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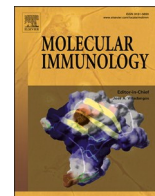
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## Review

# Playing hide and seek: Tumor cells in control of MHC class I antigen presentation

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## ABSTRACT

MHC class I (MHC-I) molecules present a blueprint of the intracellular proteome to T cells allowing them to control infection or malignant transformation. As a response, pathogens and tumor cells often downmodulate MHC-I mediated antigen presentation to escape from immune surveillance. Although the fundamental rules of antigen presentation are known in detail, the players in this system are not saturated and new modules of regulation have recently been uncovered. Here, we update the understanding of antigen presentation by MHC-I molecules and how this can be exploited by tumors to prevent exposure of the intracellular proteome. This knowledge can provide new ways to improve immune responses against tumors and pathogens.

## 1. Loss of tumor MHC class I is associated with poor outcome

Solid tumors are often infiltrated by a wide variety of adaptive and innate immune cells including CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells (cytotoxic T cells),  $\gamma\delta$  T cells, natural killer cells (NK), macrophages, neutrophils and dendritic cells (DCs), some of which possess tumor-specific killing capacity (Cali et al., 2017). In return, tumor cells can activate various intrinsic mechanisms and modulate extrinsic factors to successfully escape from control by the infiltrating immune system. The current belief is that immune escape represents one of multiple strategies for tumors to safeguard survival and proliferation, also known as the hallmarks of cancer (Hanahan and Weinberg, 2011). At a molecular level, these immune escape mechanisms include but are not limited to i) the interference in major histocompatibility complex (MHC; HLA in humans) mediated antigen presentation to prevent T cell surveillance, ii) the secretion of broadly immunosuppressive cytokines such as TGF- $\beta$  and IL-10 (Batlle and Massague, 2019), and iii) the expression of inhibitory receptors at the cell surface such as PD-L1 and PD-L2 which limit T cell activation (Seliger, 2019).

The success of therapeutic application of immune checkpoint antibodies (e.g. anti-PD-L1) to intervene in tumor immune suppression emphasizes the importance of the immune system to limit tumor outgrowth. A major factor for immunogenic tumors to escape this

immunotherapy is the negative modulation of antigen presentation by MHC class I (MHC-I) (Chowell et al., 2018; Gettinger et al., 2017; Restifo et al., 1996; Sade-Feldman et al., 2017; Zaretsky et al., 2016). Even without immunotherapy, the loss of effective MHC-I antigen presentation by tumor cells is associated with poor clinical outcome in the majority of human cancer types (reviewed by (Garrido et al., 2016; Leone et al., 2013)). Of note, MHC class II (MHC-II) is in principle not expressed by healthy non-hematopoietic cells, but surprisingly often by their malignant counterparts (solid tumors). Tumor-specific CD4<sup>+</sup> T cells are present in such tumors (Linnemann et al., 2015; Yossef et al., 2018), which explains the need of tumors to downregulate MHC-II or shift their antigenic landscape (Marty Pyke et al., 2018). It is currently unclear why non-hematopoietic cells upregulate MHC-II in the transformation process and unknown whether they subsequently downregulate MHC-II. In leukemia however, downregulation of MHC-II transcription is regularly induced by immunotherapy in the form of stem cell transplantation (Christopher et al., 2018; Toffalori et al., 2019). In addition, MHC-II expression by different tumor types is associated with favorable outcome, also in response to anti-PD-1 or anti-PD-L1 immunotherapy (reviewed by (Axelrod et al., 2019)). Much more is known about alterations in the MHC-I pathway, which is therefore the focus of this review.

