T cell immunity against MHC-Ilow tumors in mouse models

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Chapter 1

General Introduction

Cancer and the immune system

The first indication for an important role of the immune system in the treatment of cancer was in 1893. William Coley, a surgeon in New York, cured a patient with an inoperable tumor by injecting a mixture of bacteria and bacterial lysates[1]. Since then, many studies have been performed to clarify the involvement of the immune system to prevent tumor development and induce tumor regressions. One of the breakthroughs in the field was the finding that blocking the inhibitory receptor CTLA4, expressed on T cells, was effective in inducing regressions of established tumors in mice[2].

In humans, the success of multiple cancer immunotherapeutic strategies support the role of the immune system in the induction of tumor regression. The use of checkpoint inhibitors, against CTLA4 and/or PD-1, has shown to be very powerful in inducing a potent T-cell mediated anti-tumor response in a large number of patients[3-5]. In advanced melanoma patients, checkpoint inhibitors are now approved for therapy and more studies are ongoing to evaluate the effect of checkpoint blockers in other types of cancer. Another type of immunotherapy in the treatment of cancer is vaccination of patients to stimulate tumor-specific T-cell reactivity. Vaccinating patients against the human papilloma virus inducing cancer[6,7] has been successful but in other types of cancer, the success of vaccination, is limited so far. Also, the injection of *in vitro* pre-activated tumor infiltrating lymphocytes (TIL) has resulted in some complete regressions of large tumors, again underscoring the involvement of the immune system in tumor clearance[8-10].

In 2002, the concept of immune editing was proposed, defining three stages of interaction between immune cells and tumor cells: elimination, equilibrium and escape[11]. In the first stage, immune cells detect and eliminate (pre)cancerous cells, a process called tumor immune surveillance. Using genetically modified mice, various immune cells, chemokines and effector molecules have shown to be involved in this tumor immune surveillance; including T cells and IFNγ signaling, as $Rag2^{-/-}$ mice and $Rag2^{-/-}$ x $STAT1^{-/-}$ mice develop significantly more tumors, than wildtype mice[12]. Other cells mediating immune surveillance include NK cells, NKT cells, γδ T cells and effector molecules TRAIL, NKG2D and type I IFN signaling[13,14]. As the tumor progresses, a state of equilibrium emerges, in which the tumor undergoes additional genetic changes as a result of immune pressure and then enables escape from the immune system to grow out further. In humans it is more difficult to provide prove for immune surveillance, but indirect evidence suggests importance of immune surveillance in preventing tumor development as well. People who are immune-suppressed after an organ transplant have increased frequency of some tumor types[15,16]. Also people infected with the HIV virus have increased risk of developing cancer, again suggesting immune cells are involved in the prevention of tumorigenesis[17]. Moreover, an intratumoral immune contexture consisting of activated $CD8^+$ T cells, T_h1 $CD4^+$ T cells and M1 macrophages is associated with improved clinical outcome[18-20]. Together with the large amount of studies demonstrating successful eradication of tumors upon application of immunotherapeutics, the significance of the immune system in the fight against cancer has been well established.

Tumor-specific T cells

The majority of the immunotherapeutic strategies involves the (re)activation of tumor-specific T cells. $CD4^+$ and $CD8^+$ T cells develop in the thymus, where tightly regulated processes ensure T cells to enter the periphery when they have a sufficient affinity and are not reactive towards self-antigens[21,22]. Once naïve T cells enter the periphery, they circulate between the blood and secondary lymphoid organs until they encounter their cognate peptide in the context of a major histocompatibility (MHC) molecule. $CD4^+$ T cells recognize antigens typically between 14 and 20 amino acids long in the context of MHC class II (MHC-II) molecules, whereas peptide recognized by $CD8^+$ T cells are 8-10 amino acids long and are presented in MHC-I molecules. Three signals are required to induce proper activation of naïve T cells: 1) interaction between the T cell receptor (TCR) and peptide:MHC complexes, 2) co-stimulation and 3) cytokine signaling. T cell activation also induces the expression of different chemokine receptors on T cells, enabling migration of the T cells to the site of inflammation where the corresponding ligands are secreted. After a successful immune response, the majority of antigen-specific T cells are deleted, leaving behind a small population of memory T cells able to quickly respond upon re-encounter of the antigen.

