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# Rôle des Pioneer Translation Products (PTPs) dans la réponse immunitaire anti-tumorale.

Alison Pierson

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Par

**Alison PIERSON**

Role of Pioneer Translation Products (PTPs) in the anticancer immune  
response.

**Thèse présentée et soutenue à Gustave Roussy Cancer Campus, le 20 décembre 2017 :**

**Composition du Jury :**

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**“Be brave. Take risks. Nothing can substitute experience.”**

**Paulo Coelho**

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## Abbreviation

3'ss	3' splice site
5'ss	5' splice site
ACT	Adoptive Cell Transfer
ALV	Antigen Loss Variant
AP	Antigenic Peptide
AS	Alternative Splicing
B-LCLs	B-lymphoblastoid cells
BMDC	Bone Marrow Dendritic Cell
BPS	Branch Point Sequence
Breg	B regulatory
CBP	Cap-Binding Protein
cDCs	conventional DCs
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocytes
DAMPs	Damage Associated Molecular Patterns
DCs	Dendritic Cells
DRibbles	Defective Ribosomal products-containing autophagosome-rich blebs
DRiPs	Defective Ribosomal Products
eIF2	eukaryotic Initiation factor 2
ER	Endoplasmic Reticulum
ESE	Exonic Splicing Enhancer
ESR	Exonic Splicing Repressor

GTP	Guanosine Triphosphate
hnRNPs	heterogeneous nuclear Ribonucleoproteins
HPV	Human Papilloma Virus
ICOSL	Inducible T cell Co-Stimulator Ligand
IFN- $\gamma$	Interferon- $\gamma$
Ig	Immunoglobulin
ILCs	Innate Lymphoid Cells
IRES	Internal Ribosomal Entry Site
IS	Immune System
ISE	Intronic Splicing Enhancers
ISR	Intronic Splicing Repressor
LC-MRM	Liquid Chromatography- Multiple Reactions Monitoring
Leu-tRNAi	Leucine-bound tRNA initiator
LMPs	Low Molecular weight Proteins
MDSC	Myeloid Derived Suppressor Cells
Met-tRNAi	methionyl-initiator tRNA
MHC-I	Major Histocompatibility Complex class I
MHC-II	Major Histocompatibility Complex class II
MICA	MHC-I Chain-related protein A
MICB	MHC-I Chain-related protein B
MIPs	MHC class I associated peptides
MRE	miRNA Response Element
mTECs	medullary Thymic Epithelial Cells
MUG	Methylumbellifery $\beta$ -D-galactopyranoside

MVBs	Multivesicular Bodies
NA	Neuraminidase
NK	Natural Killer
NKT	Natural Killer T cell
NMD	Non-sense Mediated Decay
NP	Nucleoprotein
Ova	Ovalbumin
PABP	Poly(A)-Binding Protein
PAP	Prostate Acid Phosphatase
pAPCs	professional Antigen Presenting cells
pDCs	plasmacytoid DCs
PIC	Pre-Initiation Complex
PLC	Peptide Loading Complex
pMHC	peptide-MHC
PTC	Premature translation-Termination Codon
PTPs	Pioneer Translation Products
RAG	Recombination Activating Gene
RBS	RAS-Binding Domain
RDPs	Rapidly Degraded Proteins
RLU	Relative Light Units
RNAP	RNA polymerase
RPM	Ribopuromycylation
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
SLiPs	Short Live Proteins

SLP	Synthetic Long Peptide
snRNA	single nucleotide Ribonucleic Acid
snRNPs	small nuclear Ribonucleoproteins
sORFs	short Open Reading Frames
SP	Short Peptides
SSA	Spliceostatin A
TA	Tumor Antigens
TADC	Tumor-Associated Dendritic Cell
TAM	Tumor-Associated Macrophages
TAN	Tumor-Associated Neutrophils
TAP	Transporter Associated with Antigen Processing
TA-PTPs	Tumor-Associated PTPs
T <sub>CM</sub>	T central memory
TCR	T-Cell Receptor
TECs	Thymic Epithelial Cells
T <sub>EFF</sub>	T effector
TEIPP	T cell Epitopes associated with Impaired Peptide Processing
T <sub>EM</sub>	T effector memory
TEM	Transmission Electron Microscopy
TGFβ	Transforming Growth Factor-β
Th	T helper
TILs	Tumor Infiltrating Lymphocytes
TLR	Toll-Like Receptor
TME	Tumors Microenvironment

TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
TPPII	Tripeptidyl Peptidase II
Treg	T regulatory
Ub	Ubiquitin
VSV	Vesicular stomatitis Indiana virus
WT	Wild Type
$\beta$ 2m	$\beta$ 2-microglobulin

## Introduction

### 1. Major histocompatibility complex class I and II (MHC-I and -II) antigen presentation pathways

#### A - A brief history of histocompatibility

The notion of histocompatibility emerged from graft rejection study in the early 20<sup>th</sup> century. One of the first descriptions of the histocompatibility notion arose from the work of the British immunologist Peter Gorer and the American biologist George Snell, who independently performed tumor cells transplantation experiments in mice and identified genes that were determining both in the transplantation success and failure. Among those histocompatibility genes, they identified one locus that appeared to be dominant in the fate of the transplant and which they indirectly showed to encode for the antigen 2. This locus was named H-2 by the two researchers<sup>1</sup>. The following findings highlighted one chromosomal region that appeared to have a major role in the transplant rejection compared to other minor players in the same process and referred as the major histocompatibility complex, or MHC. This region was described as encompassing many genes that determine the immunological identity of each individual.

In parallel, the French immunologist Jean Dausset studying leukocyte-agglutinating antibodies in transfused patients, defined the first human leukocyte antigen called MAC, later renamed HLA-A2, and suggested that one major chromosomal region comprises the MHC-related genes like in mice<sup>1</sup>. Later, it was discovered that the human MHC maps to the chromosome 6 short arm<sup>2</sup> and three main regions were described: (i) the class I region containing the HLA-A, -B and -C genes which encode the class I molecule heavy chain (ii) the class II region containing the HLA-DR, -DQ and -DP genes, each of which containing A and B genes coding for the  $\alpha$  and the  $\beta$  chains respectively, and (iii) the class III region containing genes encoding for the complement components. In mice, the “classical” MHC-I molecules were called H2-D, K and L and the MHC-II molecules I-A and I-E. At that time, major features of the MHC were discovered: its primary role in transplant rejection, its genetic complexity and its highly polymorphic loci.

First studied and discovered as a transplantation antigen, the MHC or HLA system has since been closely linked to the immune response<sup>3</sup>. The discovery of T lymphocytes in the 1960s

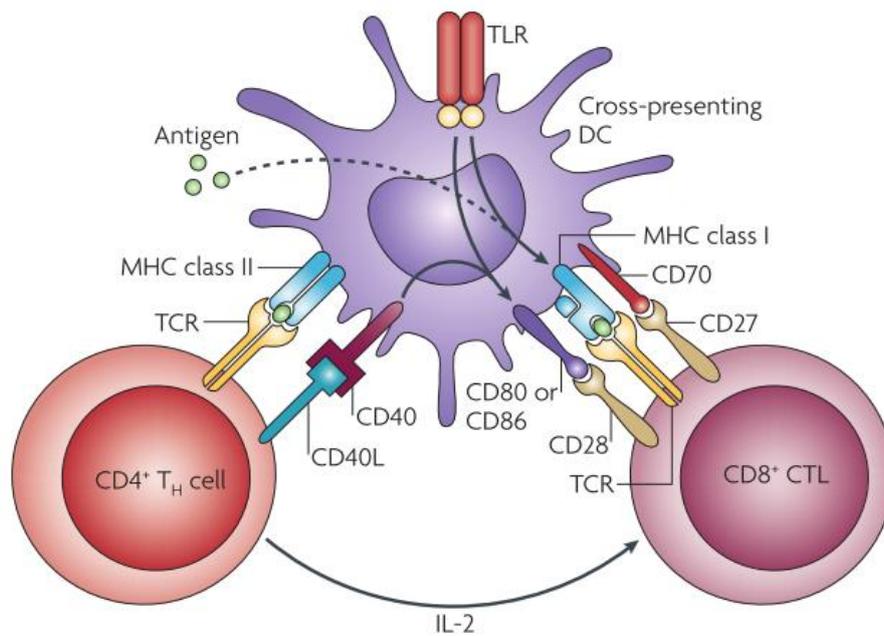
was quickly followed by the discovery of their implication in the recognition of antigens associated to MHC-I molecules<sup>4</sup>. Among many interesting studies, one can cite the work of McDevitt and colleagues who looked at the spleen cell response to synthetic peptides and showed that there is an antigen-specific polymorphism in between mice with different H-2 types<sup>5</sup>. Along with this finding, P.C. Doherty and R.M. Zinkernagel work on cytotoxic T cell response in mice infected with the lymphocytic choriomeningitis virus introduced the MHC restriction concept<sup>6</sup>. The latter refers to the ability of T cells to recognize a specific antigen presented by MHC class I molecules depending on the haplotype. A similar restriction mechanism was discovered by A.S. Rosenthal and E.M. Shevach on antigens presented on MHC class II molecules at the cell surface of macrophages in the guinea pig model, a process which is necessary for antigen-specific T helper cell activation and expansion<sup>7</sup>. In 1985 and during the following years, E.R. Unanue, who worked on the Hen egg-white lysozyme protein, and H. Grey, who worked on the ovalbumin protein, significantly contributed in the discovery of antigenic peptides as ligands for MHC-II molecules and of antigen processing<sup>8-10</sup>. In 1985, Townsend and colleagues reported similar findings on MHC-I molecules, highlighting the recognition of epitopes from degraded proteins by cytotoxic T lymphocytes on influenza infected cells<sup>11</sup>. The first MHC-I molecule crystal structure release by P. Bjorkman the same year was a precious tool in the understanding of peptide-MHC interaction as well as peptide regions recognized by T-cells<sup>12</sup>. Then, during the 90s century, HG Rammensee and colleagues focus their work on the characterization of MHC-I naturally presented peptide motifs and created the currently widely used SYFPEITHI database for MHC ligands<sup>13,14</sup>. In 1994, L.F. Stern *et. al* provided the HLA-DR1 crystal structure in complex with the influenza virus haemagglutinin peptide HA306-318, which helped to interpret in details the interactions between peptides and MHC-II molecules<sup>15</sup>.

The first steps in the vast field of antigen presentation were rewarded in 1980 when George D. Snell, Baruj Benacerraf and Jean Dausset jointly won the Nobel Prize in Physiology or Medicine “for their discoveries concerning genetically determined structures on the cell surface that regulate immunological reactions”. The MHC system, at the core of the recognition of self vs. non self during the adaptative immune response, has since been studied in many diseases such as viral or bacterial infection, cancer and autoimmune diseases, being a key element for the understanding of their progression.

## **B -The MHC class I and class II antigen presentation pathways: main roles and general features**

Beyond the central and the most studied role of the MHC-I presentation in immunosurveillance, which will be the focus of this introduction, MHC-I antigen presentation was shown to be involved in odorant-based mate selection<sup>16</sup> and in neuronal function<sup>17</sup>. Indeed, the MHC-I molecules expressed in neurons participate in the synaptic plasticity of the developing and mature mammalian central nervous system (CNS), and were found upregulated in Parkinson 'disease<sup>17,18</sup>. While the underlying mechanisms are poorly understood, these observations emphasize the fact that the immune system and the CNS share similarities that may allow them to act in concert to maintain the homeostasis of the body. In addition, the multifunctionality of the MHC-I molecules highlight the "non-immune" role of MHC-I presentation and also suggest the importance of the non-peptide binding part of the molecules in their function.

MHC-I and MHC-II antigen presentations share a similar function in the adaptive branch of the immune response which is to present at the cell surface antigenic peptides that can be recognized by CD8+ T cells and CD4+ T cells, respectively. MHC-I molecules are constitutively expressed by all nucleated cells. The expression of MHC-II molecules is restricted to thymic epithelial cells (TECs) and professional antigen presenting cells (pAPCs), including B cells, macrophages and dendritic cells (DCs) but their expression can be induced in other cell types such as fibroblasts, astrocytes, endothelial or epithelial cells upon specific cytokines stimuli, particularly IFN- $\gamma$ <sup>19</sup>. The recognition of peptides bound to MHC-I and -II molecules on pAPCs is central to the activation of naïve T cells and their proliferation (**Figure 1**). The ones presented at the surface of almost all cells and recognized by activated T-cells participate in the recognition and the elimination of non-self<sup>20</sup>. Acquisition of the peptide is achieved differently by the two MHC molecules. On one hand, peptides bound to MHC-I molecules come from endogenous sources, reflecting the intracellular events such as viral or bacterial infection, or cell transformation. On the other hand, MHC-II molecules present peptides from exogenous sources, providing a sample of the extracellular environment. These two pathways were first strictly segregated with the cytosolic proteasomal antigen processing for the MHC-I and the endosomes/lysosomes antigen processing for the MHC-II. However, several interplays between both pathways have been described and extend the possible source of peptides for both MHC-I and -II molecules.



**Figure 1: Cross-priming of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by dendritic cells (DCs)<sup>21</sup>**

One of them is the cross-presentation pathway which is characterized by the ability of APCs to take up extracellular antigens and to transfer them to the MHC-I pathway. One of its main roles is to specifically activate naïve CD8<sup>+</sup> T cells<sup>22</sup>. In that case, cross-presentation is called cross-priming. Cross-priming process appears to be performed only by specific subsets of DCs or DCs that have acquired specific functions<sup>23</sup>, and upon several environmental conditions such as TLRs activation, T helper recognition and pro-inflammatory cytokines presence. DCs can take up infected/transformed cell fragments and hence are essential for the response against pathogens or tumors as well as for the success of vaccination with protein antigens or synthetic long peptides. Internalized elements that ended in phagosomes can reach MHC-I molecules through different paths. They can access the cytoplasm to be processed by the proteasome and to enter the MHC-I pathway. Several mechanisms that allow the access of these peptide precursors to the cytoplasm have been proposed such as their transfer through leaky phagosomal membranes or through a membrane pore created by elements of the ER dislocation machinery present in the phagosome<sup>24</sup>. Besides, phagosomes that contain the MHC-I loading machinery have been isolated and shown to be able to import and cross-present exogenously added peptides, hence providing a peptide-loading location different from the conventional MHC-I pathway<sup>21</sup>. In addition, the process of autophagy, a ubiquitous process involved in the steady state turn-over of long-lived proteins and damaged organelles,

has been suggested to be implicated in cross-presentation<sup>25</sup>. As an alternative mechanism, intercellular protein channels such as gap junctions have also been demonstrated to be used for the transfer of peptides to DCs for cross-presentation<sup>26</sup>. Extensive studies have to confirm the physiological significance of these pathways.

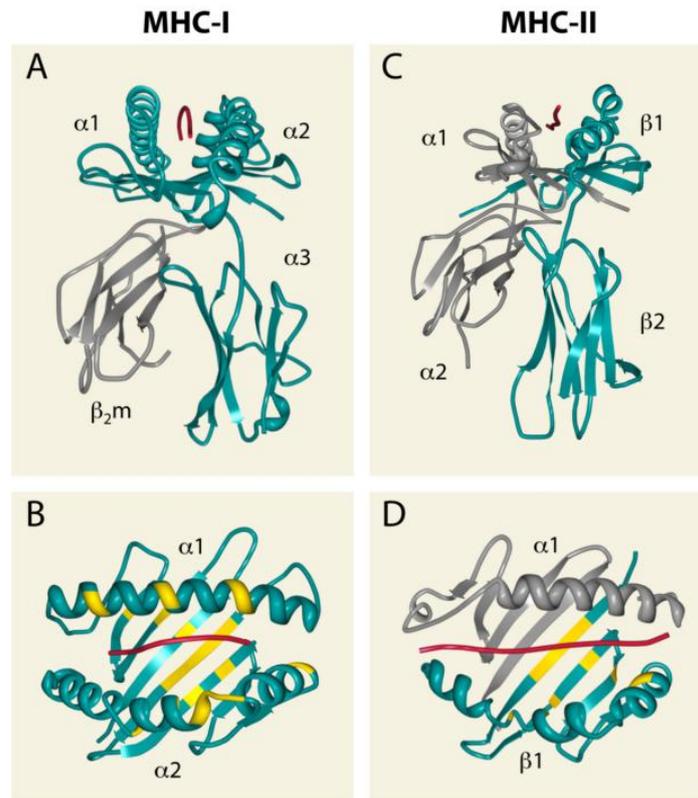
Conversely, a substantial part of the peptides presented by MHC-II molecules has been demonstrated to originate from endogenous nuclear and cytosolic proteins<sup>24</sup>. Again, autophagy can play a role in that process. For example, it has been demonstrated that APCs-like B cells infected with the Epstein-Barr virus, present antigens from endogenously expressed EBNA1 protein. The latter is delivered by autophagy to the lysosomes where it can enter the MHC-II pathway, leading to CD4+ T cell clonal-specific activation<sup>27</sup>. The role of autophagy was also highlighted for the MHC-II presentation of some self-antigens during the process of central CD4+ T cell tolerance mediated by medullary thymic epithelial cells (mTECs)<sup>28</sup>. In addition, access of some endogenous proteins to the MHC-II pathway has been shown to occur independently from autophagy. For example, alternative pathways have been suggested to present newly synthesized Env and Gag antigens to specific CD4+ T cells by HIV-infected DCs<sup>29</sup>, but the mechanism remains unclear. Interestingly, tumors cells have also been shown to present endogenously expressed peptides on MHC-II molecules. A recent study suggests that melanoma cells present an NY-ESO-1 derived antigen which pathway of processing results from its intercellular transfer between melanoma cells followed by its entry in the endocytic pathway<sup>30</sup>.

Overall, these studies highlight the fact that antigen presentation on MHC-I and MHC-II molecules does not only result from two distinct pathways but from several that could interact with each other. These observations bring even more complexity in the understanding of the pool of antigenic peptides that are expressed by cells, namely the immunopeptidome. In the next sections, we will see that our understanding of the MHC molecule structures and of the intracellular pathways, as well as the identification of self-, tumor- and viral-associated antigens bring and will bring new insights into the immunopeptidome and ultimately into the adaptative immune response. We will particularly emphasize on the direct MHC-I antigen presentation pathway and the source material that enters this pathway.

## C - MHC-I pathway step by step: what defines the MHC-I immunopeptidome?

### a. The peptide-MHC (pMHC) complex

The distinct functions between MHC-I and MHC-II molecules are partly due to their structural variations, allowing them to present different peptide repertoires and to activate separate cells. Both molecules are composed of two domains: the heavy  $\alpha$ -chain and the  $\beta$ 2-microglobulin protein ( $\beta$ 2m) for the MHC-I molecule; the  $\alpha$ -chain and the  $\beta$ -chain for the MHC-II molecule. The membrane-proximal region contains two conserved immunoglobulin (Ig) domains that support the peptide-binding unit. The latter, usually called the peptide-binding groove, is composed of two anti-parallel  $\alpha$ -helices on the top of an eight-strand  $\beta$ -sheet. The amino acid sequence of this groove is the most polymorphic part of the structure, enabling interactions of the different MHC alleles with different pools of peptides. It was observed that MHC allomorphs present largely nonoverlapping sets of peptides defining the peptide repertoire presented by one individual<sup>31</sup> (**Figure 2**). At the amino (N) and the carboxy (C) termini, the peptide forms hydrogen bonds with the two  $\alpha$ -helices. While peptides are fixed both at their N and C-termini inside the closed MHC-I molecules binding groove, peptides bound to MHC-II molecules do not have fixed termini and extend beyond the groove. As a consequence, the length of peptides presented by MHC-I molecules are usually shorter (generally described to range from 8 to 13 amino acids) than the one presented by MHC-II molecules (generally described to range from 13 to 25 amino acids)<sup>32</sup>. Some specific residues inside the groove are more important for the peptide-MHC (pMHC) complex formation and stability. For example, interactions at the F-pocket, which corresponds to the docking site of the C-terminal end of the peptide in MHC-I molecules, and at the P1 pocket, which is located at N-terminal side of the MHC-II molecule, have been shown to have a dominant impact on pMHC complex conformational and thermal stability, as well as on the immunodominance of certain peptides<sup>33,34</sup>. All the structural, biochemical, kinetic and thermodynamic information about the pMHC complex have been used for the development of binding prediction tools<sup>35,36</sup> that are widely used, for example, during the search for relevant cancer-specific antigens. Our understanding of the interactions between the peptide and the MHC molecules brings on pieces in the puzzle that elucidates how cells select the antigens they present at their surface.



**Figure 2: Structure of MHC-I and MHC-II molecules and of the peptide binding groove**

### **b. Degradation and processing**

In order to generate fragments that fit the MHC-I binding groove, polypeptides synthesized inside the cell have to be fragmented before reaching the loading compartment. This step is crucial for the activation of CD8+ T-cell by APCs and for their ability to target infected or transformed cells. Changes in the kinetics of peptide degradation<sup>37,38</sup> as well as in the cell catalytic activity<sup>39</sup> have been shown to greatly impact the subsequent cytotoxic T lymphocytes (CTL) response and therefore provide a way for modulating it.