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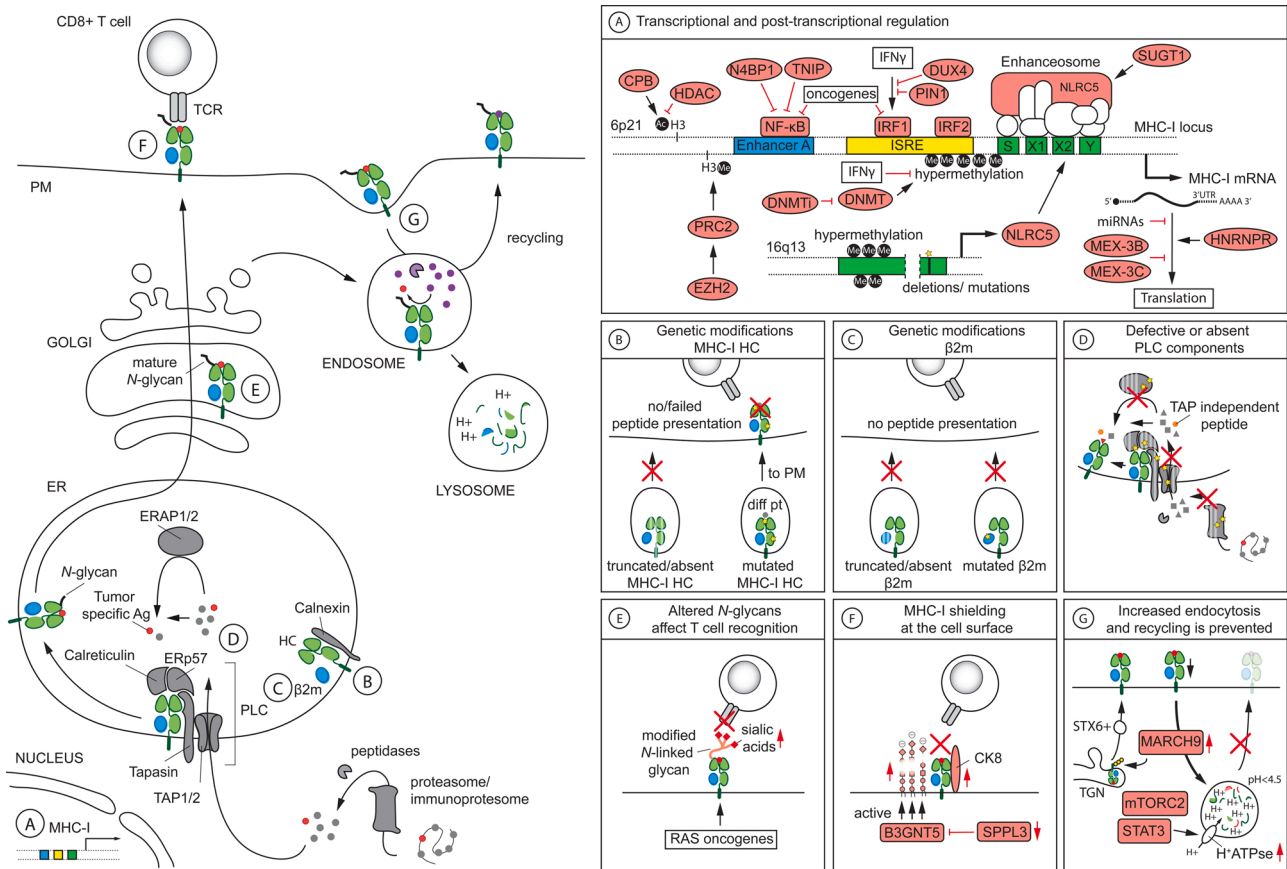
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2. MHC-I peptide processing and presentation

MHC-I molecules are ubiquitously expressed at the cell surface of all nucleated cells, although not at equal levels. For example, immune cells like mature DCs are specialized antigen presenting cells and express up to 20-fold more MHC-I than other somatic cells (Ackerman and Cresswell, 2003; Wu et al., 2016). MHC-I expression obviously starts at the transcriptional level, which is controlled at the MHC-I promoter region by NF-κB (binds to Enhancer A region), interferon (IFN)-γ induced IRF-1 (binds to the ISRE element) and a complex called the enhanceosome, which consist of NLRC5, RFX-complex (RFX5/RFXAP/RFXANK), ATF1/CREB and the NFY-complex (NFYa/b/c) (binds the SXY-module) (Jongsma et al., 2019) (Fig. 1A). The resulting MHC-I mRNA is either used for translation or degraded after MEX-3C-mediated ubiquitination (Cano et al., 2012). The MHC-I heavy chain is translated by ER associated ribosomes by virtue of an N-terminal signal sequence. In the ER, the synthesized polymorphic MHC-I heavy chain interacts with the chaperone calnexin, which supports assembly of the heavy chain and a light chain named β-2-microglobulin (β2 M), as occurring within 4 min after translation (Neeffes et al., 1993). This MHC-I heterodimer is now ready for loading with peptides originating from degraded proteins, signal sequence fragments or misfolded defective translation products (DRiPs) (Anton and Yewdell, 2014; Wei and Cresswell, 1992; Yewdell et al., 1996). These DRiPs are derived from prematurely terminated or misfolded proteins, which may be increased under pathogenic conditions.

The peptide fragments to be loaded in MHC-I are produced by the proteasome or immunoproteasome (LMP2 and LMP7) and often further trimmed to single amino acids by cytosolic peptidases that will eliminate many of these peptides before they can be translocated to the ER (Ferington and Gregerson, 2012; Michalek et al., 1993; Reits et al., 2003; Rock et al., 1994). Some of the remaining peptides can then be transported into the ER by the heterodimeric TAP1/TAP2 transporter (Parcej and Tampe, 2010). Inside the ER lumen, ERp57, calreticulin, tapasin and the MHC-I heterodimer have gathered around the TAP transporter, forming the peptide loading complex (PLC) (Cresswell et al., 1999). ERp57 stabilizes the peptide free MHC-I molecule, while tapasin interacts with TAP coupling peptide translocation to peptide loading (Wearsch and Cresswell, 2007). The length of peptides to be stably loaded onto MHC-I complexes is usually 8–12 amino acids, but TAP allows translocation of peptides from 8 amino acids to over 40 amino acids that then require further trimming (Momburg et al., 1994). Peptides entering the ER have different fates. They can be trimmed to either or not fit MHC-I or otherwise are efficiently removed by transport into the cytosol where cytosolic peptidases trim or completely destroy such peptides (Roelse et al., 1994). Peptide trimming in the ER is performed by the aminopeptidases ERAAP/ERAP1 and ERAP2 till an optimal length to fit in the MHC-I peptide binding groove (Saric et al., 2002; Saveanu et al., 2005; Serwold et al., 2002). In fact, most peptides can bind with equal on-rates but then leave MHC-I when considered not perfectly fitting. MHC-I undergoes a conformational change every time a