There are multiple types of $CD4^+$ T cells, including the suppressive regulatory T cells (T_{regs}) and various T helper (T_h) cells, which can be further divided in several subsets characterized by the expression of transcription factors and cytokine profile[23]. In an anti-tumor immune response, $CD4^+$ T cells with a T_h1 phenotype are important through their production of TNFα and IFNγ, thereby activating and recruiting myeloid cells and cytotoxic $CD8^+$ T cells to the tumor[24]. $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTL) can directly induce killing of tumor cells by expression of perforins and granzymes or membrane-bound death receptors. IFNγ, a type II IFN produced by CTL as well as T_h1 cells and NK cells, has multiple anti-tumor effects, both indirect and direct. Indirect effects include the recruitment and activation of various immune cells, the upregulations of MHC-I and co-stimulatory molecules on antigen-presenting cells (APCs), and also direct effects on tumor cells, such as induction of apoptosis and inhibition of cell growth[25]. Therefore, $CD8^+$ T cells are indispensable for the eradication of tumor cells.

Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) able to provide all three signals required for optimal T cell activation. DCs express several pattern recognition receptors (PRR) recognizing conserved molecular structures of pathogens, such as components of the bacterial wall or viral nucleic acids. These receptors include the family of Toll-like receptors (TLR), many of which are expressed by DCs[26]. Upon encountering a TLR ligand or another stimulus, DCs become activated and start to phagocytose pathogens and dead (tumor) cells, process them intracellularly and present peptides in MHC molecules to induce T cell activation. Activation of DCs induces upregulation of co-stimulatory molecules and increases their migratory capacities allowing them to migrate to lymphnodes to meet with naïve T cells. The potent capacity of DCs to induce a tumor-specific T cell response has been adopted for therapeutic purposes[27]. These include the activation of DCs *in vivo* through injection of DC stimuli, or by *ex vivo* activation of patient-derived DCs with tumor lysates[28-30].

Antigen processing for MHC-I

In all cells of the body, endogenous, cytosolic proteins are degraded by the proteasome to generate smaller peptides, between 4 and 20 amino acids[31]. Peptides of sufficient length are transported to the endoplasmic reticulum (ER) via the peptide transporter associated with antigen processing (TAP) (Figure 1). In the ER, peptides are loaded in MHC-I molecules and loading efficiency is increased by the peptide loading complex (PLC), consisting of chaperones tapasin, ERp57 and calreticulin and an MHC-I/β2m molecule. Tapasin connects TAP to the MHC-I molecules and the other two chaperones further stabilize the complex[31-33]. Aminopeptidase enzymes in the ER can further trim peptides which are too long to fit in the peptide-binding groove of MHC-I molecules. A well-known peptidase is ERAAP: ER aminopeptidase associated with antigen processing[31,34]. Once the peptide is bound in the peptide-binding groove, the peptide:MHC-I complex is released from the ER and moves to the cell surface, allowing scanning by $CD8^+$ T cells.

Dendritic cells can present peptides derived from exogenous sources in MHC-I molecules, upon take up of infected or dead cells, a process known as cross-presentation[35,36]. This process is important for the induction of a cytotoxic T cell response against tumor cells. Two pathways have been proposed describing how the exogenous antigen enters the MHC-I pathway. In the vacuolar pathway, antigens are further degraded in the phagosome by proteases and the low pH and ER resident molecules associated with the phagosome enable the binding of the peptide in MHC-I molecules to be presented in MHC-I on the cell surface[35]. According to the cytosolic model, exogenous antigens are released from the phagosome, enters the cytosol and then follows the 'endogenous' pathway via the proteasome and TAP-mediated transport into the ER. Since several different DC subsets exist, researchers tried to define the 'cross-presenting' DC type that is responsible for T cell induction. In mice, the $CD8\alpha^+$ DC subset is most potent in cross-presenting antigens and in humans this is the BDCA-3^+ DC subset[37,38]. In mice, the importance of the $CD8a^+$ DC subset, characterized by the transcription factor Batf3, is essential in the induction of an anti-tumor T cell response. Deletion of this population results in com-