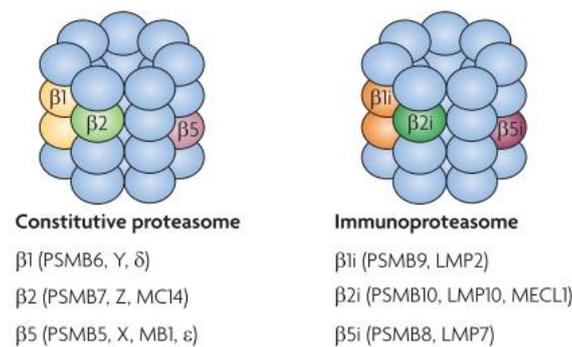
It is well established that the ATP-dependent proteolytic system called the 26S proteasome, which recognizes ubiquitin-tagged proteins, largely contributes to the generation of peptides for the MHC-I pathway. The 26S proteasome is composed of a 20S core barrel, which contains four stacked rings of seven subunits, and one 19S multisubunit complex at each of its ends. The active proteolytic components are located in the two inner rings of the barrel containing the  $\beta$ -subunits. Catalytic subunits have been particularly identified as the  $\beta_5$ ,  $\beta_2$  and  $\beta_1$  that cut after the C-terminus of hydrophobic (chymotrypsin-like activity), basic

(trypsin-like activity) or acidic (caspase-like activity) amino acids, respectively<sup>40</sup>. Of note, either basic or hydrophobic residues at the C-terminus of the MHC ligands are required by all MHC allotypes while none have been described to allow an acidic residue at this location. The proteasomal system possesses a certain level of plasticity which can favor or prevent the production of certain antigenic peptides. During the immune response, secretion of the interferon- $\gamma$  (IFN- $\gamma$ ) or the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induces the replacement of the proteasome  $\beta$ -subunits by functionally different counterparts named low molecular weight proteins (LMPs) or  $\beta$ i. The assembly of these new molecules in the 20S proteasome forms the immunoproteasome. In dendritic cells, lymphocytes and thymic epithelium, the immunoproteasome is constitutively expressed. Its catalytic subunits, LMP2, MECL1 and LMP7 have enhanced chymotryptic activities while they abrogate the caspase-like activity<sup>41</sup>. As a consequence, the immunoproteasome generates epitopes quantitatively and qualitatively different from proteasome ones and with more suitable C-terminal amino-acids (**Figure 3**).

Another regulatory complex, the proteasome activator PA28, has been shown to associate with the 20S proteasome. It can either binds at both ends of the 20S proteasome (PA28-20S-PA28) or at only one of its ends with the 19S regulator at the other one (19S-20S-PA28) forming the hybrid proteasome that has the ability to process polyubiquitinated substrates in an ATP-dependent manner<sup>42,43</sup>. The PA28 complex can be formed by the PA28 $\beta\alpha$  heteroheptamer or by the PA28 $\gamma$  or the PA28 $\alpha$  homoheptamers. The expression of PA28 $\beta\alpha$  is induced by interferon- $\gamma$  and has been observed to enhance the production of some of the MHC-I epitopes recognized by CTL<sup>44,45</sup>. In addition, PA28 $\beta\alpha$  KO mice, while showing a normal immune response to influenza infection, showed impaired ability to process and present some antigens such as TRP2 melanoma-specific peptide<sup>46</sup>. Some studies showed that PA28 $\beta\alpha$  binding modifies the cleavage mechanism of the active sites and promotes the three catalytic activities of the proteasome. These changes were shown to result in optimized antigen presentation for some studied epitopes<sup>47,48</sup> and to facilitate peptide release from the proteasome<sup>49</sup>.

Conversely, PA28 $\gamma$  binding to the 20S proteasome has not been clearly linked to the generation of peptides for the MHC-I presentation pathway. Its expression is not enhanced upon IFN- $\gamma$  stimulation. PA28 $\gamma^{-/-}$  mice showed no impairment in the generation of viral antigen repertoire upon influenza infection and in cytotoxic T cell reactivity after recombinant vaccinia virus infection compared to WT mice. However, PA28 $\gamma^{-/-}$  mice showed reduced number of CD3+ CD8+ T cells and defect in clearing pathogenic fungal infection<sup>50</sup>. In

addition, PA28 $\gamma$  only promotes the trypsin-like activity of the proteasome<sup>51</sup>. Interestingly, it is mainly present in the nucleus and has been observed associated with the 20S proteasome in an active form in nuclear speckles<sup>52</sup>, which are subnuclear domains enriched in components of the pre-mRNA splicing machinery. From these observations our team investigated the role of the nuclear PA28 $\gamma$  (or REG $\gamma$ ) associated with the 20S proteasome in the MHC-I antigen presentation and, as we will see later in more details, demonstrated that it negatively impacts the presentation of specific antigens derived from pioneer translation products.



**Figure 3: Subunit composition of the active sites of the constitutive proteasome and the immune proteasome.**<sup>43</sup>

While ubiquitin-dependent proteasomal degradation was extensively described, alternative paths such as ubiquitin(Ub)-independent proteasomal degradation has also been involved in the generation of MHC-I peptides<sup>53</sup>. Using a Ub-activating enzyme E1 inhibitor, J. Wei *et al.* strongly support the contribution of Ub-independent pathway in antigen presentation depending on class I allomorph, cell type, source protein and peptide context<sup>54</sup>. In addition, although the proteasome is responsible for the generation of most of the peptides presented by the MHC-I molecules<sup>55</sup>, a proteasome-independent pathway has been suggested to involve the cytosolic tripeptidyl peptidase II (TPPII) aminopeptidase leading to the presentation of a different pool of peptides, and has also been observed in the paper cited above<sup>54,56</sup>. These studies support the use of multiple pathways for antigen presentation.

Because the proteasome generates fragments from 2 to 25 amino acids, it may release N-extended precursors too long to bind MHC-I molecules. These precursors can be trimmed at their N-terminus by aminopeptidases either in the cytosol or in endoplasmic reticulum<sup>57</sup>. Among them, the TPPII has been identified as necessary for the presentation of specific

peptides on MHC-I molecules while it has no or negative impact on others<sup>58</sup>. In addition, the cytosolic leucine aminopeptidase, the bleomycin hydrolase and the puromycin-sensitive aminopeptidase have been shown to generate mature epitopes from precursors when studied separately outside the cell. However, their precise role in the generation of peptides has to be further investigated. In the ER, it has been proven in many studies that the ER aminopeptidases ERAAP 1 and 2 bring an important contribution to the MHC I repertoire and to the concept of immunodominance<sup>59,60</sup>.

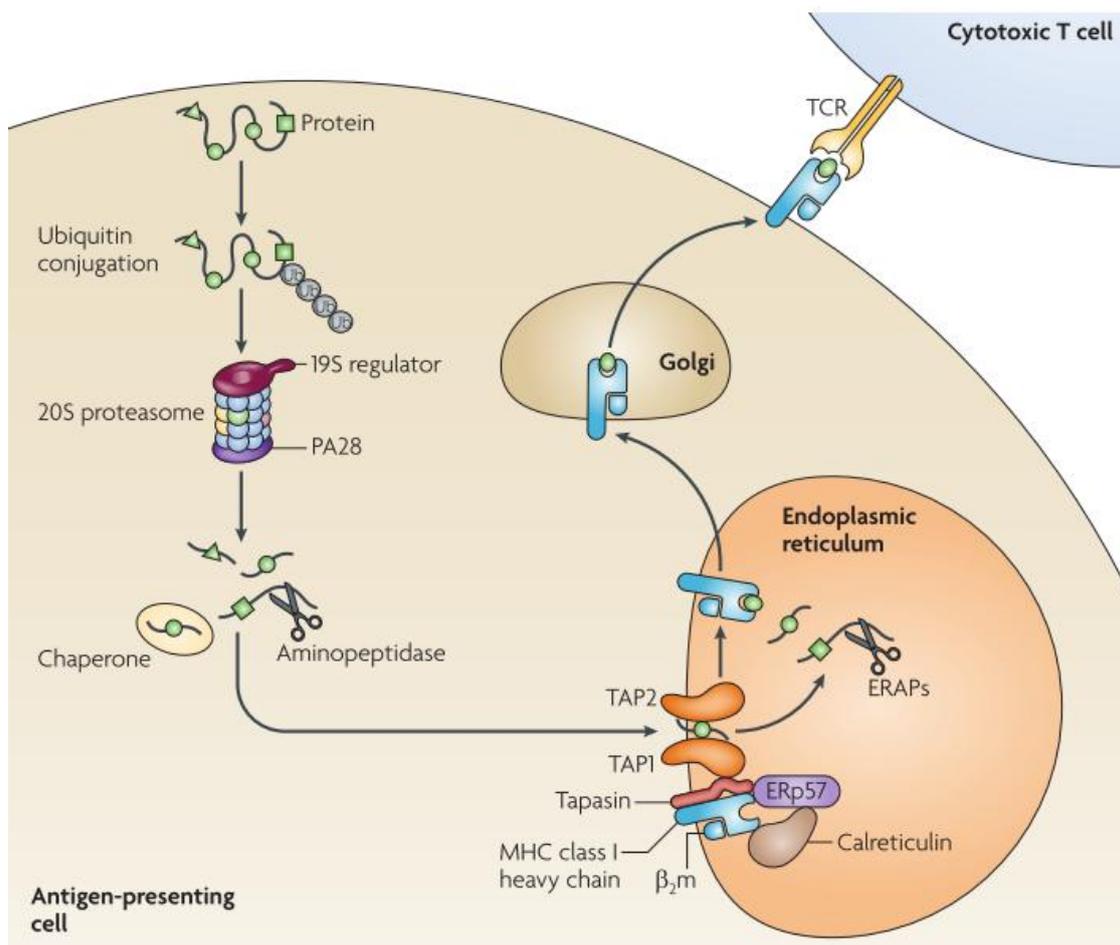
Overall, these studies shed light on important players during peptide shaping and on the ability of cells to adapt their processing path in different contexts. They show that changes in the activity and kinetics of enzymes dedicated to peptide shaping along the MHC-I pathway can considerably impact the immunopeptidome and hence the adaptative response during pathogen infection, tumor development or auto-immunity.

### **c. Loading and formation of the complex**

Intermediates produced by proteolysis have to enter the endoplasmic reticulum (ER) in order to form a complex with empty complete MHC-I molecules present in the lumen of the ER, anchored to its membrane and stabilized into the peptide loading complex (PLC). Their transport to the ER is performed by the transporter associated with antigen processing (TAP), composed of two non-covalently associated subunits, TAP1 and TAP2, forming a pore that opens and expels the peptide into the ER in an ATP-dependent manner. Proteasome generated peptides have been shown to have a less than 10 seconds half-life and should then rapidly reach and bind TAP in order to avoid complete degradation<sup>61</sup>. The binding of the peptide to the nucleotide-binding domain of each TAP subunit is ATP-independent and has been shown to be determined by the peptide N- and C-terminal residues. In fact, some characteristics such as hydrophobic and basic residues at the C-terminus or positively charged residues at position 1-3 are inclined to bind to TAP while proline is disfavored at position 2<sup>62</sup>. Therefore, the entry to the ER constitutes another step in the selection of the peptides for presentation and is in accordance with some peptide selection specificities of the proteasome and the MHC-I molecules. The TAP molecule is included into the PLC, consisting in the assembly of molecules involved in the formation of the pMHC complex. Among them, the type I glycoprotein tapasin encoded by an MHC-linked gene recruits MHC-I- $\beta$ 2m dimers to TAP. In addition, it links with the oxidoreductase ERP57 that interacts with the lectin chaperone calreticulin. All these components facilitate the recognition, translocation and loading of high

affinity peptides on the MHC-I molecules. Free peptides in the ER lumen that did not succeed in binding to the MHC-I groove are rapidly translocated back to the cytoplasm by the SEC61 translocon. The stable MHC-I molecule-peptide complexes are finally presented at the cell surface through the secretory pathway. As exceptions prove the rules, TAP-independent pathways have also been described<sup>63,64</sup>, allowing cells to present antigenic peptides upon TAP impairments such as currently observed upon viral infections or in tumors.

**Figure 4** provides an overview of the most commonly described degradation, processing and loading steps along the MHC-I antigen presentation pathway.



**Figure 4:** Antigen processing in the MHC class I-restricted pathway.<sup>43</sup>

#### d. The source of polypeptides

When and how intracellular polypeptides enter the direct MHC-I antigen-processing pathway?

The knowledge about the source of polypeptides that enter the MHC-I degradation-presentation pathway was largely gained from the study of viral infection and tumors partly because: (1) antigens presented at the cell surface derived from known viral protein and tumor-associated proteins and recognized by CTL were identified and hence it was possible to follow their presentation in different settings; (2) virus and cancer cells developed mechanisms to interact with and to impair the presentation machinery and hence provided tools to identify main actors and mechanisms along the pathway. In addition, known immunogenic proteins such as the ovalbumin (Ova) protein were used as model for the study of MHC-I antigen presentation. In fact, valuable tools were developed around the discovery of immunogenic epitopes of Ova such as the *lacZ* T-hybridoma cells B3Z<sup>65</sup>, the ovalbumin-derived peptide SIINFEKL bound to the MHC class I allele H-2K<sup>b</sup> specific 25-D1.16 antibody or the OT-1 and OT-2 transgenic mice that generate CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes specific for the Ova 257-264 and the Ova 323-339 epitopes respectively.

The first studies focused on the importance of the proteolytic pathway in order to elucidate which substrates are used by proteases and described proteins retroviral as a substantial source of MHC-I antigens. In 1988, Townsend *et al.* demonstrated that by enhancing the degradation of viral proteins in vaccinia infected cells the antigen presentation of certain viral peptides can be restored in the late phase of infection. Hence, they concluded that there was a relationship between the degradation of proteins in the cytoplasm and the expression of viral antigens at the cell surface<sup>66</sup>. However, showing that not all epitopes were affected, they already suggested a diversity of mechanisms by which the antigens were generated. Some years later, K. L. Rock *et al.* used the proteasome inhibitor MG115 to demonstrate that MHC-I presentation of the SIINFEKL antigen from the cytoplasmic Ova relies on proteolysis of this protein when it is turned-over<sup>67</sup>. In addition, they showed that the ubiquitin conjugation of the Ova in the cytosol is required for an efficient presentation of the SIINFEKL, highlighting the role of the ubiquitin-dependent proteolytic pathway in antigen presentation from full length proteins<sup>68</sup>.

In contradiction to some of these findings, it was quickly observed that antigens presented at the cell surface were not strictly related to the abundance of the source protein and the rate of its degradation. In a kinetics study of the presentation of the MHC-I peptide N on Vesicular stomatitis Indiana virus (VSV) infected cells, it has been shown that viral peptide can be recognized by specific T cells within 45min after infection contrasting with the stability of the full-length protein containing the N epitope<sup>69</sup>. Furthermore, some studies suggested that

generation of antigens for the MHC-I pathway does not necessarily require the expression of a functional protein but that antigens could be generated from a distinct translational mechanism. Among them, T. Boon and his colleagues isolated genes that encode antigens presented by mouse tumor variants, which are rejected by syngeneic mice. Then, they transfected subgenomic promoterless fragments of these genes in a non-eukaryotic expression vectors and observed that it sensitized transfected cells to CTL lysis with apparent absence of gene expression<sup>70,71</sup>. Similarly, in a viral infection model of the influenza virus in mouse cells, a study showed that the transfection of a mutated form of the virus nucleoprotein (NP) leads to the recognition of the cells by NP-specific T cells while there was no detectable expression of the protein<sup>72</sup>.

In addition to these findings, several studies have described unknown peptides recognized by tumors, viral or allogenic specific T cells that are not encoded in a conventional reading frame but in a cryptic translation reading frame<sup>73</sup>. For example, in 1994, it was demonstrated that the mouse tumor cells RLO\*1 present at their surface the pRL1b peptide recognized by specific CTL and which derives from the 5' untranslated region of the c-akt oncogene<sup>74</sup>. Besides, the tumor infiltrating lymphocytes TIL586 from melanoma-bearing patients, which induce tumor regression after an autologous transfer, were shown to recognize an antigenic peptide derived from an alternative open reading frame of the gp75 gene<sup>75</sup>.

From these observations emerged the “pepton” hypothesis developed by T. Boon in 1989, which provided an explanation of the presence of these peptides at the cell surface. Briefly, they described peptons as few hundred base pair regions of the cellular genome that are transcribed by a polymerase that does not need promoter sequences and which give rise to “pepton-RNAs”. They are translated into peptides that are independent from the corresponding full-length protein and are used as substrates for proteases of the antigen presentation pathway, perhaps as part of a normal housekeeping process of the cell. Interpreting these observations from a different perspective, J. Yewdell, L.C. Anton and J. R. Bennink<sup>76</sup> proposed in 1996 that the major source of antigens are defective products from protein synthesis that can be either premature termination products or misfolded full-length proteins quickly directed to the cell degradation systems such as the proteasome. These elements were called the Defective Ribosomal Products (DRIPs). Since then, our understanding of DRIPs and all the associated concepts has evolved, providing us with more information about the source of antigenic peptides for the direct MHC-I pathway.

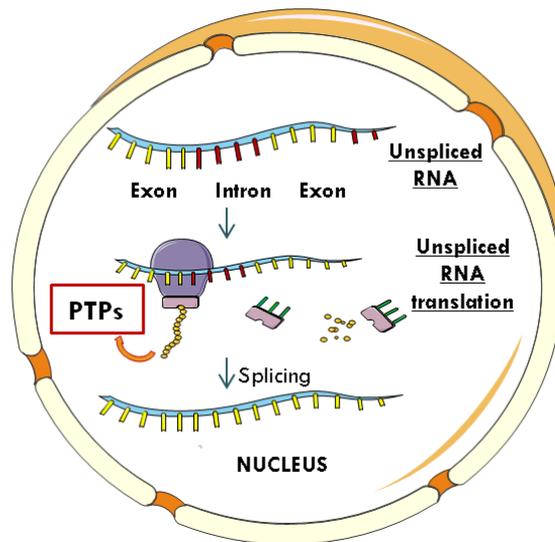
## **D - Source of polypeptides for the MHC-I pathway: the contribution of DRIPs and PTPs to the immunopeptidome and the associated concepts**

### **a. What are DRIPs and PTPs?**

Above information suggest two sources of peptides presented by the MHC-I molecules: turned over proteins and degradation of newly synthesized and deliberately defective proteins. Growing studies and improvement of tools brought insights into the still unclear mechanism of polypeptides generation. At the heart of it are the Defective Ribosomal Products (DRiPs) and the pioneer translation products (PTPs), which support and are supported by associated concepts such as non-conventional, nuclear or pioneer translation as well as alternative, defective or non-mature RNAs.

In a 2014 review, J. Yewdell and L.C. Anton broaden their definition of the DRIPS including “defective polypeptides arising from alternative/defective mRNAs, ribosomal frame shifting and downstream initiation on bona fide mRNAs, and all other errors that occur in converting genetic information into proteins”<sup>77</sup>. Moreover, they suggested the presence of a specific subset of ribosomes that is dedicated to the generation of DRIPs, which is called the immunoribosome<sup>78</sup>. The evolution in the definition emphasized the role of the mRNA structures and the translational mechanisms. To illustrate this, S. Apcher *et. al* first studied the Epstein Barr virus strategy to suppress antigen presentation by regulating its mRNA translation. They showed that the mRNA translation initiation rate and not the protein stability was determinant in the subsequent antigen presentation<sup>79</sup>. In a following study, they demonstrated that the cap-dependent translation initiating eIF4E protein is not required for the presentation of either the SIINFEKL or the MBP epitopes from different gene constructs while it is required for the expression of the corresponding full length protein. Hence, they suggested that the initiation of the translation event that governs the production of full length products is distinguishable from the one that governs antigen production<sup>80</sup>, building on the peptons hypothesis. Moreover, they showed that this translational event share characteristics, such as the requirement of the eIF4G protein, with the pioneer round of translation performed by the cell as an mRNA quality control event that can potentially redirect the mRNA to the non-sense mediated decay (NMD) pathway for degradation. During this step, the cap-binding protein (CBP) heterodimer CBP80-CBP20 binds to the 5'-m7GpppN cap structure and allows the recruitment of one or more ribosomes<sup>81</sup>. Hence, they introduced a novel class of peptides that are produced during a pioneer translation event, called the pioneer translation products

(PTPs)<sup>80</sup> (**Figure 5**). In the attempt to better characterize this translational event, S. Apcher *et al.* inserted the SIINFEKL and the MBP epitopes into different gene locations and showed that both were expressed at the cell surface similarly when inserted intronically versus exonically. Polypeptides produced from these gene constructs, i.e. the PTPs, were detected on nascent ribosomes in the nuclear compartment. Overall, they suggest that a major portion of MCH-I pathway substrates is synthesized in the early steps of mRNA maturation, before introns are spliced, by a non-canonical translation mechanism within the nucleus<sup>82</sup>.