**Fig. 1. Schematic overview of MHC-I antigen presentation.** MHC-I heterodimers are assembled in the ER from a MHC-I heavy chain and β2 m supported by calnexin. Cytosolic peptides, generated by the (immune)-proteasome enter the ER lumen through the TAP1/2 transporter. In the ER these peptides can be further processed by ERAP1/2 and loaded on the MHC-I heterodimer with help of the PLC, which consists of Calreticulin, ERp57, Tapasin and TAP1/2. The peptide loaded MHC-I heterotrimer is further modified by the addition of a N-glycan, which is processed in the ER and the Golgi. At the cell surface, MHC-I can present its peptide to CD8<sup>+</sup> T cells. At a certain point in time, MHC-I gets internalized after which it is either recycled back to the cell surface to present a newly acquired peptide or degraded in the lysosome. Right panels: Tumor cells can modulate different steps of the pathway: on a transcriptional or post-transcriptional level (A), by genetically modifying the heavy chain (B), β2 m light chain (C) or PLC components (D), by post-translational mechanisms affecting the N-glycan (E), affecting the accessibility at the cell surface, e.g. by upregulation of nSGSLs (F) and affecting MHC-I endocytosis and recycling (G).

peptide binds until a high affinity peptide is caught (Garstka et al., 2015). The resulting stable MHC-I trimers are released from the ER quality control system for transport to the plasma membrane to finally present an intracellular peptide to CD8<sup>+</sup> T cells (Neeffjes et al., 2011) (Fig. 1).

### 3. Are all MHC-I molecules equal?

During viral infections, MHC-I molecules present virus-derived peptides to initiate anti-viral T cell immune responses. Given the high capacity of viruses to adjust their genome for their own benefit, one may expect that sequences coding for immunogenic antigens should have been depleted from their genomes by now. This may have occurred if mammalian species would have had one single MHC-I protein, however this is considerably more difficult since many mammals express multiple MHC-I genes that are also polymorphic within the population. For example, humans have protected themselves by duplications within the MHC-I locus on chromosome 6, which yielded three genes (HLA-A, HLA-B and HLA-C) on each copy of the chromosome, giving rise to the expression of three to six different classical MHC-I proteins, dependent of the polymorphic HLA-types of the biological mother and father.  $\beta$ 2 m is not polymorphic. Of note, MHC-I is by far the most polymorphic human gene with more than 20,000 different alleles across the human population (Robinson et al., 2020), although some HLA-types are dominant in different human populations. HLA-A2 -for example- is present in about 60 % of the Caucasian population and it is believed that this could be the result from some infection in the past that was best controlled by HLA-A2 positive human. Along the same lines, HLA-B57 is claimed to protect people from HIV and HLA-B57 positive people will likely become dominant in populations with a strong selection pressure due to HIV infection (Martin and Carrington, 2013). How does polymorphism translate into immune responses? Non-synonymous MHC-I polymorphisms concentrate in the peptide-binding groove. The amino acid characteristics in the peptide-binding groove determine the charge and size of pockets where peptide amino acid side chains dock into. Consequently, amino acid polymorphisms alter the peptide binding sites of MHC-I resulting in the binding of different peptides (Falk et al., 1991; Trowsdale, 2005). From a single virus -for example- HLA-A2 will present certain peptides. A virus may alter the amino acids required for binding to HLA-A2 and thus escape immune surveillance but the virus is then still recognized by the immune system of another individual who presents another set of viral peptides by another allele, for example HLA-A1. As a result, polymorphism protects the population and not necessarily the individual albeit the expression of three to six different HLA alleles per individual certainly helps. In cancer cells, some HLA alleles will present many immunogenic tumor-specific peptides, while others will not present any immunogenic antigens at all. For example downregulation of a single HLA-allele in a Burkitt lymphoma and a lung carcinoma, HLA-A11 and HLA-B39 respectively, was sufficient to escape CD8<sup>+</sup> T cell recognition (Gavioli et al., 1992; So et al., 2005). Variation in peptide-selection is not the only difference between HLA-allotypes, because also differences are found in expression levels, their dependence on the PLC for peptide loading and the rate of transport to the cell surface. First, HLA-A and -B are usually highly expressed, HLA-C levels are much lower due to poor peptide loading or low HLA-C gene expression (Neeffjes and Ploegh, 1988; Neisig et al., 1996). However, the low expression of peptide HLA-C complexes is still sufficient to activate some tumor-specific T cells (Tran et al., 2015). HLA-C has another function as well as it conveys signals through inhibitory leukocyte and killer cell Ig-like receptors (LIRs and KIRs) to suppress undesired immune cell activation (Saverino et al., 2000; Valiante et al., 1997). Next, HLA-A and HLA-C bind much more efficient to the PLC than HLA-B (Neisig et al., 1996). Yet, HLA-B molecules are loaded with peptides, showing that the PLC is not required for peptide loading. Finally, HLA-B transport to the cell surface is more rapidly than for HLA-A and HLA-C (Neeffjes and Ploegh, 1988; Peh et al., 1998), indicating that the PLC

most likely delays the loading process while facilitating high affinity peptide selection by tapasin (Zarling et al., 2003).

