelsen för tumörimmunologi och mekanismer för hur tumörer undkommer immunförsvaret. Nya och mer effektiva behandlingsmetoder behövs och vissa immunoterapeutiska strategier har potential även om resultaten från kliniska prövningar hittills har varit otillfredsställande. Peptider som ingår i peptidvaccin selekteras vanligtvis baserat på hur bra en peptid binds av en viss HLA-

molekyl utan att ta hänsyn till vilka andra HLA molekyler individen har. Vi föreslår nu att en sådan behandling borde utformas efter varje individ och baseras på kunskap om individens hela uppsättning av HLA. Genom att bestämma vilka HLA-varianter som varje patient har, hur beroende av tapasin de är, och samtidigt studera uttrycket av tapasin skulle man kunna förbättra valet av peptider för peptid-vaccin eftersom man bättre skulle kunna förutsäga vilka HLA varianter som är troliga att existera på cell ytan.

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