**Figure 5:** Formation of pioneer translation products

**b. The immunopeptidome: correlation with the genome, the transcriptome and the proteome?**

**i. General features of the self- immunopeptidome**

Depending on the cell type, the number of MHC class I molecules at the cell surface has been evaluated from 1 to  $5 \times 10^5$  in complex with about  $10^4$  different peptides<sup>83</sup>. By comparing the protein and the MHC class I associated peptides (MIPs) number and the size, it was estimated that only 2% of the proteome is represented at the cell surface. Close to that calculation, a recent study on B-lymphoblastoid cells (B-LCLs) estimated that the MIPs covered only 10% of exomic sequences expressed in these cell lines<sup>84</sup>. In addition, it was shown that the immunopeptidome does not derive from a specific chromosomal region but from genes shared among all chromosomes as well as from a variety of proteins present in all cell compartments<sup>85-87</sup>.

Therefore, the MIPs repertoire and the mechanisms underlying its generation are complex and actually hard to predict. Our current definition of self vs. non-self remains unclear. In the next paragraphs, we will see how studies show that the MIPs repertoire is not generated from a random selection of the proteome and what is our current understanding of the mechanisms underlying its biogenesis.

## ii. Immunopeptidome and proteome

Currently, a few authors<sup>88,89</sup> consider that protein retirees substantially provide substrates for the MHC-I pathway. Certain studies showed that the requirement of retirees and rapidly degraded proteins (RDPs) differs according to the stress applied to the cell<sup>77</sup>, as for example during irradiation where retirees are rapidly recruited to the MHC-I pathway<sup>90</sup>.

RDPs, which are targeted<sup>91</sup> or not<sup>53</sup> to the ubiquitin-dependent proteasomal system, were in many studies shown to largely contribute to the immunopeptidome<sup>92,85</sup>. Improvement in mass spectrometry techniques was central in a deeper understanding of the correlation between the immunopeptidome and the rate of translation based on large scale-analysis. Combining the stable isotope labeling by amino acids in cell culture (SILAC) technique with pulse-chased experiments and MIPs elution in human tumor cell lines, it was demonstrated that many of MIPs are derived from newly synthesized molecules that degrade rapidly after synthesis<sup>93</sup>. Moreover, it was shown that the impact of proteasome inhibitors on antigen presentation may be due in part to their effect on the synthesis rate modulation<sup>94</sup>. In line with these observations, a study on vaccinia virus infected cells used the LC- multiple reactions monitoring (MRM) approach to tandem mass spectrometry in order to monitor viral peptides appearance at the cell surface upon infection. Researchers showed that the nature of viral epitopes presented throughout the infection is very dynamic and that the timing of their appearance correlates with the corresponding protein synthesis initiation<sup>95</sup>. Therefore, protein synthesis dynamic seems central in determining the MIPs.

Among rapidly degraded products, the question of their nature as well as DRIPs and PTPs contribution to the immunopeptidome still remains. In that attempt, one global MIPs analysis of a human cell line from mass spectrometry coupled to proteome database accounted the significant contribution of DRIPs as misassemble surplus subunits of large protein complexes<sup>96</sup>. However, in order to better gauge the participation of products from pioneer round of translation, downstream initiation, out-of-frame translation or premature termination of translation, one can argue that the MIP repertoire should be explored from the information

given upstream from the proteome, i.e from the transcriptome or the ribosomal-bound products. Large scale analysis strategies that coupled the MIP proteomic with both customized transcriptomal and proteasomal databases revealed new and important information about MIPs biogenesis.

iii. From the genome to the immunopeptidome: implication of the transcriptome and the metabolic state.

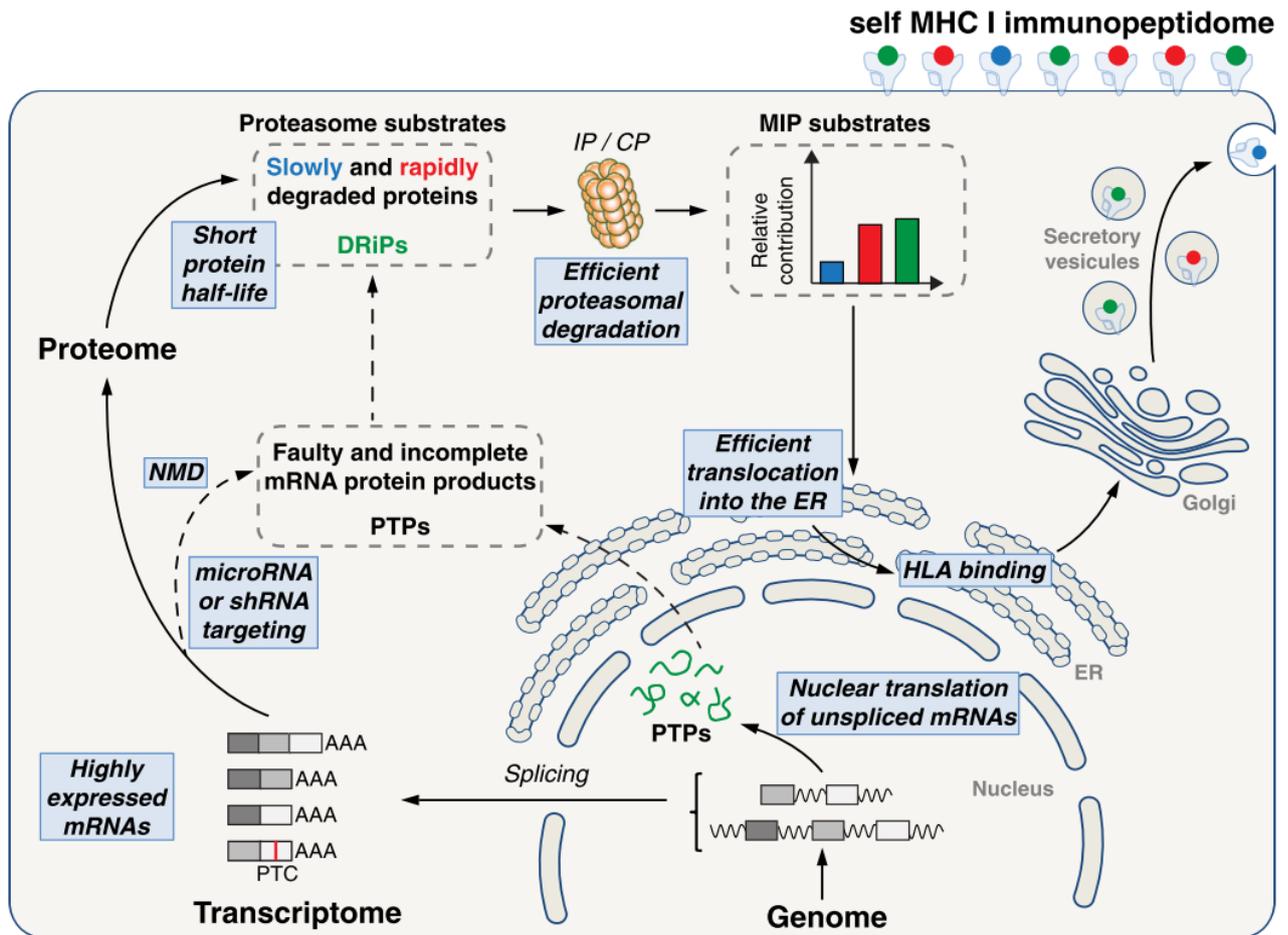
As previously seen, protein abundance was shown not to be strictly linked to MIPs<sup>93</sup>. While at first conflicting results concerning the correlation between the MHC-I peptide repertoire and the associated transcript abundance were observed<sup>86,97</sup>, it is currently assumed that transcript abundance is, among other parameters, linked to MIPs generation<sup>84,98</sup>. In addition, several experiments have demonstrated that the MIPs reveal a cell-type specific signature in accordance with the cell function. For example, researchers who studied the B-LCLs MIPs and their source genes observed enrichment in genes implicated in immunoglobulin production<sup>98</sup>. Another study from C. Perrault and colleagues showed that over 40% of mouse thymocyte MIPs are different from the same mouse dendritic cell MIPs<sup>99</sup>. Following these findings, they demonstrated that intracellular biochemical and metabolic events shape the MIP repertoire. They suggest that changes observed after treatment with the mTORC1 inhibitor rapamycin account for changes in the transcriptional sub-network that ultimately affect the transcripts at the source of MIPs<sup>100</sup>. In accordance with this observation, it was observed that HIV-infected cells present peptides derived from self-origin that appeared only upon infection<sup>101</sup>. As no change was observed in related transcripts abundance, change in postranscriptional mechanisms was suggested to be the cause of these apparitions.

Looking at the MIPs from an even wider perspective, C. Perrault and colleagues performed high-throughput proteogenomic on B-LCLs from 18 patients with 27 HLA-A and HLA-B allomorphs in order to identify features that could influence the MIP generation<sup>84</sup>. They first observed that MIPs are generated from only 59% of the entire protein coding gene set and that from 1 to 64 MIPs are generated per gene. In addition, even if HLA allomorphs present almost completely different sets of peptides, these were shown to derive from the same genomic regions. From a system level perspective, they suggested that some RNA features facilitating the translation such as a higher number of exons and shorter 5'UTR, as well a higher gene expression, were enriched in MIP source transcripts. Supporting the contribution of the NMD pathway to the generation of the MIPs, transcripts with an NMD biotype, defined

by the presence of single nucleotide variants predicted to lead to a premature termination codon generation, were also found enriched. This feature favors the contribution of PTPs and DRIPs to the MIPs generation and provides some insights into their nature. In addition, disorder and degradation motifs facilitating proteins proteasomal degradation were also prevalent. Finally, linking the genome to the MIP, it was demonstrated that peptides derived from polymorphic genomic region present in some but not all subjects on a given HLA allele and that elicit an allow-immune response account for 12% of the MIP-coding exome<sup>102</sup>.

Hence, the MIP repertoire was described as flexible and in accordance with the cell metabolism and transcriptional regulations, as well as with gene expression and genetic polymorphism. In addition, the intrinsic composition of transcripts was shown to influence their contribution to the MIP repertoire. **(Figure 6)**

Interestingly, one transcriptional mechanism that shapes the MIPs has been described by a study of HLA-A and -B associated peptides from EBV-infected B lymphocytes from 4 patients<sup>98</sup>. This study clearly linked MHC-I associated peptides with three factors: the biological pathways the genes they are derived from are involved in, the transcripts abundance and whether or not they contain a miRNA response element (MRE). They suggest that miRNAs-mediated protein synthesis destabilization, slowing-off elongation, dropping-off ribosome, or degrading the nascent polypeptide, is one mechanism by which DRIPs or PTPs are generated. A similar mechanism was suggested in mouse tumor cells treated with short hairpin RNA specific to mRNAs that encode immunogenic epitopes. In fact they showed that treatment increased targeted peptide presentation and subsequently prevented tumor growth<sup>103</sup>. However, the previously cited study on B-LCLs failed to find such a feature in transcripts that give rise to MIPs, suggesting that this correlation may not happen in all cell types and that further experiment should be performed to confirm this hypothesis<sup>84</sup>.



**Figure 6:** Overview of the major processes involved in the genesis of the MHC-I associated peptides<sup>83</sup>.

iv. Alternative translational mechanisms

The above cited findings highlight the translational event as one key step in the generation of MHC-I antigenic peptides. Translation occurs in three main steps that are the initiation, elongation and termination. The conventional initiation step described is cap-dependent and involves the mature mRNA recruitment to the 43S pre-initiation complex (PIC). The PIC is composed of the methionyl-initiator tRNA (Met-tRNA<sub>i</sub>) in complex with the eukaryotic initiation factor 2 (eIF2) bound to guanosine triphosphate (GTP). Its fixation to the mRNA occurs at the 5' end cap structure upon the eIF4F complex (including eIF4E and eIF4G cap-binding proteins as well as the eIF4A RNA helicase) and poly(A)-binding protein (PABP) action. Once attached, the complex scans the 5'UTR until the Met-tRNA<sub>i</sub> matches with an AUG codon preferentially included in the Kozak consensus (5' (A/G)CCAUGG 3'), leading to the GTP-dependent recruitment of the 60S subunit to form the 80S initiation complex that starts the translation<sup>104</sup>.

In 1999, S. Malarkannan *et al.* from N. Shastri's laboratory first suggested that translation initiating at "near cognate" triplets, differing from AUG initiation codon by a single amino acid, provides peptides for the MHC-I pathway and described a translation event starting at a CUG initiation codon<sup>73</sup>. Translation initiation at a CUG codon was partly explained by the possible pairing between the CUG with the Met-tRNAi. However, the same group demonstrated later the existence of a novel translational initiation mechanism independent from the canonical Met-tRNAi one that initiates translation at CUG start codon using a leucine-bound tRNA initiator (Leu-tRNAi) and that follows an alternative eif2A-dependent pathway<sup>105</sup>. The importance of this translational mechanism was in some extent demonstrated in a transgenic mice model expressing the H60 antigen derived from CUG codon-initiated translation, which was shown to induce central tolerance and prime CD8+ T cells<sup>106</sup>. In addition, several peptides present among the MIPs of human cancer cells or infected cells and deriving from CUG or other non-AUG translation initiation have been described and shown to be relevant in disease progression<sup>107-109</sup>. For example, in human renal cell carcinoma, a peptide translated from a CUG initiation codon from the VEGF protein was found over-expressed and CD8+ T cells were enriched in clones directed against this peptide<sup>110</sup>. Recently, N. Yang and colleagues studied the SIINFEKL antigen presentation of cells infected with an engineered influenza A virus. Taking advantage of a splicing event required by the virus to generate two distinct mRNAs with different canonical open reading frames, they demonstrated that a non-canonical translation initiated at a CUG codon in the cytoplasm is involved in the SIINFEKL presentation, and hence participates in viral immunosurveillance<sup>111</sup>.

Besides, other non-conventional events were described such as the translation of alternative reading frames or "non-coding" mRNA sequences, i.e. introns, intron-exon or exon-UTR junctions, 3' or 5'UTRs, short open reading frames or long non-coding RNA. Many immunogenic peptides generated by such translations in either cancer or virus-infected cells, in mice or humans, were discovered as MIP source and reviewed in several journals<sup>112-114</sup>. Their relevance for the immune response against tumors or pathogens in mouse or humans was demonstrated in many cases<sup>115-120</sup>. As previously observed, the study of these cryptic MIPs can bring some insight into the mechanism underlying their translations, which are however currently not well understood. For example, one recent study shows that tumor-infiltrating lymphocytes from melanoma patients who remained relapse-free after adoptive cell transfer recognized two antigens over-expressed by melanoma cells. Both these antigens were shown to be produced from a polycistronic mRNA containing multiple short open

reading frames (sORFs) in an internal ribosomal entry site (IRES)-dependent manner, which is a cap-independent translational mechanism<sup>121</sup>. Another study proposed that translation initiation downstream the primary AUG in an alternative reading frame in the human insulin mRNA could be at the source of an antigen presented by beta cells and recognized by CD8+ T cells; a mechanism leading to autoimmunity<sup>122</sup>. Among the alternative translational mechanisms, the above described pioneer translation that takes place on pre-spliced mRNA and that generates PTPs<sup>82</sup> provides one explanation for the presence of peptides derived from intronic or intron-exon junction sequences. This model is based on a mRNA ribosomal scanning that happens earlier than the conventional described mechanism and that requires the eIF4E but not the eIF4G proteins.

Observation from separate studies that some MIPs are translated from non-conventional translation mechanisms participated to the questioning of whether these events were randomly or systematically occurring in eukaryotes. Studies were facilitated by the ribosome sequencing method, allowing large scale sequencing of mRNA fragments bound to the translational machinery and providing a signature of active translation<sup>123</sup>. Results from ribosome sequencing along with large scale analysis of the human proteome using mass spectrometry analysis<sup>124,125</sup> recently confirmed non-conventional translations as a common feature of eukaryotes<sup>126-128</sup>. Regarding the generation of MIPs, researchers wondered in what extend cells present peptides from alternative translation. A recent large scale study combining elution of MIPs from B-LCLs and customized database generated from six-frame translation of RNA sequencing data estimated that around 10% of the MIPs are generated from non-conventional translation<sup>129</sup>.

This last study together with the above cited independent experiments establish a new characteristic of the MIPs repertoire, which is to reflect all the coding possibility of the genome (much greater than the currently described exome). In addition, these overall results confirm the presence of complex translational regulation mechanisms, which may in part explain how cells elect peptides that will end up at the cell surface, and which provide insight into the definition of DRIPs and PTPs. Finally, they accentuate the need to refine and create suitable databases for more comprehensive definition of the MIPs either on different cell types or during transplantation, cancer development or viral infection.

## v. Nuclear translation

As discussed above, while many translational mechanisms can generate MIPs, the one favored in one particular situation for the generation of MHC-I source peptide is not known. Along with the requirement of specific RNA features, stimuli and components of the translational machineries, intracellular localization of translation can also be one key parameter for the generation and the selection of antigenic peptide precursors.

A few studies put forward a role for nuclear translation in the generation of MIP precursors. Brian P. Dolan *et al.* took advantage of their modified influenza A virus, which upon infection makes the cell express the neuraminidase (NA) mRNA along with the SIINFEKL coding sequence inserted into the NA stalk, to indirectly show distinct cellular locations for the process that generates the NA-SIINFEKL protein and the one that generates the precursor polypeptide of the K<sup>b</sup>-SIINFEKL detected at the cell surface. In fact, treatment with RNA polymerase II inhibitor DRB inhibits the nuclear export of the NA mRNA. While the NA-SIINFEKL expression was shown to be greatly impacted upon treatment, the K<sup>b</sup>-SIINFEKL expression at the cell surface was shown to be relatively much less affected, suggesting a role for nuclear translation in the MIP precursor generation<sup>130</sup>. As already mentioned, the PTPs model developed in HEK cells strongly supports nuclear translation. Along with the observation that the intronic-SIINFEKL sequence is translated in the AUG standard ORF and is expressed at the cell surface, S. Apcher *et al.* showed that the blockade of mRNA nuclear export leads to an increase in the expression of the intronically and exonically derived SIINFEKL peptide at the cell surface. Second evidence towards the generation of peptides in the nucleus came from the observation that splicing inhibition promotes the expression of the SIINFEKL antigen at the HEK cell surface. In addition, by combining the proximal-ligation assay with the ribopuromycylation (RPM) method, allowing actively translating ribosome localization and quantification by immunofluorescence<sup>131</sup>, they could observe nascent PTPs on ribosomes in the nuclear compartment<sup>82</sup>. While nuclear translation is not commonly recognized<sup>132</sup>, these two studies provide both evidence and a biological reason for it to happen. In addition, nuclear translation is supported by other authors such as A. David *et al.* who first developed the RPM method and observed nuclear translation in the nucleoplasm and the nucleolus<sup>133</sup>. The notion of compartmentalized antigen processing was shown in a VV-infection model where antigens derived from abundant full length protein and from DRIPs did not compete for cell surface presentation<sup>134</sup>. Although the compartments were not defined in

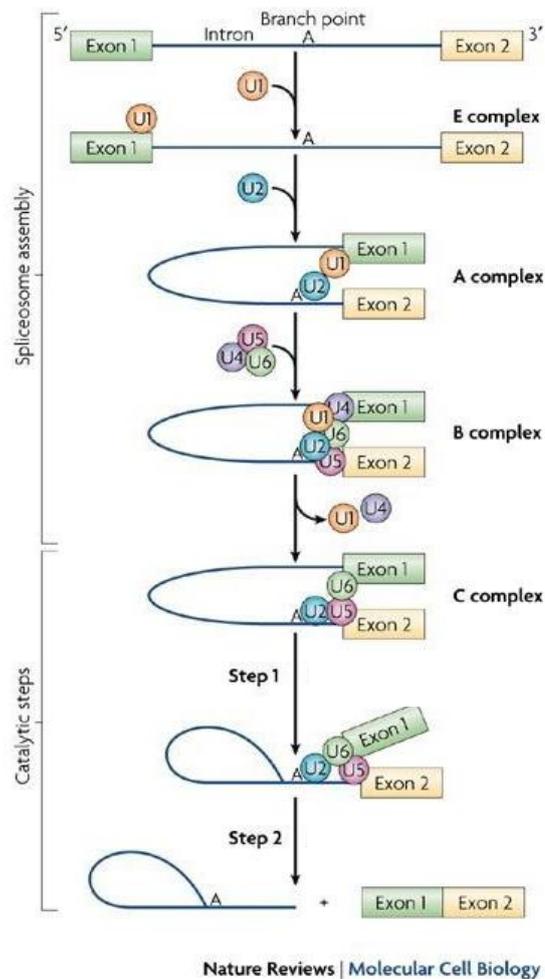
that study, authors discussed the possibility of a translational event directly coupled to nascent RNA chains, which suggests nuclear translation as a mean to select antigen precursors.

## vi. Splicing

We have just seen that gene expression is one important feature for MIP generation. While no clear links are currently demonstrated between MIP generation and splicing, the latter is one crucial mechanism for gene expression regulation, acting in concert with the NMD pathway<sup>135</sup>. Describing the PTPs model, S. Apcher *et al.* opened a narrow window on the splicing potential role in MIP generation by showing that intron and exon-derived SIINFEKL presentation at the HEK cell surface is positively impacted upon isoginkgetin splicing inhibitor treatment, supporting the role of pre-spliced mRNA translation at the source of MIPs<sup>82</sup>. We will here briefly introduce the splicing process and some splicing inhibitor molecules that provide potential tools for the MIP source study as well as for its modulation in diseases in which the splicing is impaired.