MHC-I molecules are even more complex. Within one major HLA-I type, differences in molecular and physiological behavior can be found. For example, specific HLA-variants are highly associated to the development of auto-inflammatory diseases, such as the linkage disequilibrium between HLA-B27.5 and ankylosing spondylitis. The epistatic involvement of an ERAP1 variant implicates that the antigen presentation function of these HLA-B27 molecules is required for the onset or propagation of this disease (Evans et al., 2011; Herberths et al., 2006). Some other alleles, including HLA-A2, have a preference to bind hydrophobic peptides. This leads to increased presentation of signal peptides that do not even require TAP for peptide loading compared to other HLA allotypes (Wei and Cresswell, 1992).

All these fundamental mechanisms behind MHC-I antigen presentation enable immunological control of tumor cell outgrowth, but unfortunately tumor cells respond by negatively controlling MHC-I antigen presentation pathways through specific and more broad manners leading to their immune escape. We will discuss such tumor immune escape mechanisms for conventional components, but also for various recently identified mechanisms of MHC-I regulation.

### 4. Modulation of MHC-I antigen presentation pathway by tumors

Since MHC-I molecules present a reflection of the intracellular proteome, tumor cells with aberrant proteomes, including tumor-specific antigens are in principle subjected to CD8<sup>+</sup> T cell recognition and elimination. Such immune pressure allows for specific outgrowth of tumor cells with defects in MHC-I antigen presentation. MHC-I expression in cancer has been extensively studied (reviewed by (Leone et al., 2013)). The numerous investigations of primary and metastatic tumor lesions demonstrated that MHC-I expression as detected by microarray, RNAseq or immunohistochemistry is regularly affected in many different cancer types, in some cases up to 90 % of the tumors (Cornel et al., 2020; Leone et al., 2013). These analyses further show that the low MHC-I expression is often correlated to poor expression of transcriptional regulators, PLC components,  $\beta$ 2 m and other regulators of the pathway, such as the immunoproteasome subunits LMP2 and LMP7 (Ayshamgul et al., 2011; Cathro et al., 2010; Leone et al., 2013; Ogino et al., 2003). Reduction of LMP2 and LMP7 expression may alter the HLA associated peptide repertoire, possibly preventing the presentation of immunogenic tumor-specific peptides (Favole et al., 2012). Finally, several tumors limit antigen presentation in ways that have not yet been extensively explored, including preventing the processing and presentation of DRiPs or through glycosphingolipid-mediated shielding of cell surface MHC-I (Jongsma et al., 2021; Pont et al., 2019). The outcome of downregulation of any of the above mentioned factors is always similar, functional MHC-I mediated antigen presentation is disturbed, rendering tumor cells poor targets for the immune system. Two different types of tumor-induced defects affecting MHC-I antigen presentation can be distinguished: i) irreversible defects (or hard mutations) and ii) reversible defects (or soft mutations) (Garrido et al., 2010).

#### 4.1. Irreversible MHC-I pathway defects in tumors

Genetic defects in tumors are currently irreversible. The different types of genetic alterations that have been reported in tumors to affect MHC-I antigen presentation mainly include point mutations, base pair insertions or deletions (indels) and larger deletions, which is referred to as loss of heterozygosity (LOH) in cases of full gene deletions (Clancy, 2008). Point mutations and indels can have various transcriptional or translational consequences, such as induction of alternative splicing or frameshifted open reading frames resulting in truncated and/or dysfunctional proteins.

#### 4.1.1. Truly irreversible mutations

The MHC-I heavy chain and  $\beta 2$  m light chain are obviously essential for antigen presentation. Therefore genetic mutations and deletions in the MHC-I genes on chromosome 6 and  $\beta 2$  m on chromosome 15 have irreversible effects on antigen presentation (Fig. 1B and C). There are various ways to genetically manipulate MHC-I expression. To give some examples, a splice site mutation in a MHC-I allele caused the deletion of the MHC-I  $\alpha 1$ -domain (Wang et al., 1999) and indel mutations in the first exons of the  $\beta 2$  m gene led to a frameshifted open reading frame often containing a premature stop codon (Benitez et al., 1998; Gattoni-Celli et al., 1992; Hicklin et al., 1998; Perez et al., 1999; Zaretsky et al., 2016). Point mutations in the MHC-I exon 2 or 3 (encoding for anchor residues in the peptide binding groove) generated a full length protein at the cost of peptide presentation, while mutations in exon 4 altered binding of CD8, which is an important coreceptor for T cell activation (Shukla et al., 2015).