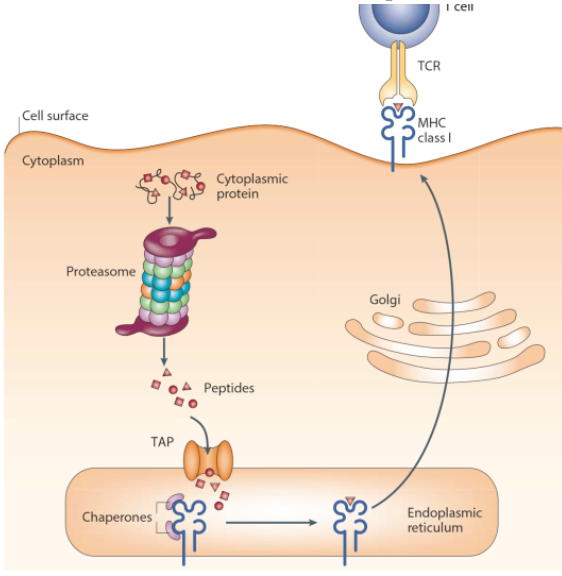

Figure 1: MHC-I antigen presentation pathway
Cytoplasmic proteins are degraded by the proteasome and peptides are transporter in the ER via the peptide transporter TAP. Upon binding with MHC-I molecules, the complexes are released from the ER and presented on the cell surface to $CD8^+$ T cells. Figure adapted from Yewdell et al., NRI (2003)

MHC-I downregulation in cancer

Just like viruses, tumor cells also manipulate the MHC-I processing pathway to escape from CD8$^+$ T cell mediated killing. In a metastatic melanoma patients who underwent multiple types of immune therapies, ten metastasis were resected; all eight regressing metastasis expressed high levels of HLA-I, whereas the two regressing metastases had lost this expression, suggesting that loss of HLA-I promoted tumor outgrowth[41]. Another recent study analyzed tumor material from four metastatic melanoma patients, treated with PD1 inhibiting antibodies, who initially responded well to the treatment, but later relapsed[42]. Exome sequencing on progressive material revealed mutations affecting the MHC-I expression; one patient had mutations in beta-2-microglobulin (β2m) and tumors of two patients had mutations in the JAK/STAT pathway, making cells irresponsive to IFNγ signaling. This was also seen in patients who failed to respond to CTLA4 antibody treatment[43].

The peptide transporter TAP in the ER membrane is the bottleneck of the MHC-I processing pathway, as it forms a bridge between the cytoplasm and ER lumen. It belongs to the family of ATP-binding cassette (ABC) transporters, and is a heterodimer consisting of TAP1 and TAP2 spanning the ER membrane and its expression is induced upon IFNγ stimulation[44,45]. In line with its pivotal role in the MHC-I pathway, TAP deficiency leads to a strong reduction of peptide:MHC complexes and is therefore a frequently used target to evade immune recognition by viruses and tumor cells. Several viral proteins block TAP function: ICP47 from the herpes simplex virus, UL49.5 from the bovine herpes virus and BNLF2A from the Epstein-Bar virus[46-48]. Loss of TAP is frequently found in different tumors, ranging from 10 to 70% and TAP impairment is associated with metastasis and poorer prognosis[49,50]. Loss of TAP1, TAP2 and tapasin are a prognostic factor for poorer survival in ovarian carcinoma[51]. Also loss of other components involved in the antigen processing machinery are often mutated and are associated with poorer prognosis and metastases as illustrated by many studies, such as reduced levels of tapasin in colorectal cancer which is associated with reduced CD8$^+$ T cell infiltrate and poorer survival[52].

Immune defense against MHC-I^{low} cells

Natural killer (NK) cells are well-known for their killing of MHC-I^{low} cells, as postulated by the missing-self theory[53,54]. However, their efficacy in the treatment of tumor cells have been limited so far. Some years ago, a novel category of CD8$^+$ T cells was identified, specifically recognizing peptides emerging on TAP-deficient cells.

TAP-independently processed peptides

Mice deficient for TAP1 have almost a complete absence of CD8$^+$ T cells, related to the lack of peptide presentation to developing CD8$^+$ T cells in the thymus[55]. Interestingly, the residual CD8$^+$ T cells selected in the absence of TAP are a diverse repertoire cells, and show self-reactive as well as alloreactive responses[55-57]. In line with a very small population of CD8$^+$ T cells in TAP1$^{-/-}$ mice, both human and mouse TAP-deficient cells still express residual peptide:MHC complexes on their cell surface. Several TAP-independently processed and presented ligands of the vaccinia virus (VACV) have been identified, some of which could induce a CTL response in HLA-A2transgenic mice[58]. Also signal peptides are well-known to be processed independent of TAP and as these peptides preferably bind into HLA-A2, expression of this HLA molecule is relatively unaffected by loss of TAP[59]. Comparison

of peptides presented on TAP-deficient versus TAP-proficient human cells revealed that in TAP-deficient cells, there is a relative abundance of signal peptides presented on the TAP-deficient cells in line with their TAP-independent processing[60,61]. Also non-signal peptide sequences were eluted from TAP-deficient cells, some of which depended on proteasome processing[61]. Peptides presented on TAP-deficient cells had a decreased MHC-I binding affinity compared to those presented on TAP-proficient cells[61]. This could be due to the alternative binding motive of the peptides in the binding groove of the MHC-I molecule[60].