Splicing removes introns from pre-mRNA and assembles the protein coding exons into mature mRNA. This event is catalyzed by the spliceosome, a dynamic RNA-protein complex consisting of five small nuclear ribonucleoproteins (snRNPs U1, U2, U4, U5 and U6) and around 180 auxiliary proteins<sup>136</sup>. Many similar sites within a transcript exist, and the splice site selection process is complex and not completely known. Among the first events in the splicing process is the snRNP U1 recruitment to intron/exon junction 5' end splice site (5'ss). The snRNP U2 is recruited to the 3' splice site (3'ss) after the binding of several factors to the branch point sequence (BPS) and the polypyrimidine-rich tract, resulting in the pre-spliceosomal A complex formation. Its recruitment to the pre-mRNA is one of the key steps that trigger additional interactions, leading to the tri- U4/U5/U6 complex incorporation and forming the catalytic spliceosome. The latter then rearranges and proceeds to splicing (**Figure 7**). *Cis*-regulatory elements and *trans*-acting factors promote the spliceosome recruitment to the mRNA and participate in the splice site selection. Among splicing factors, the best characterized are serine-arginine (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind to intronic or exonic splicing enhancers (ISE or ESE) which promote the use of adjacent splice site, or to intronic or exonic splicing repressor (ISR or ESR) which have the opposite effect<sup>137</sup>. Their activity is often context-dependent and can vary depending on the binding factors and on the location they bind<sup>138,139</sup>. The dynamic recruitment of these proteins favor the selective usage of potential 5' and 3' splice sites, promoting alternative splicing

(AS). The latter can result from exon exclusion, intron retention, mutually exclusive exons or alternative 5'ss or 3'ss usage<sup>137</sup>. Hence, AS favors the formation of several mRNA isoforms from one pre-mRNA, potentially leading to different protein isoform formation that regulate many functions in the cell<sup>140,141</sup>. In addition, AS can also promote premature stop codon appearance that directs mRNAs to the NMD<sup>135</sup>.



**Figure 7: Spliceosome assembly and catalytic steps**<sup>142</sup>

Alteration of splicing is observed in diverse cancer types. While their overall role in tumor initiation and progression is still unclear, altered tumor suppressor or oncogenes have been identified as a result of aberrant splicing<sup>143,144</sup>. Hence, splicing emerged as a promising therapeutic target in cancer and several compound inhibiting the splicing were developed. For example, the SSA and the pladienolide B were shown to inhibit the SF3B spliceosome subunit, which is a component of the U2 snRNP, and to ultimately prevent the early spliceosome assembly<sup>145,146</sup>. Several derivatives of these compounds were synthesized for

their use in pre-clinical or clinical studies in cancer<sup>147,148</sup>. Besides, other less studied molecules such as the biflavonoid isoginkgetin and the madrasin molecules were shown to prevent the stable recruitment of the U4/U5/U6 tri-small nuclear ribonucleoprotein, resulting in accumulation of the prespliceosomal A complex<sup>149,150</sup>. As we will see later, our group provided evidence for a role of the splicing inhibitor isoginkgetin in PTPs-derived antigen presentation in cancer.

The intricate regulation of the MHC-I and –II antigen presentation reflects the overall high complexity of the immune response during the development of auto-immunity, infection or tumors as well as during thymic selection. Every new insight into PTPs and DRIPs nature as well as their mechanism of production, processing, binding and transport can provide new means to elaborate efficient therapies to support the immune system in its fight against diseases. In the next section, we will focus on the link between MHC-I antigen presentation, T-cell specific response and cancer, and on some anti-tumoral immunotherapeutic strategies currently under development.

## **2. MHC-I antigen presentation in cancer**

### **A - The anti-tumor immune response**

#### **a. Cancer immune surveillance and editing**

In order to conceptualize the complex mechanisms underlying tumors development, D. Hanahan and R. Weinberg published in 2000 the hallmarks of cancer, which described the six following biological processes: resistance to cell death, limitless replicative potential, self-sufficiency in growth signal, evasion from growth suppressors, induction of angiogenesis and activation of invasion and metastasis<sup>151</sup>. Our view of neoplastic disease however widened and two new crucial concepts emerged: reprogramming of energy metabolism and immune evasion<sup>152</sup>. This last new hallmark stated in 2011 reflects many years of research on the interactions between the tumor and the immune system (IS), and their essential role in the tumor fate.

In 1959, Lewis Thomas suggested that the IS maintains tissue homeostasis by preventing the growth of neoplastic cells. The idea that the immune system can target cancer cells has

evolved hand to hand with the concept of histocompatibility and doubts remained in the middle of the 20<sup>th</sup> century about whether tumor graft rejections were the result of mechanisms driving allograft rejection or tumor-specific rejection. With the use of inbred mice strains, it was observed that either chemically- or virally- induced tumors provoke immunological reactivity when injected into syngeneic mice, demonstrating a tumor-specific rejection<sup>153</sup>. Following these observations, Sir Macfarlane Burnet proposed in 1970 the immunosurveillance concept characterized by the immune system ability to detect and eliminate potentially dangerous tumor with no “clinical hints of its existence”<sup>154</sup>. Thierry Boon and colleagues first described tumor antigens in mouse tumor cell lines<sup>70,71</sup> and in melanoma patients<sup>115</sup>, and discussed their potential for immunotherapy development<sup>155</sup>. While the first immunodeficient mouse models failed to demonstrate immunosurveillance, the development of mice model lacking IFN- $\gamma$  responsiveness (STAT<sup>-/-</sup> or IFNGR1 receptor<sup>-/-</sup>), perforin (perforin<sup>-/-</sup>) or T, B and NKT cells (RAG 2<sup>-/-</sup> mice) showed enhanced susceptibility to methylcholanthrene-induced tumors or spontaneous tumors compared to wild-type mice<sup>156</sup>. In 2002, Gavin Dunn *et al.* extensively reviewed the studies demonstrating immunosurveillance and highlighted the fact that tumors transplanted into immunocompetent mice show different abilities to grow depending on the immune environment where they have first grown, which convey the idea of tumor immune selection. To explain the intricate host-tumor interactions they proposed the cancer immunoediting model, comprising three main processes called the 3Es: Elimination, Equilibrium and Escape<sup>156</sup> (**Figure 8**).

The elimination phase refers to the described immunosurveillance and corresponds to the elimination of nascent neoplastic cells by both the IS innate and adaptative branch<sup>157</sup>. In the equilibrium phase, the IS efficiently controls the proliferation of tumor cells without disease clinical appearance. It has been observed that equilibrium can occur over a very long period of time in mice, throughout most of their lifetime<sup>158</sup>. In some clinical cases in humans it was shown that immunosuppressed recipients of organ transplantation developed malignancies that originate from the foreign organ, suggesting that tumors cells were kept dormant by the immunocompetent donor<sup>159</sup>. While still unclear, some mechanisms participating in keeping tumor cells dormant have been observed such as the cytokines IFN- $\gamma$  and TNF secretion by T-helper 1 cells<sup>160,161</sup>, or the IL-23 positive role and the IL-12 negative one<sup>162</sup>. In addition, spontaneous metastasis dormancy in mice was shown to be dependent on T lymphocyte response<sup>163</sup>. During the equilibrium phase, the immune system can shape tumor immunogenicity and variants that have the potential to escape immune recognition can

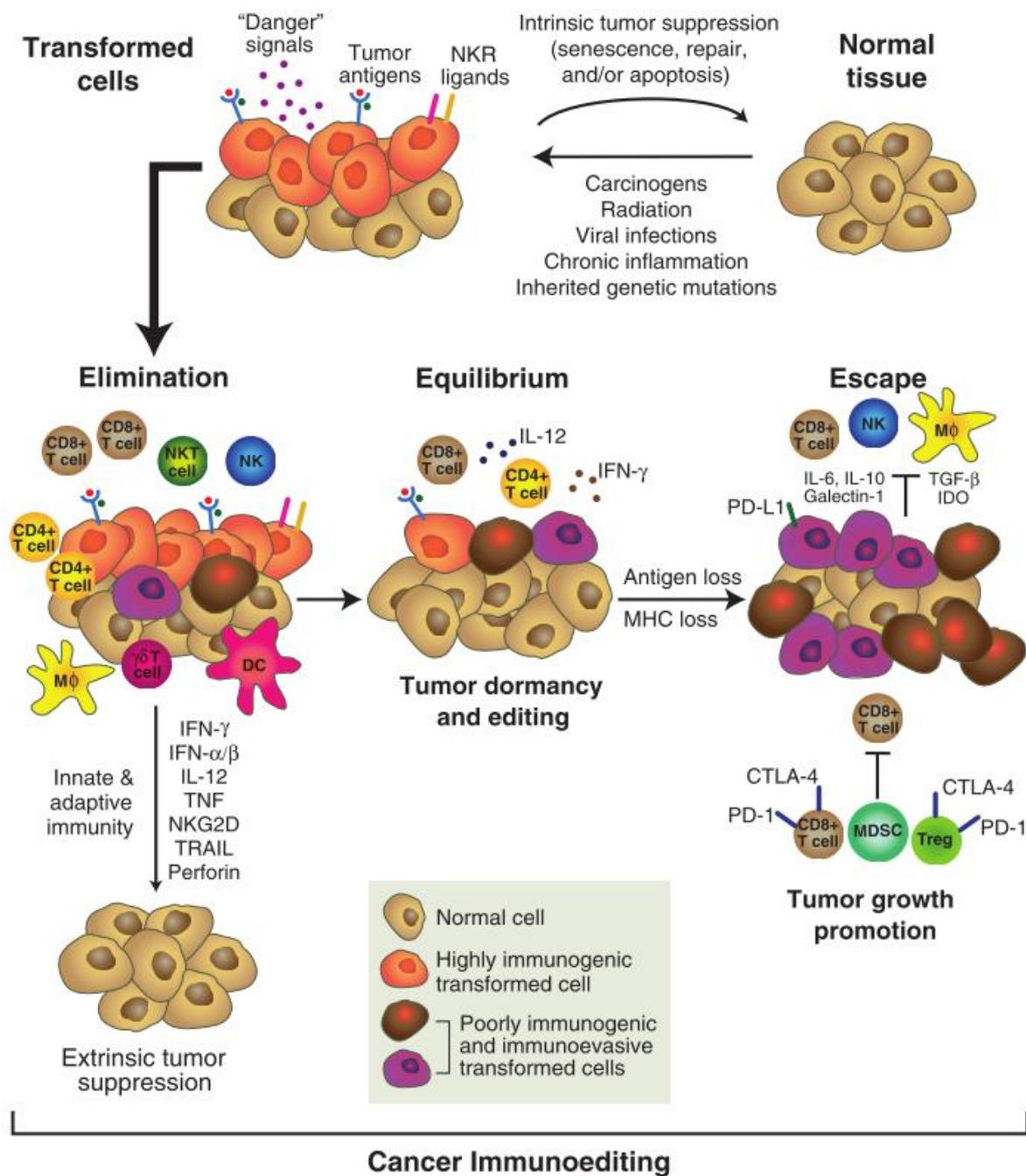
emerge. In the escape phase, edited cells with reduced immunogenicity fail to be controlled by the IS, extensively proliferate and become clinically apparent. Mechanisms that underline immune escape can be categorized as follow: reduced cancer cell immune recognition, increased resistance to cytotoxic effectors of immunity and established immunosuppressive microenvironment<sup>164</sup>. Tumor editing, however, does not end at the first clinical observations. The response observed after immunotherapeutic treatments such as checkpoint inhibitors or vaccines prove that even at disease late stage the tumor is still editable by the IS and can potentially be driven to equilibrium or elimination, as well as re-evade the immune control when patient relapse or when metastases appear.

From this model emerged the dual role of the immune system which can either prevent or favor tumor development<sup>165</sup>. It is often represented as a balance with pro-tumoral effects on one hand and anti-tumoral effects on the other hand, and the vast field of cancer-immunology strives to decipher the mechanisms that tilt the balance one way or the other.

#### **b. The major immune players: pro- or anti- tumor activity?**

The analyses of tumor microenvironment (TME) have provided important information on the immune cells involved in the pro- or the anti- tumoral response. Two main TME profiles have been observed<sup>166</sup>. The first one is a T-cell inflamed TME highly infiltrated by CD8<sup>+</sup> T effector cells, macrophages, some B cells and plasma cells, and in which gene expression profiling shows a specific signature for T cell activation-related chemokines<sup>167</sup>, APC activation markers and type I IFN<sup>168</sup>. The second is a non T-cell-inflamed TME that shows no spontaneous immune infiltration evidence and that generally does not respond to several immunotherapeutic treatments such as cancer vaccines, checkpoint blockade and adoptive T cell transfer<sup>169</sup>. In line with these observations, certain immune markers presence or absence in cancer patients has proven to have prognostic and predictive values for treatment outcomes and patient survival in several cancer types. Immune markers in cancer are therefore extensively studied in order to be able to state an accurate “immune profile” for each individual and adapt their treatment<sup>170</sup>. In addition, the mechanisms underlying those immune profiles can promote the development of innovative immunotherapies that could counteract non-inflamed TME or relapse and help to refine the current therapeutic strategies tested. In that section, we will briefly talk about the current state of knowledge on the immune cell role

in the pro- and anti-tumor response and inflammation, keeping in mind that stromal cells also play an important role in that process<sup>171</sup>.



**Figure 8: Cancer immunoediting<sup>172</sup>**

i. Innate immune response

The innate immune response is commonly characterized by its ability to react quickly upon threat, its non-specificity to a particular foreign element, and its indispensable role in

supporting the adaptive response. Innate immune cells can be derived from the lymphoid and the myeloid lineages and have an essential role in shaping the TME.

### Innate lymphoid cells (ILCs)

Natural killer (NK), natural killer T (NKT) and  $\gamma\delta$  T cells are the best described innate lymphoid cells (ILCs) found in various tumor microenvironments. ILCs are characterized by their ability to secrete and respond to a variety of cytokines through the numerous receptors they express. They differ from T and B cells of the adaptive immunity by their lack of recombination activating gene (RAG)-dependent rearranged antigen receptor, making their reaction non antigen-specific<sup>173</sup>. NK cell activity results in a balance between activating and inhibiting receptor stimulation. By recognizing cell surface stress ligands, NK cells can exercise a direct tumoricidal activity toward cancer cells. Conversely, receptors such as KIR or CD94–NKG2A prevent NK activation upon MHC-I molecule recognition. This mechanism prevents them from killing healthy cells and hence enables them to eliminate tumors that downregulate MHC-I expression<sup>174</sup>. Infiltration of NK cells in the TME in patients with colorectal carcinoma, gastric carcinoma or squamous cell lung cancers has been correlated with favorable prognosis<sup>175–177</sup>. In addition, their contribution to tumor growth control has been demonstrated in several mouse models<sup>178</sup>. NKT cells and  $\gamma\delta$  T cells can recognize tumors in a TCR-dependent manner and can also have cytolytic activity. NKT cells recognize tumor-derived glycolipids and  $\gamma\delta$  T cells recognize phosphoantigens or stress-induced molecules such as MHC-I chain-related protein A (MICA) or protein B (MICB)<sup>179</sup>. The invariant NKT subset has been demonstrated to control tumor growth during immune surveillance<sup>180,181</sup> whereas the II NKT subset has been suggested to be immunosuppressive<sup>182</sup>. Similarly,  $\gamma\delta$  T cells have been shown to have a positive role in the anti-tumor response in many cases but also to adopt a tumor growth promoting Treg profile ( $\gamma\delta$  Tregs) under certain circumstances<sup>178,179</sup>.

### Macrophages and neutrophils

In the myeloid lineage, tumor-associated macrophages (TAMs) as well as tumor-associated neutrophils (TANs) are polarized toward distinct phenotypes that can have anti- or pro- tumor effects. Two TAM phenotypes with opposite effects on tumor growth are commonly defined. The anti-tumor M1 macrophages are characterized by their IL-12<sup>high</sup> and IL-10<sup>low</sup> cytokine production and are able to attract and activate Th1 lymphocytes through MHC-II antigen presentation. On the other hand, pro-tumor M2 macrophages have an IL-12<sup>low</sup> and IL-10<sup>high</sup>

cytokine profile; they suppress anti-tumor T-cell response through different mechanisms and recruit Tregs<sup>183</sup>. TAM have mainly been found to have an M2 phenotype and have been associated to poor prognosis in cancer patients<sup>184-186</sup>. Several strategies to convert M2 macrophages into M1 macrophages in cancer have been tested such as addition of IFN- $\gamma$  at the tumor site, activation of TLR9 combined with anti-IL10 antibody treatment, overexpression of miR-155 or treatment with a low dose of anti-VEGFR2<sup>187-189</sup>. TAM and TAN have equivalent plasticity. It has been shown for example that transforming growth factor- $\beta$  (TGF- $\beta$ ) inhibition can drive the pro-tumorigenic N2 neutrophils phenotype to the anti-tumorigenic N1 phenotype that express immune stimulating factors and that can have a cytotoxic activity toward cancer cells<sup>183</sup>.

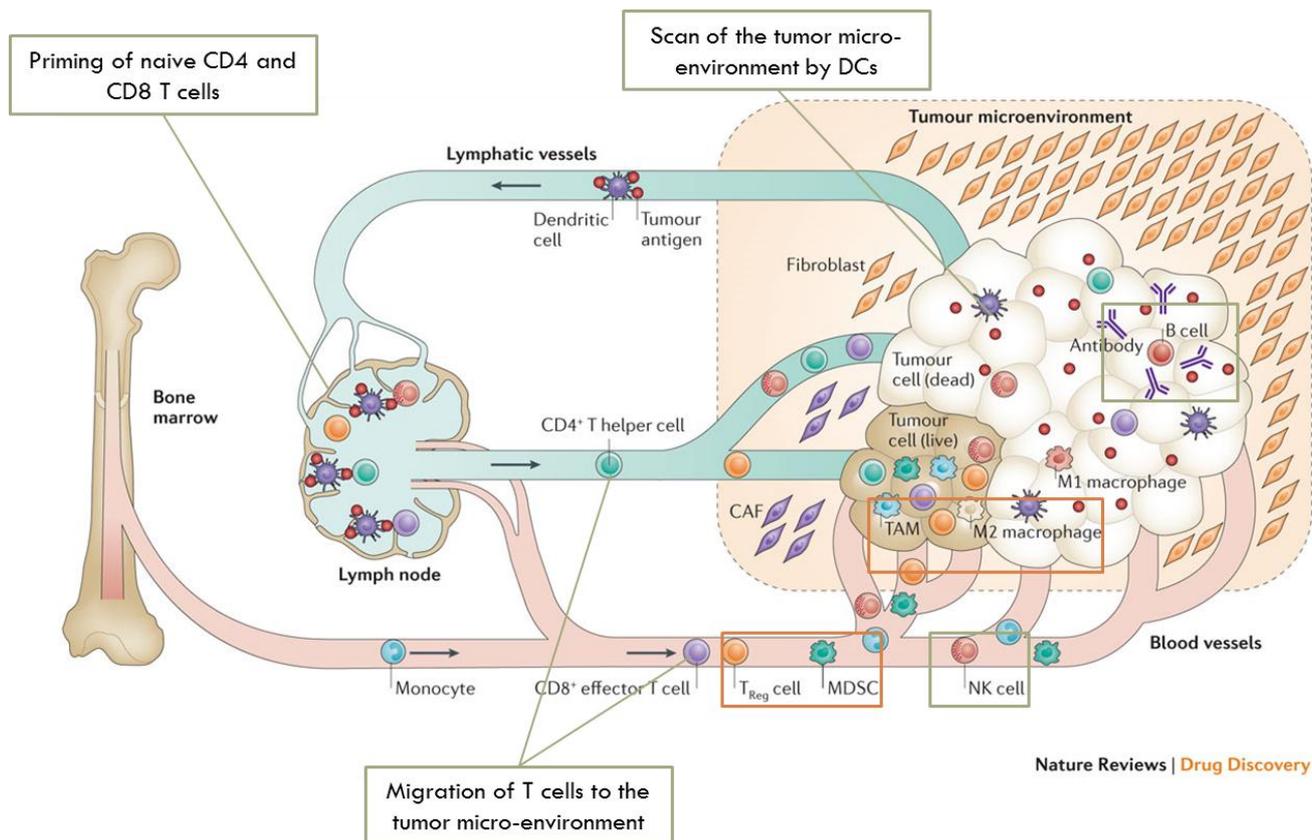
#### Myeloid derived suppressor cells (MDSCs) and mast cells

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature immune cells that have been shown to accumulate in cancer. In human patients, they have been identified accumulating in the peripheral blood and lymphoid tissues as a consequence of aberrant myelopoiesis and, as their name suggests, they are mostly immunosuppressive. In fact, MDSCs suppress T cell activation and cytotoxicity, induce the differentiation and expansion of Tregs, and inhibit NK cell activation<sup>190</sup>. They also promote angiogenesis and tumor-metastasis. Mast cells are granulated cells that form a population of white blood cells with diverse morphologies, surface receptor and mediator contents and are well described during allergy and in autoimmune diseases. Peritumoral and intratumoral mast cells have been observed in solid and hematologic tumors and in most cases show pro-tumorigenic action. However, again, mast cells action toward tumors is dual and depends on the stimuli they encounter. Interestingly, in melanoma, prostate or pancreatic cancers, mast cells appear to switch their activity depending on the stage of tumor initiation and growth, showing that their anti-tumor action can be reversed<sup>191</sup>.