Because of the relative random nature of genetic mutations, in many cases single chromosomes are affected, which may not be sufficient for tumor immune escape. LOH also allows functional protein expression from the remaining chromosome. Nevertheless,  $\beta 2$  M LOH results in a marked reduction of  $\beta 2$  M and consequently MHC-I expression (Maleno et al., 2011; Sade-Feldman et al., 2017). Since the other  $\beta 2$  M gene is usually mutated this then results in a complete absence of functional  $\beta 2$  M and full elimination of MHC-I antigen presentation (Bernal et al., 2012; del Campo et al., 2014). In the case of the polymorphic MHC-I heavy chain, mutations or LOH can delete one or more unique HLA-I alleles from a tumor, which directly reduces the diversity of tumor-specific antigens presented (Garrido and Algarra, 2001; Shukla et al., 2015).

#### 4.1.2. Irreversible mutations with immunological opportunities

Genetic mutations were also detected in other components of the MHC-I antigen presentation, including the PLC members tapasin, Erp57, TAP and calreticulin (Cerami et al., 2012) (Fig. 1D). Examples include frameshift deletions and point mutations in the TAP1 gene resulting in a truncated protein or affecting the functionality of its ATP-binding domain resulting in poor peptide transport (Chen et al., 1996; Seliger et al., 2001). Frameshift mutations in the calreticulin gene led to a protein with a C-terminus lacking its KDEL sequence, which is essential for its ER localization (Arshad and Cresswell, 2018). The absence of calreticulin in the PLC leads to suboptimal peptide loading of MHC-I and consequently inefficient antigen presentation (Howe et al., 2009).

In contrast to the lack of T cell recognition of tumor cells genetically depleted of MHC-I heavy and light chains, mutations in other genes may maintain or even generate opportunities for T cell mediated tumor control when different antigenic peptides are presented. Genetic defects in members of the PLC modulate but do not completely abolish MHC expression because i) expression of various HLA-I alleles is independent of several PLC members as mentioned before, ii) even PLC-dependent HLA-I alleles are usually surface expressed albeit at a low level (de Waard et al., 2021b; Rizvi et al., 2014). Moreover, the PLC components modulate the peptides presented by MHC-I. Defective TAP transport for example leads to the presentation of an altered peptide repertoire containing more TAP-independent peptides, so called T cell epitopes associated with impaired peptide processing (TEIPP) (Doorduyn et al., 2016; Marijt et al., 2018; van Hall et al., 2006). This is not always advantageous to tumor evasion. Although these TEIPPs are usually non-mutated self-peptides, some of them may be selectively presented by TAP-deficient tumor cells and should then be considered neo-epitopes that can be recognized by the immune system. Thus genetic defects in PLC components even provide opportunities for immunotherapeutic targeting of tumor-specific antigens presented by the remaining MHC-I molecules, although careful evaluation of off-target reactivity against healthy cells with natural low PLC expression is required (de Waard et al., 2021a).

#### 4.1.3. Circumventable irreversible mutations

Finally, MHC-I regulatory factors that are not directly influencing peptide loading are also often mutated in cancer. These include the genes encoding the enhanceosome (e.g. NLR5) or other components involved in the expression of MHC-I or PLC genes (e.g. IRF2) (Kriegsman et al., 2019; Yoshihama et al., 2016) (Fig. 1A). For example, mutations were found in the NLR5 gene and JAK1/2 (IFN pathway component) leading to protein loss-of-function in tumors (Shin et al., 2017; Yoshihama et al., 2016). It is important to evaluate how irreversible these mutations really are in relation to MHC-I antigen presentation, since the therapeutic induction of other, similar or parallel pathways may compensate for some lost functions. For example, the MHC-I diminishing effects caused by the irreversible loss of IRF2 can be overcome *in vitro* by activating IFN signaling pathways to induce IRF1 in cancer cells (Kriegsman et al., 2019). There are plenty of such opportunities in the field to develop targeted treatments with the goal to make irreversible mutations reversible, some of which are described below.

#### 4.2. Reversible MHC-I pathway defects in tumors

Next to structural irreversible mutations, tumor cells can also contain many reversible defects affecting MHC-I antigen presentation. The negative effect of a large group of reversible defects on antigen presentation can, as the name suggests, potentially be restored by a treatment (cytokines or specific inhibitors) since they are often driven by altered expression levels of MHC-I regulators. We will discriminate here between tumor-driven interference in transcriptional, post-transcriptional and post-translational processes. Here, we define the transcriptional regulatory defects as i) disturbed promoter activation ii) epigenetic dysregulation and iii) oncogene activation. Each of these can affect the expression of MHC-I heavy chain,  $\beta 2$  m and other proteins controlling the different steps of the antigen processing and presentation. Some of this latter group of proteins are encoded by genes located in the MHC-I locus on chromosome 6 and their expression is often correlated with the expression of MHC-I heavy chains, as reported for LMP2, LMP7, TAP subunits and tapasin (Ozcan et al., 2018; Yoshihama et al., 2016).