In TAP-proficient cells, the leader sequence of classical MHC-I molecules is the main peptide bound in the non-classical MHC-I molecule HLA-E and its mouse homologue Qa-1^b [62]. Loss of TAP results in an alternative peptide repertoire presented in these monomorphic MHC-I molecules. Peptide elutions of TAP-deficient K562.HLA-E cells showed a broad repertoire of more than 500 peptides eluted from HLA-E [63]. The majority of these TAP-independently processed peptides were 9-mers and had a binding motive very comparable to the HLA-A2 motive, with their anchors at position 2 and 9. Also peptides eluted from Qa-1^b on mouse TAP-deficient RMA-S cells revealed a broad peptide repertoire, ranging from 8 to 18 amino acid in length[64]. So in the absence of TAP, an alternative repertoire of self-peptides is presented on both classical and non-classical MHC-I molecules. This alternative peptide repertoire was called TEIPP: T cell epitopes associated with impaired peptide processing[65]. These peptides are derived from housekeeping proteins which are ubiquitously expressed by all somatic cells, but are only presented on TAP-deficient cells (Figure 2).

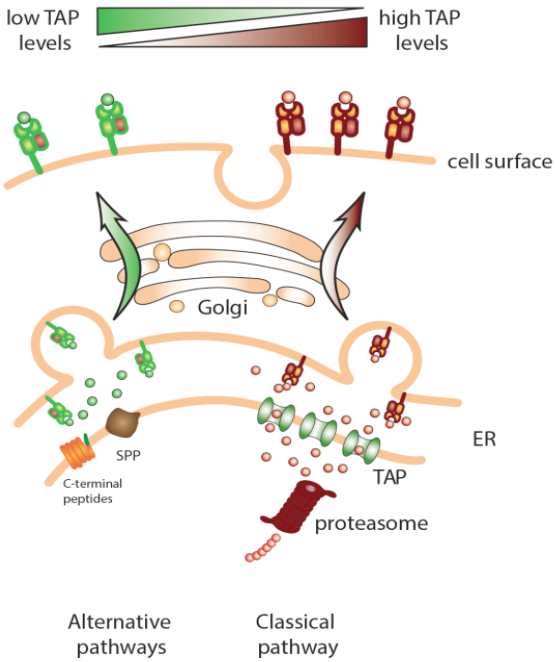

Figure 2: Alternative antigen-processing pathways
In cells with defects in the antigen-processing pathway, such as the peptide transporter TAP, an alternative peptide repertoire emerges on the cell surface following alternative pathways. This includes the SPP-mediated release of C-terminal peptides from housekeeping protein. Adapted from Oliveira et al., *Frontier in Immunology* (2015)

TEIPP-specific T cells

The first human TEIPP antigen was identified in a patient with non-small-cell lung carcinoma (NSCLC). $CD8^+$ T cells isolated from this patient recognized an epitope from the C-terminal part of the preprocalcitonin (ppCT) signal peptide, presented in the context of HLA-A2. Inhibition of the proteasome or TAP did not affect T cell recognition, showing that the peptide was processed by an alternative mechanism[66]. Instead, blocking of the signal peptide (SP) or signal peptide peptidase (SPP) led to complete abrogation of T cell recognition. Restoration of TAP function abrogated the recognition of tumor cells by the ppCT-specific $CD8^+$ T cells[67]. Also in healthy donors, $CD8^+$ T cells specific for TAP-independently processed epitopes are present[68]. Immunizations of mice with TAP2-deficient RMA.-S.B7 cells and subsequent *in vitro* stimulations led to the isolation of several TEIPP-specific $CD8^+$ T cells clones, restricted for both classical H2-D^b and K^b molecules, as well as the non-classical Qa-1^b molecules[65]. Recognition by these TEIPP T cell clones depended on CD3 and CD8 signalling and was irrespective of the origin of the target cells[65]. These peptides may be processed and presented via an alternative pathway. Analysis of a K^b-restricted TEIPP-specific T cell clone revealed that the particular TEIPP epitope recognized was processed by the proteasome and metalloproteases, as blocking these in TAP-deficient tumor cells resulted in loss of T cell recognition[69]. The first identified mouse TEIPP was recognized by a D^b-restricted T cell clone and peptide elutions combined with a synthetic peptide library identified the peptide to be a C-terminal epitope of the ceramide synthase Trh4 (or Lass5)[65]. This fatty acid transporter is a transmembrane protein spanning the ER membrane and the epitope recognized by T cells protrudes into the ER lumen[70] (Figure 3).