#### Dendritic cells

As mentioned in the first part of the introduction, dendritic cells are professional antigen-presenting cells (pAPCs) that activate the adaptive immune response through antigen presentation, expression of co-stimulatory signals (CD80/CD86) and T cell proliferation and differentiation-inducing cytokine secretion. In addition, in some circumstances, they can modulate the innate immune cell response without T-cell activation. Many DC subsets have been described in mouse and in human and their role during anti-tumor response is under deep

investigation. Currently described steady state DCs subset are plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs have been shown to be specialized in the production and secretion of type I interferon and to have an overall reduced ability to cross-present antigens. In human breast and ovarian cancers, infiltration of pDCs to tumors has been associated with poor prognosis<sup>192-194</sup>. Tolerogenic pDCs in some cancer types have been observed with reduced capacity for type I IFN production partly due to tumor cell-derived TGF $\beta$  and TNF secretion, with reduced secretion of pro-inflammatory cytokines and chemokines in response to toll-like receptor (TLR) stimulation or with enhanced expression of inducible T cell co-stimulator ligand (ICOSL) favoring Treg cell expansion<sup>195</sup>. However, vaccination with pDCs loaded with tumor antigens in melanoma patients has been demonstrated to induce CD4+ and CD8+ T cell response<sup>196</sup>. In addition, *in vivo* immune response against established tumors induced by TLR-9 activated pDCs have been observed in mice and was shown to imply NK cell-mediated tumor cell lysis followed by cDCs activation and T-cell cross-priming<sup>197</sup>. Among cDCs are lymphoid tissue residents and migratory DCs. It was recently suggested that tumor-associated dendritic cells (TADCs) distinguish into 3 main subsets<sup>198</sup> which are: pre-cDC-derived cDC1s, pre-cDC-derived cDC2s and monocyte-derived Mo-DCs. cDC1s migratory CD103+ and resident CD8 $\alpha$ + DCs are dependent on IRF8, BATF3, and ID2 transcription factors and play a major role in CD8+ T cells priming<sup>199,200</sup>. In cancer, CD103+ DCs have been shown to traffic tumor antigens to the draining lymph node in a CCR-7 dependent manner in mouse melanoma models and this trafficking has been described as essential for T-cell priming<sup>201,202</sup> (**Figure 9**). In addition, the presence of a Baft3-dependent DC subset, referred as CD141 DCs in melanoma patients, correlates with a protective anti-melanoma response<sup>203</sup>. cDC2s differentiate into migratory or resident CD11b+ cells, depend on IRF4 and ZEB2 transcription factors and have been identified to display enhanced MHC II presentation and to play a dominant role in CD4+ T cells activation. However, anti-tumor role of these cells have not been clearly identified<sup>200</sup>. Despite the observation of efficient T-cell priming during cancer development, DC dysfunction is observed in most cancers and is thought to be induced by tumor progression and tumor-derived signals that polarize immunocompetent DCs into regulatory DCs, promoting cancer growth<sup>204-206</sup>.



**Figure 9: Dendritic cells in the lymph node and in the tumor microenvironment.**<sup>207</sup>

ii. Adaptive immune response

B cells

B cells are part of the humoral immunity, secreting antibodies and producing antigen-antibody complexes. They can also present antigens on MHC-II molecules after internalization by their surface immunoglobulins, express co-stimulatory molecules and secrete cytokines. B cells are a heterogeneous population and, such as many components of the immune system, can display pro- or anti-tumorigenic properties. When present in the TME, regulatory B (Breg) cells, which are composed of different subsets, promote tumor progression and have been suggested to be induced in response to inflammation from multiple B cells differentiation stages<sup>208</sup>. As an example of their mechanism of action, Breg secretion of IL-10 was shown to skew T cell differentiation in favor of the T regulatory phenotype and was found to suppress pro-inflammatory Th1 and Th17 cells when coupled with CD40-CD40L cognate interaction between Breg and CD4+ T cells<sup>209</sup>. Conversely, B cells in the TME can also be anti-tumorigenic and their action may lie in part in their ability to prime

CD8<sup>+</sup> T cells<sup>210</sup>. Besides, they were shown to display direct cytotoxicity to cancer cells through TRAIL/Apo-2L-dependent mechanisms upon CPG activation<sup>211</sup>. A favorable outcome of B cells tumor-infiltrating lymphocytes was observed in non-small cell lung carcinomas and in ovarian, breast and cervical cancers<sup>212,213</sup>.

## T cells

Subsets in the T cell population share the antigen-specific T-cell receptor (TCR) expression and a few key signaling molecules such as CD3. As previously mentioned, naive cytotoxic CD8<sup>+</sup> T cells and CD4<sup>+</sup> T helper cells are activated upon recognition of MHC-I and MHC-II antigens presented by pAPCs, respectively, and differentiate into effector or memory T cells. They display distinct phenotypes which were associated with pro- and/or anti- tumoral effects depending on their polarization, the tumor type in which they are studied and on how they are characterized<sup>214</sup>.

CD4<sup>+</sup> T helper (Th) cells exhibit various biological functions including the regulation of CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) and B cell-dependent antibody production, and the innate immunity component activation or inhibition. Th cells can differentiate into different subsets defined by their secreted cytokine and expressed transcription factor profile. The four best described subsets are Th1, Th2, Th17 and T regulatory (Treg) cells. The Th1 response mainly involves IL-2, which recruits and retains CTLs at the tumor site, IFN- $\gamma$ , which induces tumor cell MHC-I and MHC-II expression and stimulates reactive oxide/nitrogen species production by macrophages, and TNF- $\alpha$ . Th1 is therefore associated with anti-tumoral effects<sup>214-216</sup>. The Th2 cells secrete IL-4, IL-5, IL-10 and TGF- $\beta$ , and have been shown to exert a predominant pro-tumoral activity, however being able to promote cancer cytotoxicity through eosinophils recruitment to tumor<sup>215,217</sup>. Th17 cells express IL-17 and can differentiate into Th1 or Treg cells. They were reported in many human and mice TME but their role regarding tumor progression is ambiguous, partly accounted by their high plasticity<sup>218</sup>. CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> lymphocytes, also called Treg cells, exert potent immunosuppressive functions. They have been shown to reduce immunotherapy and tumor vaccine efficacy by suppressing antigen-specific T-cell activation and expansion<sup>219</sup>. Specific CD4<sup>+</sup> T cell subset have been reported to directly kill tumor cells through the recognition of MHC-II antigens presented on tumor cells<sup>220-223</sup>, emphasizing the multifaceted role of Th cells in the anti-tumor response.

Many studies have highlighted the major role of CD8<sup>+</sup> tumor infiltrating lymphocytes (TILs) in the anti-tumor response. A positive correlation between the frequency of CD8<sup>+</sup> TILs and cancer-free survival has been shown in patients with breast, lung, melanoma, colorectal, and brain cancer<sup>224-227</sup>. Compared to CD4<sup>+</sup> T cells, no CD8<sup>+</sup> T cells subsets have been described; however, different T cell fates have been identified and studied for their role in the anti-tumor response. T effectors (T<sub>EFF</sub>) express high levels of IFN- $\gamma$ , TNF- $\alpha$ , perforin, and granzymes, displaying cytotoxic activity upon antigen-specific recognition at the tumor cell surface<sup>228,229</sup>. In addition, T central memory (T<sub>CM</sub>) and T effector memory (T<sub>EM</sub>) cells have been shown to display antigen-specific anti-tumor activity in humans<sup>230,231</sup>. Nevertheless, the relative contribution of each of these cell types to the CD8<sup>+</sup> T cells anti-tumor response is unclear<sup>232</sup>. In addition, CD8<sup>+</sup> TILs often display dysfunctional properties *in vivo* that contribute to tumor evasion. Exhausted T cells have reduced cytotoxic activity, proliferative potential and low IFN- $\gamma$  expression and have been suggested to result from chronic antigen exposure and from an overall immunosuppressive microenvironment<sup>233</sup>. They express inhibitory receptors which are targeted by some immunotherapies, such as anti-PD1 or anti-CTLA4 antibodies, in order to reactivate cytotoxic activity<sup>234</sup>. In addition, immunosuppressive CD8<sup>+</sup> T regulatory cells, characterized by the loss of the co-stimulatory molecule CD28 and different from CD4<sup>+</sup>FOXP3<sup>+</sup> cells have also been described in tumors<sup>235,236</sup>.

CD8<sup>+</sup> T cell contribution in anti-tumor immunity will further be described in the following sections, with emphasis on MHC-I associated antigens recognized by CD8<sup>+</sup> T cells and the development of tumor vaccines.

## **B -The cancer immunopeptidome: dynamic and implication in the development of immunotherapies**

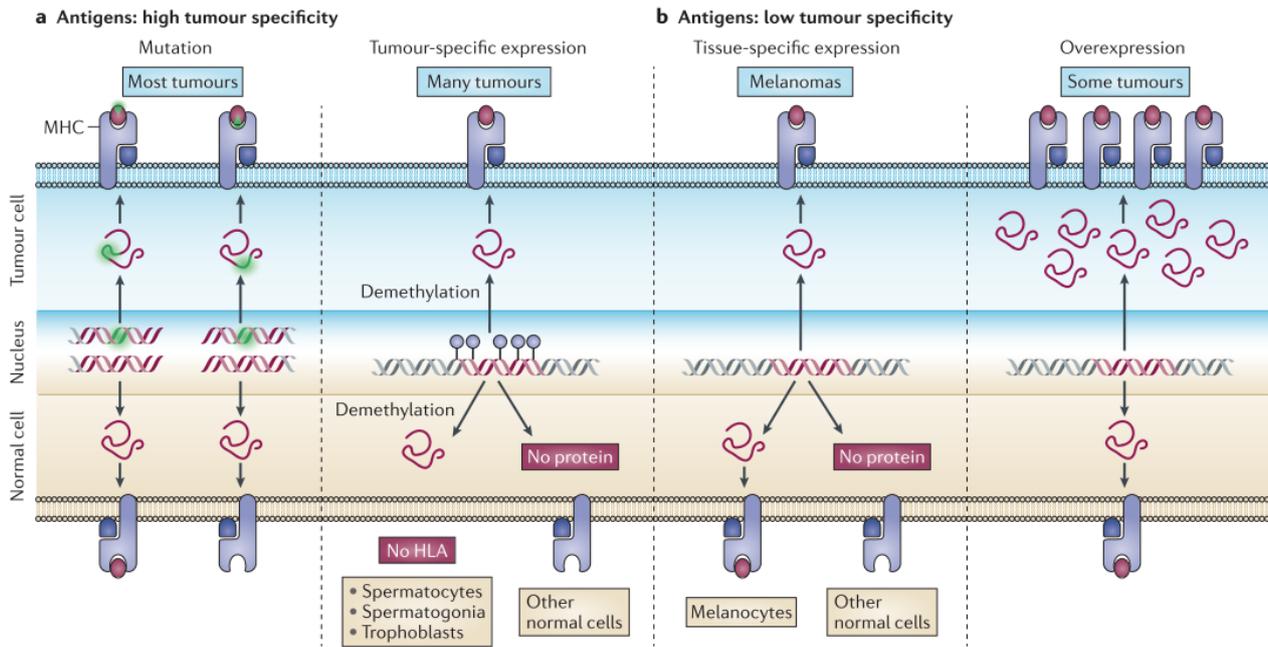
### **a. The tumor-associated immunopeptidome**

The cancer cell genome sequence, gene expression and metabolic and transcriptional states are highly modified in comparison to normal cells, deeply impacting the immunopeptidome. MHC-I peptides recognized by T-cells on cancer cells, called tumor antigens (TA), are the subject of extensive research. Their identification aims at developing passive immunotherapies with the use of TA-targeting antibodies or active immunotherapies with the use of TA-targeting therapeutic cancer vaccines. Since their discovery in the eighties, many TA have been identified. Pierre G. Coulie and colleagues proposed classifying them in the

two following categories: antigens of low tumoral specificity and antigens of high tumoral specificity<sup>237</sup> (**Figure 10**).

The first category includes antigens derived from differentiation or from overexpressed proteins. Differentiation antigens are either silent or expressed at a low level in specific tissues. Best characterized antigens from this category are expressed by melanoma cells and melanocytes, and include antigens derived from tyrosinase<sup>238,239</sup>, Melan-A<sup>240,241</sup> and GP100<sup>242</sup> genes and presented on HLA-A2 molecules. These antigens were identified through the establishment of CTL clones from melanoma patients' peripheral blood or tumor infiltrating T lymphocytes then screened against a cDNA library derived from autologous melanoma. In addition, prostate acid phosphatase (PAP) was identified as a differentiation antigen expressed in prostate cancer and was the first US and Europe approved cancer vaccine target<sup>243</sup>. In addition, overexpressed antigens in cancer also elicit CTL responses in patients. Known examples of antigens are hTERT and surviving expressed by almost all cancers, as well as P53 and HER2 overexpressed in multiple cancers but widely studied in breast cancer<sup>244</sup>.

The second category of antigens of high tumor specificity includes antigens from cancer-testis genes, mutated genes and viral antigens. Antigens from cancer-testis genes are expressed in a wide range of tumors and have been observed to be expressed in healthy tissues only in male-germline cells<sup>245</sup>. Several cancer-testis gene families located on the X chromosome have been identified such as MAGE, CAGE, CTAG and SSX<sup>237</sup>. These cancer-testis antigens along with differentiation and overexpressed antigens are also commonly called shared antigens. The latter category refers to their ability to be expressed by medullary thymic epithelial cells during the induction of central immunological tolerance. Central tolerance suppresses high-avidity T-cells recognizing those antigens<sup>246</sup> and could hence strongly impact the subsequent anti-tumor T cell response after vaccination<sup>247,248</sup>. Biased TCR usage or leaky central tolerance mechanisms have however been described; allowing an efficient response against one differentiation antigen<sup>249,250</sup>. Antigens specific to tumors have the strong advantage of eliciting a high-avidity T cell response. Cancers caused by virus or other pathogens infection present viral antigens that can be targeted in therapeutic as well as in prophylactic vaccines. For example, EBNA1 and LMP2 antigens from EBV are used as therapeutic vaccines in several EBV- induced malignancies<sup>251</sup> while antigens from the E6 and E7 oncoproteins from the human papilloma virus (HPV) were used as target in vaccine for HPV-induced premalignant diseases such as cervical intraepithelial neoplasia or vulvar intraepithelial neoplasia<sup>252</sup>.



**Figure 10: Class of human tumor antigens recognized by T lymphocytes<sup>237</sup>**

Antigens arising from cancer-related mutated sequences are called neoantigens. While point mutations that give rise to neoantigens were first described in the nineties, their contribution to the anti-tumor response were confirmed recently when analyzing response to checkpoint blockade therapies<sup>253</sup> or adoptive transfer of autologous TILs<sup>254</sup>. These studies took advantage of next generation sequencing, proteogenomic and bioinformatics technologies for neoantigen discovery. For example, a study combining whole exome sequencing with HLA binding prediction tools from melanoma patient cancer and normal cells allowed the selection of potential neoantigens expressed by the tumor and demonstrated that adoptively transferred TILs were reactive against a set of tested mutant epitopes<sup>255</sup>. Using a similar method with additional RNA sequencing, CD8+ T cells recognizing neoantigens in two glioblastoma mouse models were identified<sup>256</sup>. Recently, CD4+ T helper cells have been shown to recognize an epithelial cancer specific mutation in one patient and to reduce tumor growth when they are adoptively transferred<sup>257</sup>, emphasizing the role of MHC-II antigens in cancer. Commonly shared neoantigens such as those derived from RAS, P53 or EGFR mutations, or BCR-ABL fusion are driver mutations and were use as target in several vaccines, but are however rare<sup>258</sup>. Mainly, neoantigens presented by tumors are different between patients, highlighting the need for the development of individualized cancer vaccination<sup>258</sup>.

## b. Immunopeptidome dynamic during immunoediting

The role of TAs in the process of immunoediting remained for a long time unclear. Previously cited experiments in immunodeficient mice revealed that T-cell activity is deeply involved in immunoediting. The identification of neoantigens and associated technologies played an important role in the understanding of these mechanisms and it was only recently demonstrated that non-edited tumors present TAs driving immunoediting. Using MCA-induced tumor in Rag<sup>-/-</sup> mice, which resemble highly immunogenic non-edited tumors, H. Matsushita *et al.* could identify one “rejection” antigen by combining the identification of tumor-specific mutations from exome sequencing with MHC-I binding prediction algorithms<sup>259</sup>. When a mixture of cells expressing for the majority the rejection spectrin-β2 mutant antigen was injected into immunocompetent mice, they showed that T-cell response against this antigen leads to the outgrowth of tumor variants lacking it, demonstrating a T-cell dependent immunoediting mechanism. Similar observations were made on non-edited oncogen-driven tumors genetically engineered to express two immunogenic antigens, where they observed a strong CD8+ T cells response specific to these antigens early during tumor development (less than 14 days), which induced the outgrowth of cells that either do not express the potent antigens or that have reduced H2-k<sup>b</sup> expression corresponding to the haplotype on which studied antigens are expressed<sup>260</sup>. Resembling the immunoediting mechanisms observed in mice tumor models, the reappearance of tumors with a loss of specific antigens has been shown in some patients along the course of immunotherapeutic treatments. For example, one analysis of a melanoma patient with a NY-ESO-1 positive tumor and vaccinated with a vaccinia/fowlpox NY-ESO-1 followed by NY-ESO-1 protein + CpG showed that progressing lesions as well as brain metastasis that appeared after treatment were NY-ESO-1 negative and MHC-I positive<sup>261</sup>. Another recent example came from the study of some identified neoantigens dynamically presented on MHC-I molecules and recognized by T-cells in two melanoma patients treated with autologous adoptive cell transfer (ACT)<sup>262</sup>. In sequential tumor lesions they observed the loss of some mutant alleles coding for neoantigens and changes in the expression of some neoantigen-coding RNAs, both corresponding with changes in neoantigen-specific T-cells in the tumor micro-environment. This suggests that T cells exert pressure under the disappearing or appearing of tumor variants with different neoantigen-expressing cells. Consistent with this model that highlights the role of neoantigens in the immunoediting mechanism, another study that followed patients with non-small cell lung cancer treated with checkpoint blockade inhibitors PDL-1 and CTLA-4

together or alone showed the emergence of tumor subclones that have lost the expression of identified neoantigens after treatment. The latter were recognized by T-cells present in the peripheral circulation. The loss of specific subclones or the deletion of chromosomal regions was identified as the mechanisms of immunogenic antigen expression loss<sup>263</sup>.

Hence, immunoediting as an immune escape mechanism during immunotherapy treatments was emphasized in human and changes in the tumor immunopeptidome appeared to have a central role in that process. Besides, these findings highlight the importance of targeting several TAs in cancer vaccines in order to reduce the likelihood of subclone outgrowth and relapse. In addition, based on the importance of immunoediting as well as the relationship between mutational burden and response to immune checkpoint blockade, neoantigen presentation seem to be a major determinant in the IS ability to control tumor growth.

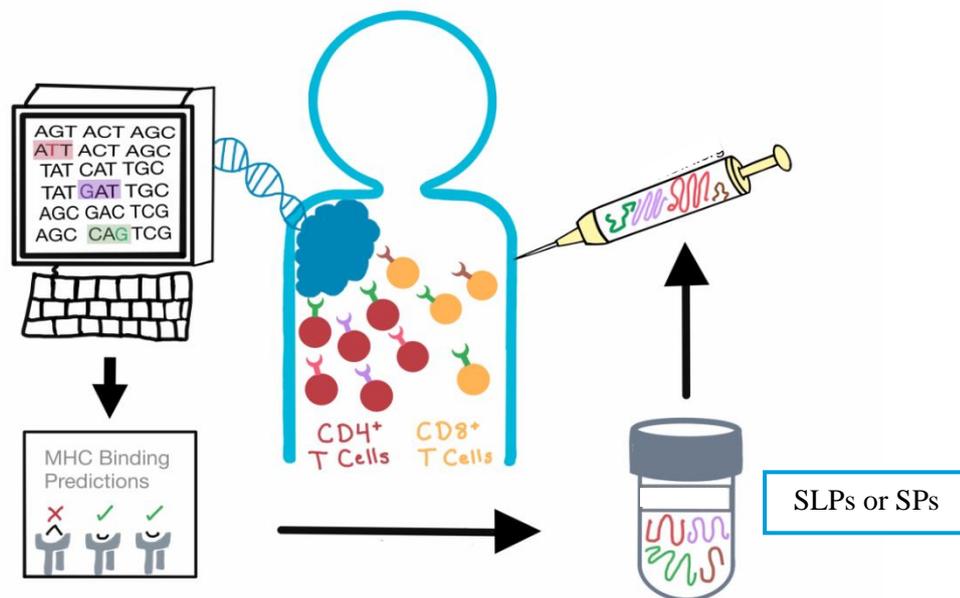
### **c. Therapeutic cancer vaccines**

The discovery of TA pivotal role and associated T-cell anti-tumor response led to the development of active immunotherapies, which were developed in various ways: DNA/RNA-encoding TA, TA-loaded dendritic cells, lysate of tumor and synthetic peptides encompassing TA vaccines. While each approach has specific advantages and drawbacks, the common goal of these vaccines is to prime effector cells to seek and destroy cancer cells.