##### 4.2.1. Disturbed MHC-I promoter activation

As mentioned above, MHC-I heavy chain expression is regulated by a transcriptional complex called the enhanceosome (including NLR5), NF $\kappa$ B signaling and IFN signaling (Jongsma et al., 2019). These transcriptional regulators are controlled by complex pathways and many positive and negative regulators have been identified over the years. For example, the nuclear cochaperone SUGT was recently identified as a positive regulator of NLR5 (Dersh et al., 2021), and N4BP1 as well as TNIP1 were identified to negatively regulate NF- $\kappa$ B signaling (Spel et al., 2018) (Fig. 1A). Furthermore the peptidyl-prolyl isomerase, PIN1, and the transcription factor DUX4 are both thought to inhibit MHC-I transcription by interfering in the IFN signaling pathway and found upregulated in cancer (Chen et al., 2018; Chew et al., 2019) (Fig. 1A). Although inhibitors or cytokines regulating these regulators have as far as we know not yet been identified, they provide potential targets to therapeutically increase MHC-I expression levels.

NF- $\kappa$ B and IFN signaling pathways are often upregulated under inflammatory conditions leading to an increase in MHC-I expression. However, recent evidence indicates that individual components of these pathways also regulate basal MHC-I expression. Some IRFs are constitutively expressed in cell lines (Wu et al., 2016) suggesting that IRFs (binding the ISRE elements) can control MHC-I expression also under non-inflammatory conditions. The expression of IRF2, in contrast to IRF1 is constitutive and quite stable in the absence of IFN stimulation (Oshima et al., 2004; Ren et al., 2015; Watanabe et al., 1991). IRF2 activates MHC-I, TAP2 and ERAP1 expression, thereby positively affecting MHC-I antigen presentation under non-inflammatory conditions (Kriegsman, Rock, 2019, J Immunol). Loss of IRF2 in cancer cells

leads to immune evasion (Kriegsman et al., 2019), emphasizing the importance of factors regulating basal MHC-I expression in tumors.

#### 4.2.2. Epigenetic dysregulation

In cancer cells, MHC-I expression is often silenced by epigenetic modifications at the MHC-I locus, including chromosome remodeling, histone modifications and DNA methylation. Such epigenetic dysregulation affects gene expression by modifying the chromatin structure. Because these modifications leave the DNA sequence intact, they are in principle reversible. Chromatin remodeling of the TAP1 promoter can be induced by the histone acetyltransferase CBP, which relaxes the DNA structure of the TAP1 promoter to allow transcription (Setiadi et al., 2007). Not surprisingly, CBP recruitment was decreased in some tumor cells (Setiadi et al., 2007). CBP acts by acetylating Histone H3, a modification that can be removed by Histone deacetylases (HDACs) in order to reduce gene expression (Fig. 1A). The fact that HDACs can be inhibited by small molecules fueled their evaluation to improve antigen presentation. Indeed, chemical HDAC inhibition induced Histone H3 acetylation, leading to enhanced MHC-I surface expression and antigen presentation in multiple tumors (Khan et al., 2008; Mora-Garcia Mde et al., 2006; Ritter et al., 2017; Sun et al., 2019; Yang et al., 2020). Another epigenetic modification affecting gene transcription is the addition of a methyl group to DNA by methyltransferases (DNMTs). Hypermethylation of the MHC-I locus and the NLRC5 promoter region inhibits transcriptional activity and attenuates MHC-I antigen presentation in various cancer types (Nie et al., 2001; Ye et al., 2010; Yoshihama et al., 2016) (Fig. 1A). DNA hypermethylation can be reversed by IFN- $\gamma$  stimulation or by DNMT inhibitors, as shown in breast cancer and melanoma cell lines (Fonsatti et al., 2007; Luo et al., 2018; Serrano et al., 2001; Vlkova et al., 2014). Hypermethylation of the MHC-I locus and the resulting attenuated antigen presentation were also induced by Polycomb repressive complex 2 (PRC2) activation (Burr et al., 2019; Dersh et al., 2021) (Fig. 1A). Gain of function mutations in the PRC2 encoding gene and its activator EZH2 are frequently observed in cancer and can be corrected by chemical inhibitors. Surprisingly also loss-of-function mutants of PRC2 were identified, which correlated with poor survival (Laugesen et al., 2016). These data suggest a broader transcriptional impact of PRC2 involving other cancer-related factors than MHC-I.

#### 4.2.3. Oncogenic pathways

Oncogenes encode for proteins in pathways important for critical functions like cell survival and proliferation. Uncontrolled activation of these oncogenic pathways has the potential to cause malignant outgrowth of cells. An example of an oncogene is NMYC, a transcription factor overexpressed in some neuroblastomas. Since NMYC suppresses NF- $\kappa$ B signaling in these cells, its overexpression downregulates MHC-I expression (van 't Veer et al., 1993). Other examples of oncogenic pathways affecting MHC-I expression are the RAS/MAPK and PI3K/AKT pathways, activated by EGF and Her2 receptor binding, and Wnt signaling. Activation of these pathways in several types of cancer downregulates MHC-I transcription usually by inhibiting IFN or NF- $\kappa$ B signaling (Fig. 1A) (Brea et al., 2016; Chandrasekaran et al., 2019; Herrmann et al., 2004; Lohmann et al., 1996; Lulli et al., 2017; Maruyama et al., 2010; Mimura et al., 2013; Seliger et al., 1998; Vertuani et al., 2009; Yang et al., 2020). Downstream of PI3K/AKT is the mTOR kinase that senses starvation and controls many processes including translation, leading to the production of radiation-specific peptides. Ionizing radiation activates mTOR which also upregulates MHC-I expression at the cell surface. This finding was the basis for radio-immunotherapy where the specificity in space (radiotherapy) is combined with specificity of the target (through immunotherapy) (Reits et al., 2006). Also the expression of other antigen presentation pathway components is affected by oncogene expression. RAS oncogene expression or KI-RAS mutations downregulate the antigen presentation components LMP2, TAP1 and tapasin in colorectal carcinomas, which can be often restored by cytokine treatment indicating that these defects are