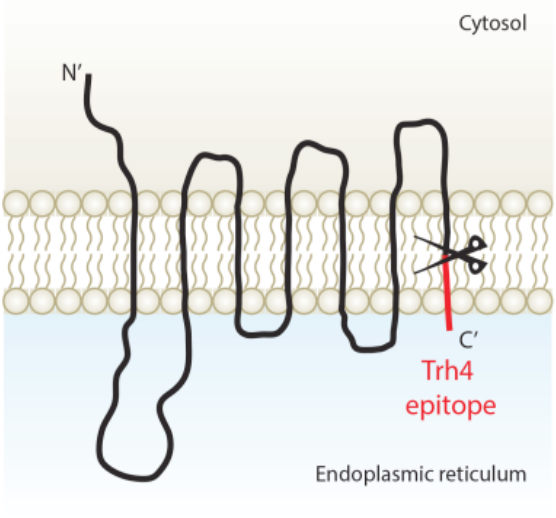

Figure 3: Prototypic mouse TEIPP antigen Trh4
The C-terminal peptide-epitope (indicated in red) protrudes the ER membrane and is released by cleavage by the signal peptide peptidase SPP, depicted by the scissor. Adapted from Oliveira et al., *JI* (2013)

Processing of the Trh4 epitope did not depend on the proteasome or TAP, but was mediated by the signal peptide peptidase (SPP)[70]. Cleavage by this enzyme led to release of the epitope in the ER lumen, allowing it to bind in D^b molecules, followed by expression on the cell surface of TAP-deficient cells. Overexpression of the Trh4 transcript in

TAP-proficient cells induced the recognition of these cells by the TEIPP- T cells clone, suggesting that the abundance of TAP-dependently processed peptides normally outcompetes the presentation of TEIPP antigens[71]. In the absence of TAP, this overload of other peptides is not present, allowing the TEIPP peptides to be presented. Importantly, the Trh4 epitope binds with high affinity and stability to the D^b molecules[71]. Trh4 is a housekeeping protein and is expressed in all cells, irrespective of TAP status[71]. However, only TAP-deficient cells induce activation of the Trh4-specific T cell clone.

Scope of this thesis

The selective expression of TEIPP peptides on TAP-deficient, MHC-I^{low} cells and the presence of $CD8^+$ T cells specific for these peptides suggest that these peptides and their specific T cells could potentially be used in the fight against immune-escaped tumors. Much is already known about the Trh4 epitope, such as its expression and alternative processing pathway. In this thesis, we aimed to unravel the characteristics and application of TEIPP-specific T cells against MHC-I^{low} tumors. In **chapter 3**, the specific binding of the epitope in the D^b molecule was analyzed using crystal structures and we show that the peptide has a unique binding conformation in the peptide binding groove. To study the thymic selection and the behavior of TEIPP-specific T cells *in vivo* in more detail, a TCR transgenic mouse model was generated based on the Trh4-specific T cell receptor. In **chapter 4**, the characterization of the mouse model ('LnB5 tg') is described. We show that the TEIPP T cells are efficiently selected in the thymus and can be potently activated in wildtype mice by peptide vaccination. In **chapter 5** the requirement for co-stimulatory molecule B7 on target cells is shown, to optimally activate TEIPP T cells in the effector phase. This is most likely needed to compensate for low MHC-I levels as a result of TAP impairment. The requirements to activate tumor-infiltrating TEIPP-specific T cells are studied in **chapter 6**. In **chapter 7** we describe an alternative immunotherapeutic strategy effective in inducing clearance of established MHC-I^{low} tumors, which does not involve TEIPP T cells. Rather, tumor-specific $CD4^+$ T cells and NK cells cooperate in inducing tumor regression.

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