Among the different strategies, synthetic peptides provide the advantage of being easy to synthesize, cost-effective, flexible in the choice of antigens and of having low risk of inducing anaphylaxis<sup>264</sup>. While many clinical studies on synthetic peptides as anticancer vaccines are currently performed, some studies targeting the MAGE-A3 antigen in melanoma<sup>265</sup> and lung cancer<sup>266</sup> or a telomerase antigen in pancreatic cancer have been stopped in phase III after showing a lack of efficacy<sup>267</sup>. The main reasons for those failures have been attributed to a poor understanding of the immunization process<sup>268</sup>, to immunosuppressive mechanisms in high tumor burden<sup>269,270</sup> and to suboptimal antigen choice. These observations are in line with the above cited studies that converge toward the pro-tumorigenic polarization of immune and adaptive cells, the loss of immunogenic antigen variants at late stages of tumor progression and to the observation that Th- and CTL-specific neoantigen expression drives high-avidity T-cell response<sup>260,261,263</sup>.

In line with that, new strategies focus on the study of adjuvants and vaccines co-treatments that efficiently trigger T cell priming and tumor infiltration while shutting down pro-tumorigenic immune response and signals<sup>271-274</sup>.

Moreover, efforts are directed toward the intrinsic formulation of the administered synthetic peptides by optimizing their size and the number and quality of epitopes included. The exact minimal peptide-epitope recognized by CD8 T cells, also known as short peptides (SPs), were the first type of peptide vaccines used in clinical trials. However, their relative efficacy compare to synthetic long peptides (SLPs), which comprise different epitope antigens in one single peptide, is a matter of debate. In fact, SPs were shown to be uptaken and presented by pAPCs that induce a suboptimal priming of CTL, such as B cells or tissue resident DCs, in non-inflamed lymphoid organs or at the vaccination site and could therefore trigger exhaustion or deletion of antigen-specific T-cells<sup>275,276</sup>. Conversely, both human plasmacytoid and monocyte-derived DCs were shown to efficiently cross-present SLPs *in vitro*, better than whole protein, while T and B cells were inefficient, supporting the use of SLPs in tumor vaccines<sup>277–279</sup>. Nowadays, vaccine strategies include several antigens in order to overcome intratumoral heterogeneity and immune evasion through the outgrowth of antigen loss variants (ALVs)<sup>258</sup>. In addition, a significant amount of SLP vaccines containing CD8+ and/or CD4+ specific TA-neoantigens are currently developed<sup>280,281</sup> (Figure 11).



**Figure 11: Personalized peptide vaccines** (from acir.org)

#### **d. Defects in MHC-I expression as a cancer immune escape mechanism**

As previously mentioned, the loss or the downregulation of a specific antigen presentation<sup>261,282</sup> and of overall or haplotype-specific MHC I presentation<sup>283–285</sup> is observed in cancer as tumor escape mechanisms from T lymphocytes response. Impaired MHC-I antigen presentation results in part from genetic and epigenetic modifications, as well as from transcriptional, translational or post-translational mechanisms. In fact, some MHC haplotypes and many components of the processing machinery (e.g. LMP2, 7 and 10, or ERAP, etc.) and of the peptide loading machinery (e.g. TAP,  $\beta$ 2m, etc.) display altered expression due to loss of heterozygosity, gene mutations, gene methylation or IFN- $\gamma$  signal transduction-pathway defects<sup>286,287</sup>. These defects were classified into reversible (“soft”) or irreversible (“hard”) when they are structural<sup>288</sup>. Several strategies are developed to counteract the appearance of these defects with the ultimate goal to potentiate the efficacy of immunotherapies such as vaccines or checkpoint blockade therapies.

Type I IFN (IFN- $\alpha$  and IFN- $\beta$ ) and type II IFN (IFN- $\gamma$ ) promote the expression of many components of the MHC-I pathway via transcriptional activation and has been shown to restore MHC-I presentation in human cancer cell lines with soft lesions<sup>289</sup>. IFN- $\alpha$  is used as a therapeutic agent in several types of cancer including melanoma, in which it enhances tumor cell production of TAP<sup>290</sup> and improves survival<sup>291</sup>. Moreover, it was observed in a mouse melanoma model containing both MHC-deficient and MHC<sup>high</sup> expressing cells that transferred CD8+ T cells targeting MHC<sup>high</sup> cells lead to the regression of both cell types through IFN- $\gamma$ -related rescue of MHC expression in deficient melanoma cells<sup>292</sup>. This experiment echoes the phenomenon of epitope spreading. The latter was for example described in a study of therapeutic vaccination in melanoma where tumor regression was driven by CTL clones targeting antigens beyond the scope of the initial vaccine target. Authors suggested that this observation was due to local production of IFN shaping the tumor antigens repertoire<sup>293</sup>. However, in several cancers, resistance to IFN signaling has been observed<sup>294,295</sup>.

Epigenetic modulation using histone deacetylase inhibitors such as trichostatin A and valproic acid have also been described as a strategy to restore some of the antigen presentation machinery component expression<sup>296</sup>. Moreover, gene therapy using viral vector replacing MHC-I related genes with “hard” lesions in cancer cells are also developed<sup>297</sup>. Interestingly,

study of the impact of chemotherapies on different human cancer cell lines revealed that gemcitabine is able to increase class-I molecule expression at the cell surface, which was also correlated with increased T-cell toxicity<sup>298</sup>. In addition, gemcitabine was shown to enhance the WT1 mRNA expression in human pancreatic cancer, resulting in enhanced WT1 antigen expression at the cell surface and sensitivity to CTL cytotoxic effects<sup>299</sup>.

Finally, defects in TAP are correlated with the MHC-I surface expression decrease and fundamental immunopeptidome alteration, leading to the appearance of antigens named T cell epitopes associated with impaired peptide processing (TEIPP)<sup>300</sup>. These TEIPP are generated from TAP- and proteasome-independent processing pathway and were observed not to be subjected to central tolerance<sup>301,302</sup>. In addition, TEIPP-specific T cells were shown to control a mouse model of TA-deficient tumor. Therefore, they emerge as a potential target for tumor immune escape variants.

While the mechanism of tumor escape through MHC-I antigen presentation needs to be further investigated, these studies demonstrate that the modulation of antigen presentation-related gene expression by different strategies can impact both the overall level of MHC-I expression and the MIP repertoire, resulting in reduced or enhanced tumor recognition.

## PhD objectives

Our growing understanding of the interactions between immune cells and cancer cells highlights tumor immune escape mechanisms as a major cause of tumor resistance to immunotherapeutic treatments and relapse. Key components of these intercellular interactions are antigens displayed at the cancer cell and at the antigen presenting cell surface and recognized by T lymphocytes. During tumor growth, the pool of presented antigens is greatly impacted and evolves rapidly, allowing tumor cells to avoid immune recognition and killing. Antigen presentation relies on extracellular stimuli as well as intracellular events, which produce, shape and select elements that will end up at the cell membrane. New insights into those events emphasized the products of alternative translations as a source of antigens, strongly enlarging the potential pool of presented peptides. PTPs are produced from the alternative translation of pre-mRNAs, enabling the presentation of antigen from theoretically ‘non-coding’ sequences. However, their role during the natural anti-cancer immune response is not yet known. Our team objectives are to study the relevance of these PTPs during immune cell activation and recognition of cancer cells and define the mechanisms by which they are produced and processed. During my PhD, I had the opportunity to work on three different projects fitting these goals.

My main project was based on the study of the MHC-I direct presentation of a PTPs-derived antigen model in several human and mouse cancer cell lines and of different molecule impact on this presentation. The ultimate goal was to find key cellular processes for the presentation of PTPs-derived antigens in cancer. During this research, we identified several splicing inhibitors positively impacting PTPs-derived antigen MHC-I presentation in cancer cells and highlighted the role of the splicing event in the anti-tumor immune response. Our findings led us to patent the IP2 splicing inhibitor for its ability to reduce mouse tumor growth in an immune-dependent manner. In addition, we will soon submit the paper presented in the next section: “Article: Splicing inhibition modulates MHC-I antigen presentation and induces anti-tumoral immune response”.

My second project was based on the work of Duvallet E. *et al.* who showed that PTPs expressed by tumor are efficiently cross-presented by pAPCs *in vitro* and *in vivo*, leading to the activation of CD8+ T cells<sup>303</sup>. After working on the purification of model PTPs and the development of a tumor-associated PTPs-based vaccine in mice with the sarcoma model MCA205, we tested this vaccine in the B16F10 melanoma model. We showed that a prophylactic model-PTPs-based vaccine can efficiently trigger anti-cancer immune response

in mice in both MCA205 and B16F10 models and patented the use of PTPs as vaccine. Moreover, we identified the potential role of melanosomes secreted by melanoma cells in PTPs cross-presentation by pAPCs. The results on PTPs-vaccine and PTPs-carrying melanosome vaccine in the B16F10 model are presented below, in the section “Proof of concept: PTPs elicit an anti-tumor immune response *in vivo*”.

Finally, I participated in the project led by Mathilde Boulpicante whose goal was to study the processing of PTPs. We identified one key negative regulator of PTPs-derived antigens presentation: the proteasome regulator REG $\gamma$ . The latter was observed to be upregulated in cancer and to induce the complete degradation of PTPs by the proteasome in the nucleus, preventing PTPs-derived antigen expression at the cell surface. The submitted paper is presented in Annexe 1- Tumors escape immunosurveillance by overexpressing a regulator of the proteasome: REG $\gamma$ .

# Article: Splicing inhibition modulates MHC-I antigen presentation and induces anti-tumoral immune response.

## Splicing inhibition modulates MHC-I antigen presentation and induces anti-tumoral immune response

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### Abstract

Tumor antigens (TA) presented on MHC-I molecules in tumors are crucial in eliciting an immunoprotective response and in the success of cancer immunotherapies but are often lost or downregulated during cancer cell immune escape. Alternative translations emerged as a rich source of TA and pioneer translation products (PTPs) are peptides produced from such a translation occurring on pre-spliced mRNA in the nucleus. We thus screened different splicing inhibitors for their ability to positively modulate TA-PTPs-derived antigen presentation in human and mouse cancer cell lines *in vitro* and identified the isoginkgetin molecule. We demonstrated that isoginkgetin acts at the PTPs production stage. We further provided the water-soluble and less toxic IP2 molecule derived from the isoginkgetin and showed that it increases PTPs-derived antigen presentation of cancer cells *in vitro*. In addition, we observed that it reduces the MCA205 mouse sarcoma and B16F10 mouse melanoma tumor growth *in vivo* in a CTL-dependent manner. Furthermore IP2 treatment creates a long-lasting anti-tumoral response. Hence we describe a new efficient “first in class” immunomodulator of the antitumor response. These findings add to the understanding of the

role of the splicing machinery in antigen production and presentation and identify the spliceosome as a druggable target for improving MHC class I immune response.

## **Introduction**

All nucleated cells present antigenic peptides (APs) at their surface through the major histocompatibility complex class I (MHC-I) pathway. APs guide CD8 cytotoxic T cells (CTL) and CD4 T helper cells surveillance of pathogenic elements and are the targets of therapeutic anti-cancer vaccines currently developed. Initial clinical trial results with therapeutic vaccines targeting tumor antigens (TA) haven't met expectations. Main failures have been associated to immunosuppressive mechanisms and to a suboptimal antigen choice<sup>304,305</sup>. One of the important events driving tumors immunoselection and correlated to poor prognosis is the tumor cell MHC-I antigen presentation loss or downregulation<sup>306,307</sup> due to extracellular stimuli or defects in some components of the pathway<sup>286</sup>. Moreover, along with the overall MHC-I antigen presentation decrease, the nature of antigens presented at the cell surface, namely the MHC-I immunopeptidome (MIP), is of critical importance for immune recognition. Immunoediting during cancer development or immunotherapeutic treatment was in several clinical cases shown to induce T-cell targeted TA specific loss in outgrowing tumors during relapse or metastasis appearance<sup>261,308,309,282</sup>. Therefore, current immunotherapeutic strategies aim at enlarging the range of targeted cancer peptides and restoring MHC-I antigen presentation.

The MIP is dynamic, flexible and reflects the cell metabolism and transcriptional regulations as well as the cell gene expression and genetic polymorphism. These features might in part be explained by the variety of peptide sources for the MHC-I pathway, currently classified in two categories, the retiree proteins and defective ribosomal products (DRiPs)<sup>310</sup>, which include products of alternative translation<sup>77,311</sup>. Our group identified the presence of a pioneer translation of precursors-mRNAs (pre-mRNAs) at the source of APs, occurring before introns are spliced, independently on the full length protein translation<sup>80</sup> and giving rise to pioneer translation products (PTPs). PTPs can therefore theoretically be derived from intronic sequence, 3' or 5' UTR regions as well as alternative reading frames and seem to play a role in the dynamic of cancer development. In fact, we showed that cancer cell presenting PTPs-derived antigen can be recognized by specific T-cells when inoculated in mice, leading to tumor growth reduction. Besides, purified PTPs containing a model epitope efficiently

promote anti-cancer immune response when injected as a peptide vaccine in mice<sup>303</sup>. In cancer patients, some antigens produced from intronic or other “un-translated” region were observed<sup>115-117</sup>.

PTP discovery emphasizes the role of splicing in AP generation. Pre-mRNA splicing is catalyzed in the nucleus by the spliceosome, a conserved and dynamic multi-protein complex composed of five small nuclear RNAs (snRNAs) interacting with over 180 proteins. A growing number of studies report that spliceosome deregulations entail aberrant splicing patterns in many cancers contributing to abnormal tumor cell proliferation and progression, and recurrent spliceosome mutations have been reported in several cancers. In recent years several microbial natural products and their synthetic analogues have been reported to inhibit the spliceosome, including pladienolides B and D, spliceostatin A, FR901464, E7107, isoginkgetin and madrasin. Beyond their reported cytotoxic activities, splicing inhibitor have been of particular interest for us because of their potential impact on PTPs production and AP presentation<sup>144</sup>.

Here we show that the biflavonoid isoginkgetin and its derivative IP2 enhance the presentation of a PTPs-derived antigen expressed in human and mouse cancer cell lines. In addition, a boost of the anti-cancer response and a long-lasting response *in vivo* were achieved upon IP2 treatment. Our work proposes a new mechanism of action of these splicing inhibitors that modulate the MIP at the cancer cell surface by enhancing the presentation of PTP-derived antigens and potentiate the anti-tumoral immune response.

## **Material and methods**

### **Cell culture, transfection, plasmids and drugs**

MCA205 mouse sarcoma cell line is cultured at 37°C under 5% CO<sub>2</sub> in RPMI 1640 medium (Life Technologies) in the presence of 1% glutamine, 1% sodium pyruvate, 1% non-essential amino-acids, 1% penicillin/streptomycin and 10% FBS (Life Technologies) under standard conditions. B16F10 mouse melanoma cell line, MRC5 human fibroblast cell line and A375 human melanoma cell line are cultured at 37°C under 5% CO<sub>2</sub> in DMEM medium (Life Technologies) containing 1% glutamine, 1% penicillin/streptomycin and 10% FBS. A549 human lung carcinoma cell line is cultured under standard conditions in DMEM/F12 + Glutamax I in the presence of 1% HEPES, 1% sodium pyruvate and 10% FBS. Stable MCA205-Globin-SL8-intron and B16F10-Globin-SL8-intron cell lines are cultured under the same condition as MCA205 and B16F10 cell lines respectively, with additional 2mg/ml G418 (Life Technologies) for selection. Stable MCA205-Ovalbumin and B16F10-Ovalbumin cell lines are cultured under the same condition as MCA205 and B16F10 cell lines respectively, with additional 0.2µg/ml zeocin (Life Technologies) for selection. The SL8/H-2K<sup>b</sup>-specific (B3Z) T-cell reporter hybridoma are cultured at 37°C under 5% CO<sub>2</sub> in RPMI 1640 medium in the presence of 1% glutamine, 0.1% β-galactosidase, 1% penicillin/streptomycin and 10% FBS under standard conditions. MCA205, A375, A549 and MRC5 cell lines are transfected with the reagent jetPRIME (Ozyme) according to the manufacturer protocol. B16F10 cell line is transfected with the reagent GeneJuice (Millipore) according to the manufacturer protocol. The plasmids YFP-Globin-SL8-intron, YFP-Globin-SL8-exon and pCDNA3-Ova have been described previously<sup>82</sup>. The pre-mRNA splicing inhibitor isoginkgetin from Merck Millipore and the madrasin from Sigma-Aldrich are dissolved in DMSO according to the manufacturer protocol. IP2 and M2P2 were synthesized from the isoginkgetin powder as described in the patent and dissolved in PBS.

### **T-cell assay**

MCA205 and B16F10 mouse cell lines are transfected with 1µg per well of the plasmid YFP-Globin-SL8-intron, YFP-Globin-SL8-exon, pCDNA3-Ova or pCDNA3 empty plasmid (negative control) for 24h. A375, A549 and MRC5 human cell lines are transfected with the plasmid encoding mouse H-2K<sup>b</sup> molecule for 12 hours followed by the transfection of the plasmid YFP-Globin-SL8-intron for 24 hours. Cells are treated with different doses of drugs

overnight. Then cells are washed three times with the RPMI 1640 medium of B3Z cells and  $5 \times 10^4$  cells are co-cultured with  $1 \times 10^5$  B3Z cells. Each condition is performed in three technical replicates. In positive control wells,  $4 \mu\text{g/ml}$  of synthetic peptide SL8 is added. Cells are then incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  overnight. Cells are centrifuged at 1200rpm for 5min, washed twice with PBS 1X and lysed for 5min at  $4^\circ\text{C}$  under shaking with the following lysis buffer: 0.2% TritonX-100, 10mM DTT, 90 mM  $\text{K}_2\text{HPO}_4$ , 8,5 mM  $\text{KH}_2\text{PO}_4$ . The lysate is centrifuged at 3000rpm for 10min and the supernatant is transferred to a 96-well optiplate (Packard Bioscience, Randburg, SA). The revelation buffer containing  $38 \mu\text{M}$  of methylumbellifery  $\beta$ -D-galactopyranoside (MUG) is added and the plate is incubated at room temperature for 3 hours. Finally, the  $\beta$ -galactosidase activity is measured using the FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany). Results are obtained in relative light units (RLU). Background RLU from negative control is subtracted and results are divided by the RLU of the positive control (corresponding to the overall H-2K<sup>b</sup> expression). The untreated condition is standardized to 1 in order to show the SL8/H-2K<sup>b</sup> expression fold change. Results are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (unpaired student t test).

## **FACS Analysis**

### **H-2Kb expression at the cell surface**

Mouse cells are treated with drugs for 18hours. Human cells are transfected with the plasmid encoding the mouse H-2K<sup>b</sup> molecule for 24 hours, followed by 18hours treatment with drugs. Cells are harvested and  $2 \times 10^6$  human cells are stimulated with  $5 \mu\text{g}$  SIINFEKL synthetic peptide for 15min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . Human and murine cell lines are incubated with human or mouse FcR block respectively for 10min at  $4^\circ\text{C}$  according to the manufacturer protocol (Miltenyi Biotec). Mouse cells are stained with anti-mouse MHC class I (H-2K<sup>b</sup>)-PerCP-eFluor®710 (AF6-88.5.5.3, eBioscience) or with the corresponding mouse IgG2a K isotype control-PerCP eFluor®710 (eBM2a, eBioscience) for 30min at  $4^\circ\text{C}$ . Human cells are stained with anti-mouse OVA257-264 (SIINFEKL) peptide bound to H-2K<sup>b</sup>-APC (25-D1.16, eBioscience) for 30min at  $4^\circ\text{C}$ . Cells are stained with DAPI prior to acquisition for dead cell exclusion. In mouse cell lines the isotype antibody is used for PerCP eFluor®710-positive gating while in human cell lines non-stimulated cells are used for APC-positive gating. Cells are analyzed using the BD LSRII flow cytometer (BD Biosciences) and data are analyzed

with the FlowJow software (V10). Results are expressed as mean  $\pm$  SD. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test).

### **Annexin V staining**

Mouse cells are treated with drugs for 18hours. Floating and adherent cells are collected, washed in PBS and resuspended in 1X Annexin V Binding Buffer with APC Annexin V (BD Pharmingen) according to the manufacturer protocol. Cells are gently vortexed and incubated 15 min at RT in the dark. Cells are stained with DAPI prior to flow cytometry analysis. Cells are analyzed using the BD LSRII flow cytometer (BD Biosciences) and data are analyzed with the FlowJow software (V10). Results are expressed as mean  $\pm$  SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test).