caused by dysregulation rather than genetic mutations in these genes (Atkins et al., 2004). Alternative ways by which oncogenes disturb antigen presentation have also been reported. For example, an activating mutation in the Ras-pathway protein BRAF induces internalization of MHC-I molecules from the cell surface towards endolysosomal compartments reducing MHC-I surface expression, which could be restored by MAPK inhibitor treatment (Bradley et al., 2015).

#### 4.2.4. Post-transcriptional modulations

Translation of mRNA encoding for MHC-I pathway components can be modulated by small (~20 nucleotides) single stranded non-coding RNAs called microRNAs (miRNAs). miRNAs bind the 5'UTR, 3'UTR or coding sequence of the targeted mRNA affecting its translation and sometimes inducing its degradation (Bartel, 2004). Therefore it is not surprising that several cancer types exhibit altered expression of miRNAs modifying MHC-I expression and antigen presentation (Fig. 1A). Within the last two years, several more MHC-I modulatory miRNAs have been added to the list as composed by Friedrich et al. (Friedrich et al., 2019). For example miR-19 was identified to negatively modulate the expression of MHC-I genes in human cancer cells (Li et al., 2020) while miR-200a-5p, miR-26b-5p and miR-21-3p target the 3'UTR of TAP1 thereby downregulating the TAP1 protein as shown in melanoma (Lazaridou et al., 2020a, b). Decreased levels of TAP1 as a result of high levels of miR-200a-5p in melanoma patients was associated to shorter overall survival and miR-26b-5p overexpression led to decreased T cell recognition. Both observations support the idea that tumors can alter miRNA expression to evade immune detection by decreasing MHC-I antigen presentation (Lazaridou et al., 2020a, b). Effects of miRNAs were often reverted using miRNA inhibitors, providing new opportunities to enhance current immunotherapies.

Post-transcriptional modification of antigen presentation is also regulated by RNA-binding proteins, such as HNRNPR and the RNA-binding E3 ligases MEX-3B and MEX-3C. All bind the 3'UTR region of MHC-I mRNA. HNRNPR enhances MHC-I mRNA stability (Reches et al., 2016), while MEX-3B and MEX3C destabilize the mRNA leading to its degradation and decreased MHC-I expression (Cano et al., 2012; Friedrich et al., 2019; Huang et al., 2018) (Fig. 1A). Importantly, MEX-3B expression by melanoma confers immune resistance during anti-PD-1 immunotherapy (Huang et al., 2018). Mechanistically, MEX-3C binds the 3'UTR of HLA-A2 mRNA via its RNA-binding KH domain, transports the mRNA towards the cytosol where it associates with de-adenylation complex CCR4-NOT. There MEX-3C ubiquitinates the catalytic subunit CNOT7 regulating its activity leading to removal of the mRNAs stabilizing poly(A) tail followed by degradation in a ubiquitin dependent manner (Cano et al., 2015). The deubiquitinating enzyme USP7 antagonizes this HLA-A2 mRNA degradation (Cano et al., 2012).

#### 4.2.5. Post-translational modulations

Human MHC-I heavy chains are modified by an N-glycan at residue Asn86 (Bjorkman et al., 1987), critical for the assembly, peptide loading and cell surface expression of MHC-I and especially HLA-B alleles (Neeffjes and Ploegh, 1988; Wearsch et al., 2011; Zhang et al., 2011). Murine MHC-I heavy chains even contain 2 N-linked glycans positioned at the two ends of the peptide binding groove. MHC-I N-linked glycans can be modulated by RAS oncogene activation, leading to increased N-glycan branching with more sialic acids (Bolscher et al., 1988) (Fig. 1E). These changes in the N-glycan may negatively affect immune recognition since shortening glycans or removing sialic acids with glycosidase inhibitors improved T cell responses (Boog et al., 1989; Neeffjes et al., 1990), although effects on other surface molecules were not excluded.

At the plasma membrane MHC-I molecules can diffuse laterally (Edidin, 2010; Gromme et al., 1999), which provides the option to interact transiently with other cell surface molecules. This diffusion -perhaps- also facilitates clustering during interactions with a TCR at the immunological synapse or to locate in superclusters with other proteins,