### **RNA preparation, RT and qPCR**

Cells are transfected with the YFP-Globin-SL8-intron plasmid for 24 hours followed by treatment with drugs for 48 hours. Cells are then harvested and total cellular RNA is extracted and purified using the RNeasy Mini kit (Qiagen) according to the manufacturer protocol. The reverse transcription is carried out with 0.5  $\mu$ g of RNA with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer protocol. The StepOne real-time PCR system (Applied BioSystems) is used for qPCR and the reaction is performed with the Power SYBR green PCR master mix (Applied BioSystems). The results are analysed using the StepOne software. Mouse gene specific primers are designed as follow: 18S mouse forward primer, 5'-GCCGCTAGAGGTGAAATTCTTG-3'; 18S mouse reverse primer, 5'-CATTCTTGGCAAATGCTTTCG-3'; Glob-SL8-exon forward primer, 5'-AGAAGTCTGCCGTTACTGCC-3'; Glob-SL8-exon reverse primer, 5'-AGGCCATCACTAAAGGCACC-3'; Glob-SL8-intron forward primer, 5'-GTATCAAGGTTACAAGACAG-3'; Glob-SL8-intron reverse primer, 5'-GGGAAAATAGACCAATAGGC-3'.

### **MTT assay**

Cells are plated in a 96-wells plate with  $1 \times 10^4$  cells per well for 24 hours. Cells are then treated with drug overnight. After removing the medium from the plate, the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) powder is re-suspended into PBS, filtered and added into the plate wells at 2,5mg/ml. The plate is

incubated for 2 hours at 37°C, with 5% CO<sub>2</sub>. It is then centrifuged and the supernatant is carefully removed in order not to disturb the precipitated formazan. 200µl/well of DMSO is added and the plate is shaken for 10 minutes. Absorbance is read at 544nm using the FLUOstar OPTIMA. Each experiment is repeated three times and the average of the three results is expressed in percentage of cell viability compare to the untreated condition ± SEM.

## **Animal studies**

### **Mice tumor challenge and drug treatment**

C57Bl/6J female mice are obtained from Harlan Laboratories. NU/NU nude mice are obtained from Charles River. At 7 weeks old, mice are injected subcutaneously on the right flank with 5x10<sup>4</sup> MCA205 wild type (WT) or 7,5x10<sup>4</sup> MCA205-Globin-SL8-intron cells, or with 5x10<sup>4</sup> B16F10 wild type (WT) or B16F10-Globin-SL8-intron cells along with matrigel (VWR). Five days after challenge, mice are treated intraperitoneally with different dose of PBS, Isoginkgetin, IP2 or M2P2. Ten and fifteen days after challenge mouse are again treated intraperitoneally with the same drug. Area of the tumor is recorded every 3 to 4 days until ethical limit points are reached. All animal experiments were carried out in compliance with French and European laws and regulations. Tumor-free mice are kept for 100 days after tumor challenge and are then injected subcutaneously on the right flank with 7,5x10<sup>4</sup> MCA205-Globin-SL8-intron cells and on the left flank with 5x10<sup>4</sup> B16F10 WT cells. Results are expressed as mean ± SEM. \*p<0.05, \*\*P<0.01, \*\*\*P<0.001 (ANOVA with Tukey's multiple comparison test comparing all groups).

## Results

### Splicing inhibition increases the presentation of antigens derived from introns and exons in cancer cells

Cancer cells develop different intracellular mechanisms that shape the MHC-I peptide pool and quantity, leading to cancer cell reduced antigenicity and T-cell recognition escape. In recent studies we have highlighted PTPs as one MHC-I peptide source and provided the first evidence for splicing inhibition to enhance PTPs-dependent antigen presentation by treating HEK cells with isoginkgetin. The latter has been reported to inhibit the spliceosome during the early stages of its assembly. To improve cancer cell antigenicity and immune recognition, we determined whether isoginkgetin was able to enhance tumor associated PTPs-(TA-PTPs) derived antigen expression and presentation by cells. For that purpose, the human melanoma cell line A375, the human lung cancer cell line A549 and the normal human fibroblast lung cell line MRC5 were transiently expressing the MHC-I H2-K<sup>b</sup> molecule and the PTPs-SL8 epitope from an intron within the  $\beta$ -Globin gene construct (Globin-SL8-intron) and were treated with different isoginkgetin concentrations for 18h. The overall H2-K<sup>b</sup> expression upon treatment was measured by B3Z activation after free SL8 peptide addition for each condition. Treatment with isoginkgetin increases intron-derived-SL8 antigen presentation in the three cell types, in a dose dependent manner (Figure 12 A). In parallel, the same experiment was performed on the mouse melanoma B16F10 and sarcoma MCA205 cell lines transiently expressing the Globin-SL8-intron construct. In accordance with the previous results, the isoginkgetin elicits intron-derived-SL8 antigen presentation increase, in a dose dependent manner (Figure 12 B). To investigate further isoginkgetin impact on PTPs presentation, both murine sarcoma and melanoma cell lines were transiently expressing the PTPs-SL8 epitope from an exon within the  $\beta$ -Globin gene construct (Globin-SL8-exon) or transiently expressing the Ova cDNA. In the latter construct, the SL8 epitope does not need to be spliced to be expressed. We observed that isoginkgetin increases exon-derived-SL8 antigen presentation in MCA205 and B16F10 cancer cells in a dose dependent manner (Figure 12 C), whereas the splicing inhibitor has no effect on the Ova cDNA-derived SL8 production (Figure 12 D). Hence, a splicing event seems to be required for isoginkgetin to impact PTPs-dependent antigen presentation. This suggests an action of isoginkgetin during the PTPs production stage and not downstream in the MHC-I antigen presentation pathway.

Similarly to isoginkgetin, madrasin inhibits the spliceosome at the early stages of assembly. To assess isoginkgetin specificity, we treated previously tested human and mouse cell lines with different madrasin concentrations. In A375, MCA205 and B16F10 cell lines, we observed enhanced intron-SL8-dependent antigen presentation comparable to isoginkgetin effect (Figure 12 E and 12 F). No impact of the treatment was seen in A549 cells while an antigen presentation decrease was observed in the human non-transformed cell line MRC5. Therefore, madrasin and isoginkgetin seem to have different specificities regarding PTPs production: while isoginkgetin positively impacts all cell types, the madrasin treatment only positively impacts certain cancer cell types and negatively impacts the non-transformed cell line, suggesting diversity in the mechanism and/or in the effectiveness of both molecules. Supporting that, isoginkgetin was found less toxic for cells compared to madrasin at concentrations where they efficiently enhance PTPs-derived antigen presentation (Tables S1 and S2). In addition, when treated with the chemotherapeutics gemcitabine or cyclophosphamide, human and mouse cell lines expressing the SL8 from an intron did not show any significant modulation of the antigen expression (Figure S1), except when MCA205 and A549 cells were treated with gemcitabine. Again these results suggest a specific action of isoginkgetin on PTPs production in all cell lines. Finally, the H2-K<sup>b</sup> molecule expression at the cell surface was assessed after isoginkgetin and madrasin treatment by flow cytometry. Isoginkgetin decreases H2-K<sup>b</sup> molecule expression in MCA205 cells, enhances their expression in B16F10 cells and does not impact human cell H2-K<sup>b</sup> expression (Figure S2). Madrasin does not impact H2-K<sup>b</sup> molecule expression in all cell lines (Figure S2). These observations are not correlated to the SL8 antigen presentation modulation observed suggesting that isoginkgetin and madrasin impact PTPs production rather than downstream components of the MHC-I pathway.

Overall, these results show that isoginkgetin boosts PTPs-derived antigen presentation in cancer cells independently of the epitope setting, i.e. in exonic or in intronic sequences, and independently of the cell lines tested. Moreover, the effects of both isoginkgetin and madrasin shed further light on the importance of splicing for MHC-I peptides generation in cancer cells. Finally, they support the idea that pre-mRNAs are a source for antigen presentation when the spliced machinery is unpaired.

## **Isoginkgetin treatment inhibits tumor growth *in vivo* when the intron-derived-SL8 epitope is expressed.**

Antigens abundance at the cell surface is one important parameter in determining the magnitude of the CD8+ T cell response<sup>312</sup>. The SL8 peptide is highly immunogenic *in vivo*. Looking at SL8-specific T-cells activation *in vitro*, we observed a change in the SL8 expression abundance at the cancer cell surface after splicing inhibition. In order to test splicing inhibition effects on cancer cells *in vivo*, we first looked at the impact of isoginkgetin treatment on the growth of intron-derived SL8 peptide-expressing tumor. For that purpose, MCA205 and B16F10 cells stably expressing the globin-intron-SL8 construct were inoculated subcutaneously in mice. At days 5, 10 and 15 after tumor inoculation, the mice were injected intraperitoneally with a define dose of isoginkgetin, and the tumor growth was monitored (Figure 13 A). In mice bearing MCA205 globin-SL8-intron (MCA205 GI) tumors, we observed a significant tumor size reduction, over 50% at day 27 after challenge when treated with 12 and 18mg/kg of isoginkgetin (Figure 13 B). The isoginkgetin impact on B16F10 globin-SL8-intron (B16F10 GI) tumor growth is lower than on MCA205 GI; however the drug still significantly slows down tumor growth (Figure 13 C). To assess the link between SL8 overexpression and tumor growth reduction *in vivo* after isoginkgetin treatment, we performed the same experiment with mice inoculated with either MCA205 or B16F10 wild type (WT) cells. No significant reduction of MCA205 WT (Figure 13 D) or in B16F10 WT (Figure 13 E) tumor growth was observed after treatment with 12 and 18mg/kg of isoginkgetin. These results suggest that the expression of an immunodominant epitope, herein the SL8 peptide, is required for isoginkgetin to impact tumor growth *in vivo*. From these observations, one could infer a role for CD8+ T cells in tumor reduction.

Therefore, we then assessed the requirement of the immune response for isoginkgetin to reduce tumor growth. Immunodeficient Nu/Nu nude mice were inoculated subcutaneously with either MCA205 or B16F10 cells stably expressing the Globin-intron-SL8 or WT, and treated with the same settings as previously described (Figure 13 A). No effect of isoginkgetin treatment was observed on the growth of each of the four tumor types (Figure 13 E-H).

Overall, these results show that tumor size reduction upon isoginkgetin treatment requires the presence of an active immune response *in vivo*, and suggest that the increase in the expression of an immunodominant epitope drives the anti-tumor immune response.

**The compounds derived from the natural Isoginkgetin product IP2 and M2P2 are water soluble, inhibit the splicing and are less toxic.**

With the aim of transferring the isoginkgetin compound to the clinic, two of its intrinsic properties were limiting: its hydrophobicity which reduces its bioavailability and its toxicity on normal and tumor cells. Therefore, derivatives of the natural isoginkgetin product were synthesized and tested for their ability to inhibit the splicing, to increase PTPs-derived antigens *in vitro* as well as to reduce tumor growth *in vivo*.

The derivatives IP2 (figure 14 B) and M2P2 (figure 14 C) were synthesized from the commercial isoginkgetin (Figure 14 A), extracted from leaves of maidenhair tree, *Ginkgo biloba L.* Route of synthesis is provided in figure S4. Briefly, the synthesis of IP2 or compound 2 (as named in the schema) was accomplished by the phosphorylation of isoginkgetin employing *in situ* formation of diethylchlorophosphite to provide compound 1. Further cleavage of the ethyl ester protective groups with iodotrimethylsilane afforded the phosphoric acid intermediate, which was immediately treated with sodium hydroxide to complete a practical route to the disodium phosphate prodrug. For the synthesis of the M2P2 molecule, the remaining two phenol groups of compound 1 were alkylated using methyl iodide to furnish compound 3. Treatment of the latter under similar conditions to prepare compound 2 from compound 1 gave the disodium phosphate prodrug 4 or M2P2, whereas its reaction under basic conditions provided compound 5.

The IP2 and M2P2 water solubility was found to be considerably higher than that of the parent compound isoginkgetin (data not shown). In addition, we tested the ability of IP2 and M2P2 to inhibit the splicing of the Globin-SL8-intron gene product in MCA205 and B16F10 cells. Interestingly, IP2 and M2P2 provide two distinct patterns of splicing inhibition in each cell line. IP2 treatment increases the presence of non-spliced RNA products in both cells but more efficiently in MCA205 than in B16F10, such as isoginkgetin or madrasin treatment does. In contrast, M2P2 treatment does not impact splicing in B16F10 cells while it has a strong impact on splicing in MCA205 compared to IP2, isoginkgetin and madrasin treatment (Figure 14 D and 14 E). Hence, IP2 and M2P2 seem to display different mechanisms for inhibiting the splicing. Importantly, we observed that the splicing pattern of IP2 is similar to the one of madrasin and isoginkgetin for the studied gene product. In addition, it has been shown that cancer cells can acquire deficiencies in the splicing machinery that benefit their growth, for example by preventing the expression of tumor suppressor genes<sup>144</sup>. These

deficiencies do not have the same nature in all tumors and therefore could explain the distinct effects of splicing inhibitors on separate tumor types. Both IP2 and M2P2 display no toxicity in MCA205 and B16F10 WT cells at the doses tested (Figure 14 F and 14 G). Overall, we have provided two new drugs that are water soluble and that can impact the splicing differently in our two model cell lines at doses that do not impact cell viability.

**Isoginkgetin derivative IP2 efficiently increases MHC-I presentation of intron-derived antigen *in vitro*, reduces tumor growth *in vivo* and extends survival.**

In order to test the potential immunomodulatory effect of IP2 and M2P2 in comparison with isoginkgetin, the two molecules were first tested for their ability to increase the MHC-I presentation of PTPs-derived antigens *in vitro*. For that purpose, MCA205 and B16F10 cells were transiently expressing the Globin-intron-SL8 construct and treated with 15 $\mu$ M or 35 $\mu$ M of IP2 or M2P2. While treatment with IP2 increases the intron-SL8-derived antigen presentation in MCA205 and B16F10 cells similarly to what we observed after isoginkgetin treatment (Figure 15 A and B, left panels), M2P2 decreases its presentation in MCA205 cells and does not impact it in B16F10 cells (Figure 15 A and B right panels). These results are interestingly correlated to the respective ability of IP2 and M2P2 to inhibit the Globin-SL8-intron gene splicing. In fact, M2P2 has no impact both on splicing and on the SL8 antigen presentation in B16F10. Conversely, M2P2 strongly inhibits the splicing in MCA205 and negatively affects the SL8 presentation. Along with these results, we showed that the expression of the H2-K<sup>b</sup> molecules at the cell surface is not affected in the cell lines treated with IP2 and M2P2 (figure S5A and S5B). Hence, it seems likely that a tight regulation of splicing is required for treatments to positively impact the presentation of intron-derived epitopes. These results show that the isoginkgetin derivative IP2 acts as a booster of the PTPs-derived antigenic presentation *in vitro* in the same way as the natural product.

The IP2 and M2P2 molecules were then tested for their anti-tumoral effect *in vivo*. As previously performed with isoginkgetin treatment, MCA205 sarcoma cells or B16F10 melanoma cells, stably expressing the Globin-intron-SL8 construct or WT, were subcutaneously inoculated in mice. At days 5, 10 and 15 after tumor inoculation, each group of mice were intraperitoneally treated with 18mg/kg of isoginkgetin, IP2 or M2P2. At this dose, a significant decrease in MCA205 GI tumor growth was observed after treatment with IP2 compare to isoginkgetin treatment, while no impact of M2P2 treatment was monitored (Figure 15 C, left panel). In addition, the reduction of B16F10 GI tumor growth was similar

after treatment with 18mg/kg of isoginkgetin or IP2, while M2P2 had no effect on growth (Figure 15 C, right panel). As IP2 treatment reduces tumor growth and is water soluble, we decided to increase the dose injected in mice. The increased dose of IP2 did not improve its anti-tumoral effect on MCA205 GI at 24mg/kg but increased it on B16F10 GI at 36mg/kg (Figure 15 C). Strikingly, isoginkgetin and M2P2 treatment did not impact MCA205 WT and B16F10 WT tumor growth, whereas IP2 treatment inhibits both tumors (Figure 15 D). We confirmed that IP2 does not induce tumor cell apoptosis even at a high dose (Figure S5 C and S5 D). Finally, IP2 treatment extends survival of mice, with more than 50% of survivors 100 days after tumor inoculation (Figure 15 E, right panel). At the same time, around 30% of mice treated with isoginkgetin are still alive (Figure 15 E, middle panel). M2P2 treatment does not impact survival (Figure 15 E, left panel).

Overall, these results suggest a correlation between the PTPs-derived antigen presentation increase observed *in vitro* and tumor growth reduction *in vivo* after treatment. Interestingly, contrary to isoginkgetin, IP2 treatment inhibits the growth of tumors that do not bear the highly immunodominant SL8 epitope derived from PTPs. The difference between both molecules can be due to their biodisponibility, which should be higher for water-soluble molecule than for hydrophobic molecule, as well as to the use of an increased treatment dose. We hypothesize that the splicing inhibitor IP2 potentiates the cell surface immunodominant epitope appearance, driving the anti-tumoral response.

### **IP2 treatment efficacy is dependent on the immune-response and creates a long-lasting anti-tumoral response.**

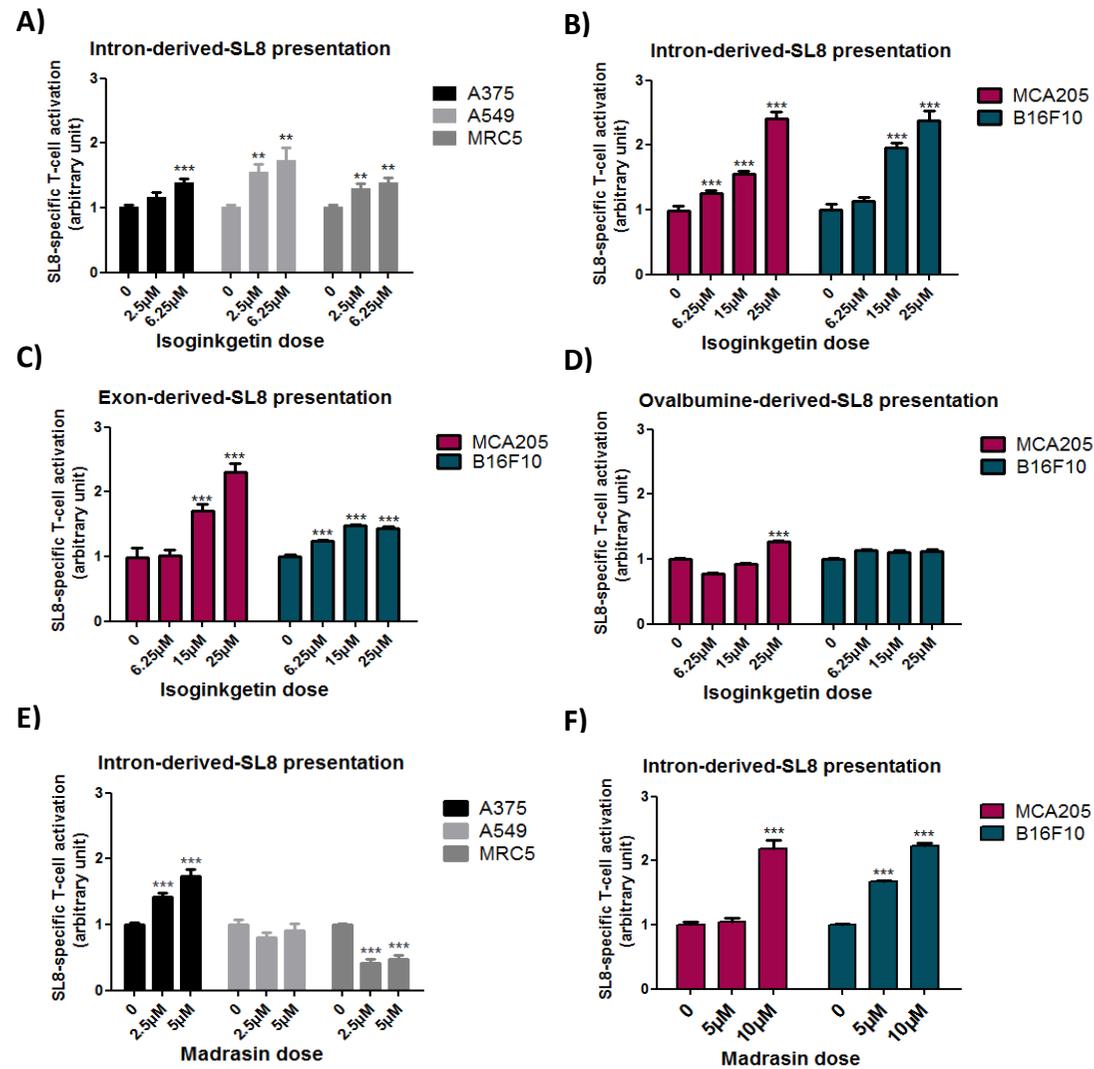
To determine the requirement of the immune system and especially of the T-cell response for IP2 efficacy against tumor, we looked at its effect in Nu/Nu athymic nude mice that lack T-cells but not B and NK cells. As previously tested with isoginkgetin, MCA205 or B16F10 cells stably expressing the Globin-intron-SL8 construct or WT were subcutaneously inoculated in mice. At days 5, 10 and 15 after tumor inoculation, each group of mice were intraperitoneally treated with the dose of IP2 which was found to be the most efficient in immunocompetent mice. Hence, MCA205GI, MCA205 WT and B16F10 GI and WT bearing mice were treated with 18mg/kg, 24mg/kg and 36mg/kg respectively. In each condition, no impact of IP2 treatment was observed on tumor growth (Figure 16 A and B).