as suggested by biophysical experiments (Edidin, 2010). Recently, MHC-I molecules were shown to interact with molecules that are largely ignored in the fields of immunology and antigen presentation, but abundantly present at the plasma membrane, the glycosphingolipids (GSLs) (Jongsma et al., 2021; Zhang et al., 2019). GSLs are hydrophobic ceramide units containing highly variable hydrophilic sugar structures (Hakomori, 1981). They are generally enriched in membrane microdomains, including lipid rafts, and affect cell surface receptor function (Coskun et al., 2011; Kabayama et al., 2007; Tagami et al., 2002) and protein-protein interactions (Kawakami et al., 2002; Todeschini et al., 2007). Receptor-ligand interactions shape the immune system, therefore it is not surprising that GSLs play important roles in innate and adaptive immunity (Nakayama et al., 2018; Zhang et al., 2019). Still, the many potential roles that GSLs can have in modulating anti-tumor immunity is underexplored because GSLs are difficult to study with the currently available toolset. Yet, we recently identified that high levels of the subtype neolacto-series GSLs (nsGSLs) interferes with the accessibility of MHC-I molecules for T cells and immune cell receptors like LIR-1 and KIR2DL2 (Jongsma et al., 2021). These nsGSL expression levels are generated by the enzyme B3GNT5, which is post-translationally regulated by the Signal Peptide Peptidase Like 3 (SPPL3) protease located in the ER and Golgi (Jongsma et al., 2021) (Fig. 1F). Overexpression of the core synthesis enzyme for GSL production, UGCG, and consequently elevated levels of a diverse repertoire of GSLs, including nsGSLs, have been observed in several tumor types including glioma, AML and adenocarcinomas (Furukawa et al., 2015; Hakomori, 1984; Jennemann et al., 2017; Wang et al., 2012; Wikstrand et al., 1991). Since these nsGSLs affect antigen presentation, they can support tumor cells to evade the immune system, at least when tumor cells produce more of these nsGSLs. Indeed, inhibition of GSL-synthesis by pharmacological inhibition of UGCG can delay tumor growth (Inokuchi et al., 1987; Jennemann et al., 2017; Radin, 1999). Not only GSLs can shield MHC-I molecules, also the cytokeratin CK8 has been shown to mask MHC-I molecules at domains involved in CD8<sup>+</sup> T cell activation (Wu et al., 2013) (Fig. 1F). Overexpression of cytokeratins has been observed in malignant tissues (Moll et al., 1982).

Cell surface accumulation of MHC-I molecules at the cell surface is controlled by MARCH9 (ubiquitously expressed) and its close homologue MARCH4 (mainly expressed in brain and placenta). Since the early 2000s, it is known that these MARCH proteins target MHC-I into the endosomal pathway for degradation in an ubiquitin dependent manner (Bartee et al., 2004; De Angelis Rigotti et al., 2017; Eyster et al., 2011; Hoer et al., 2007). Several tumors express MARCH proteins which may support immune evasion by lowering the cell surface expression of MHC-I proteins (Wang et al., 2008) (Fig. 1G). It is still unclear how MARCH activity is controlled and where in the pathway ubiquitination of MHC-I takes place. Next to its role in MHC-I endosomal targeting and degradation, MARCH9 is required at the trans-Golgi network to ubiquitinate and sort MHC-I into syntaxin 6 (STX6) positive endosomes for secretion (De Angelis Rigotti et al., 2017) (Fig. 1G). Furthermore, the de-ubiquitinating enzyme UCH-L1 promotes MHC-I recycling (Reinicke et al., 2019). Strikingly, MHC-I endocytosis can also have a positive effect on antigen presentation, namely when it is not degraded but sorted for recycling back towards the cell surface. The acidic environment of the endosomes promotes the release of the bound peptide and can load the molecule with a new peptide generated in the endocytic pathway where after the MHC-I molecule can be recycled to the cell surface to present its new peptide (Gromme et al., 1999). However tumor cells may eliminate this recycling option by decreasing the endosomal pH below pH 4.5 leading to full and definite dissociation of the MHC-I complex (Fig. 1G). Endosomal pH is controlled by the vacuolar H<sup>+</sup>-ATPase, which on its turn is regulated by signaling involving proteins such as STAT3 and mTORC2 (Liu et al., 2018). The dysregulation of these factors in tumors may result in acidification of endosomes and attenuation of the MHC-I recycling pathway.

## 5. Concluding remarks

The MHC-I antigen presentation pathway is one of the best studied pathways in biology (Fig. 1). Still, our understanding is not saturated as new players of this pathway have been identified in unbiased genome-wide knockout studies (Dersh et al., 2021; Jongsma et al., 2021). Successful antigen presentation by MHC-I requires both specific and general processes involved in transcriptional and translational control, protein synthesis and degradation. In fact, there is hardly any cell biological process not involved in MHC-I antigen presentation. The recent introduction of novel factors opens new areas for understanding and manipulation of tumor-specific immune responses. It is important to note that evaluation of tumor immune escape mechanisms usually ignores the potential differential effects on HLA allotypes or immunodominant antigens presented by (primary) tumor cells, while these are known to be crucial determinants of the anti-tumor immune response (Chowell et al., 2018; Schreiber et al., 2002). Tumors do not care which component of the pathway they manipulate as long as MHC-I expression is reduced or eliminated allowing the cells to escape from an immune response. Each of the many components required for successful MHC-I antigen presentation is a potential therapeutic target as long as this is compatible with cell survival. While we are approaching saturation of the components involved in MHC-I antigen presentation, this still presents with great surprises and the introduction in the field of immunology of molecules that are long ignored, such as glycolipids.

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