In addition, in order to assess the specific requirement of CD8<sup>+</sup> T cells for IP2 efficacy, we tested the impact of *in vivo* CD8<sup>+</sup> T cells depletion in mice. Mice were inoculated with

MCA205 GI cells subcutaneously followed by a scheduled treatment with anti-CD8<sup>+</sup> T cell antibody or with the isotype 2A3. IP2 treatment was administered as previously at day 5, 10 and 15. Interestingly, anti-CD8<sup>+</sup> T cell antibody treatment completely abrogated the anti-tumor effect of the IP2 treatment (Figure 16 C). Therefore, this result confirms that the effect of the IP2 treatment on tumor growth is dependent on the CD8<sup>+</sup> T cell response, which supports an antigen-driven cytotoxic activity against the tumor cells.

Finally, around 50% of mice inoculated with MCA205 GI and subsequently treated with IP2 as described above became tumor free after treatment. 100 days after the first tumor inoculation, these mice were re-challenged with MCA205 GI tumor cells on the right flank and with B16F10 cells on the left flank. While B16F10 tumors grew over time, MCA205 GI did not grow in mice. These results demonstrate that mice developed a long term anti-tumoral response specific to MCA205 GI tumor after IP2 treatment.

Overall these results shed light on the capacity of specific splicing inhibitors, such as isoginkgetin and IP2, to positively modulate the anti-tumoral immune response. In addition, they confirm that PTPs-derived antigens are efficiently presented and recognized by CD8<sup>+</sup>T cells *in vitro* and *in vivo* and that a change in their presentation at the cell surface in quantity or in quality can lead to a CD8<sup>+</sup> T cells response against cancer.



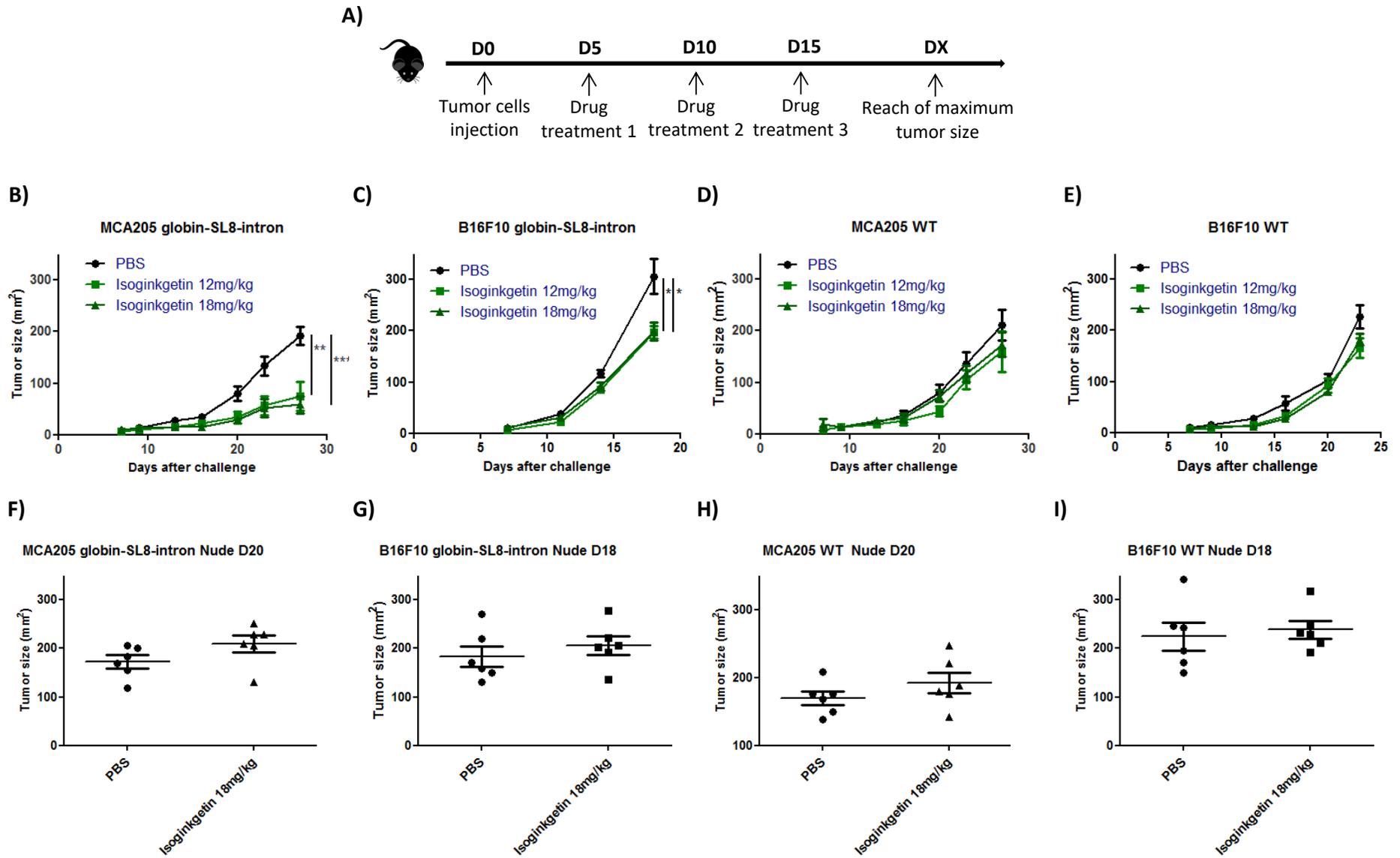
**Figure 12: Splicing inhibition increases exon- and intron-derived antigens MHC-I presentation in cancer cells**

## Figure 12

B3Z SL8-specific T-cell activation after co-culture with **(A)** human melanoma A375, human lung carcinoma A549 or normal human lung fibroblast MRC5 cell lines, all transiently expressing the intron-derived SL8 peptide and the H2-K<sup>b</sup> molecules and treated upstream with 2,5µM or 6,25µM isoginkgetin for 18 hours; or with **(B)** mouse sarcoma MCA205 or mouse melanoma B16F10 cell lines both transiently expressing the intron-derived SL8 peptide and treated upstream with 6,25µM, 15µM or 25µM isoginkgetin for 18 hours. B3Z activation after co-culture with MCA205 or B16F10 cells that both transiently express **(C)** the exon-derived SL8 peptides or **(D)** the Ova cDNA construct, which does not need to be spliced, treated upstream with 6,25µM, 15µM or 25µM isoginkgetin for 18 hours. B3Z activation after co-culture with **(E)** A375, A549 or MRC5 transiently expressing the intron-derived SL8 peptide and the H2-K<sup>b</sup> molecules and treated upstream with 2,5µM or 5µM madrasin for 18 hours; or with **(F)** MCA205 or B16F10 cell lines both transiently expressing the intron-derived SL8 peptide and treated upstream with 5µM or 10µM madrasin for 18 hours. Free SL8 peptides were added in each condition to ensure that T-cell assays were carried out at non-saturated conditions and that the expression of MHC-I molecules was taking into account in the

results. Each graph is one representative of at least four independent experiments.

Data are given as mean ± SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test).

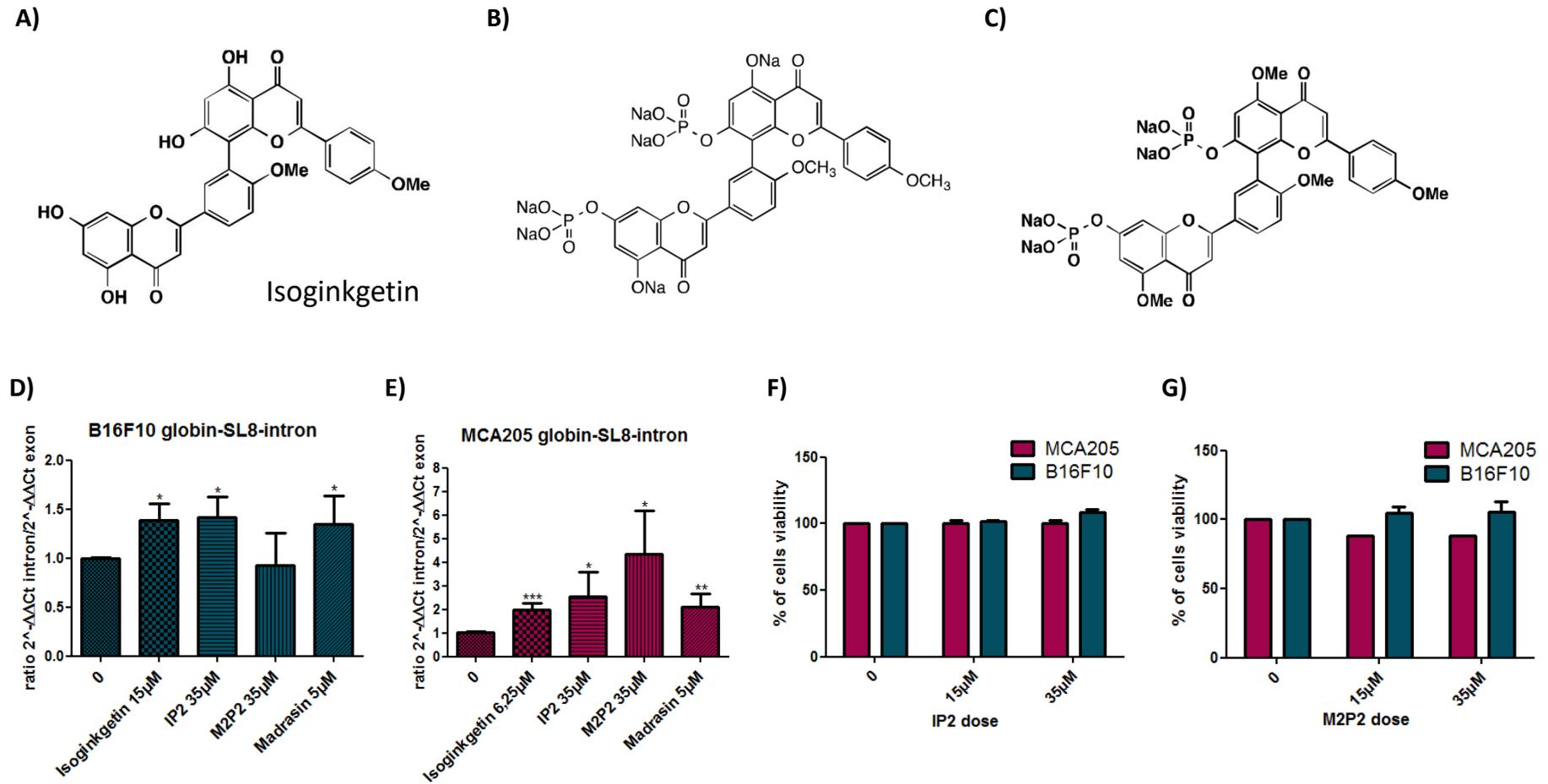


**Figure 13: Isoginkgetin splicing inhibitor reduces the growth of tumor bearing intron-derived-SL8 in an immune-dependent manner**

### Figure 13

(A) Experimental settings. Growth of (B) sarcoma MCA205 or (C) melanoma B16F10 cells that both stably express the globin-SL8-intron construct or (D) MCA205 Wild Type (WT) or (E) B16F10 WT cells that were subcutaneously inoculated in the flank of immunocompetent C57BL/6 mice thereafter injected intraperitoneally with 12mg/kg or 18mg/kg of isoginkgetin at day 5, 10 and 15 after inoculation. Tumor size was assessed every 3 to 4 days until reaching the established ethical endpoints. Each line represents the tumor size in area (mm<sup>2</sup>) of 6 mice in each group. Size in area (mm<sup>2</sup>) of (F) MCA205 globin-SL8-intron, (G) B16F10 globin-SL8-intron, (H) MCA205 WT or (I) B16F10 WT tumors subcutaneously inoculated in the flank of immunodeficient Nu/Nu Nude mice thereafter injected intraperitoneally with 18mg/kg of isoginkgetin at days 5, 10 and 15 after inoculation. Data are presented at the day before the endpoints are reached.

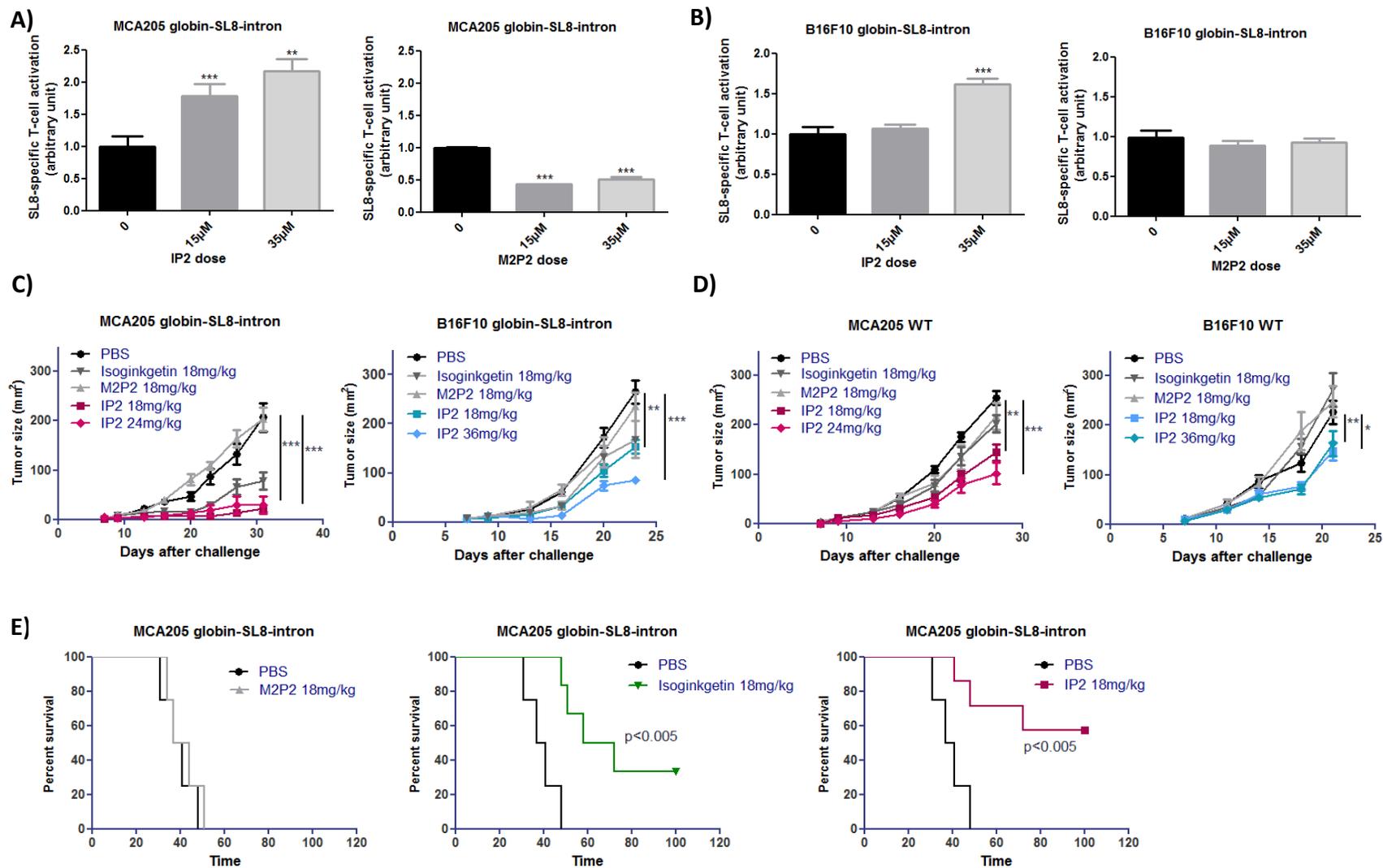
Data are given as mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01 (ANOVA with Tukey's multiple comparison test comparing all groups).



**Figure 14: Synthesis and activity of the isoginkgetin derivatives IP2 and M2P2.**

## Figure 14

Molecular structure of (A) isoginkgetin, (B) IP2 and (C) M2P2 compounds. (D) B16F10 globin-SL8-intron or (E) MCA205 globin-SL8-intron were treated with 15 $\mu$ M isoginkgetin, 5 $\mu$ M madrasin, 35 $\mu$ M IP2 or 35 $\mu$ M M2P2 for 48 hours. RNA was extracted and qRT-PCR was performed with primers amplifying the unspliced (intron) and the spliced (exon) globin-SL8-intron RNA. Data are given as mean  $\pm$  SEM of the ratio of  $2^{-\Delta\Delta C_t}$  intron and  $2^{-\Delta\Delta C_t}$  exon of at least three independent experiments. MTT assay performed on MCA205 or B16F10 cells treated with 15 $\mu$ M or 35 $\mu$ M of (F) IP2 or (G) M2P2. Data are express as mean  $\pm$  SEM of the percentage of viable cells compared to the control condition of at least three independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test).

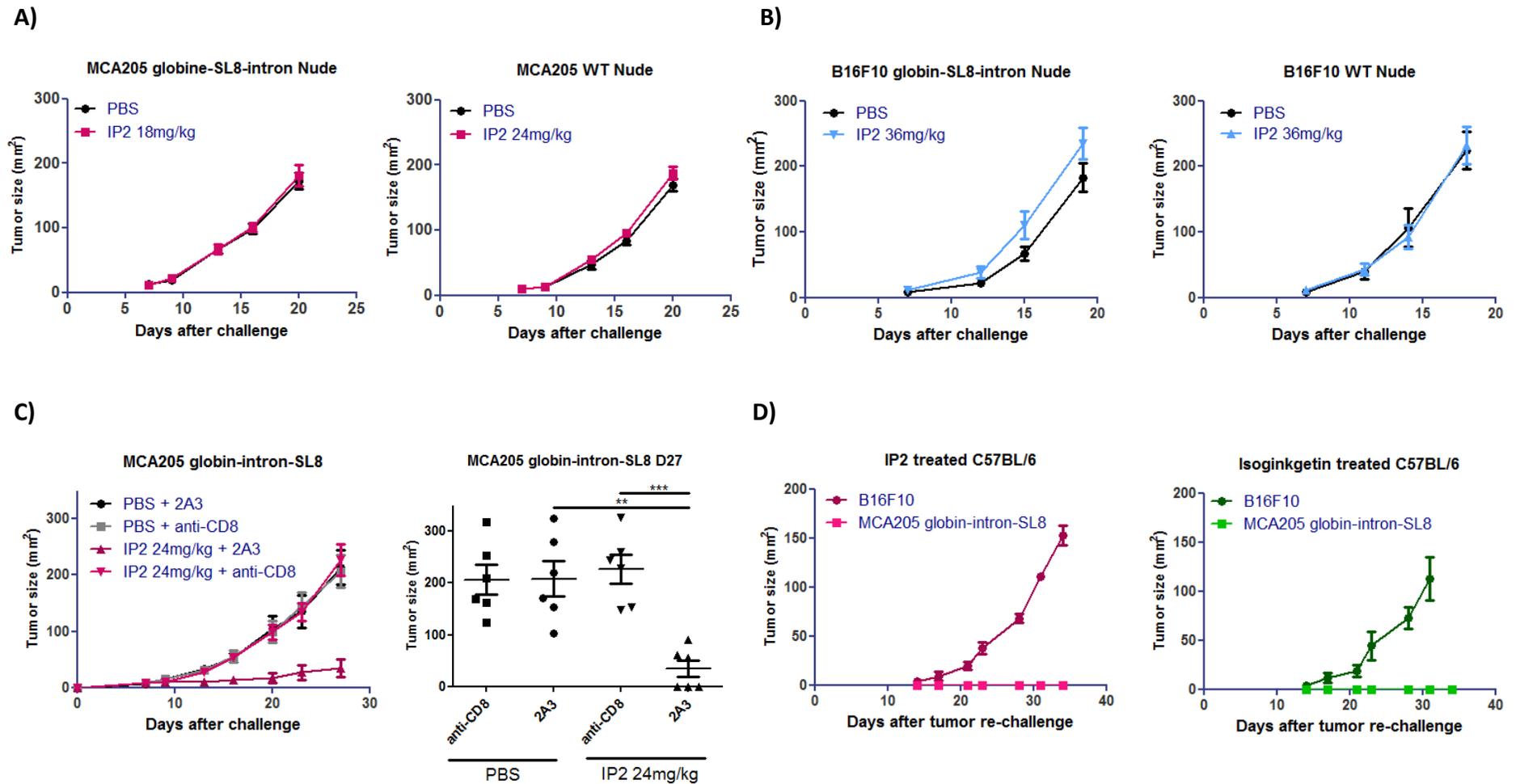


**Figure 15: IP2 treatment reduces tumor growth and extends survival.**

## Figure 15

B3Z SL8-specific T-cell activation after co-culture with mouse (A) sarcoma MCA205 or (B) melanoma B16F10 cell lines both transiently expressing the intron-derived SL8 peptide and treated upstream with 15 $\mu$ M or 35 $\mu$ M of IP2 (left panels) or of M2P2 (right panel). Data are given as mean  $\pm$  SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test). Growth of (C) MCA205 (left panel) or melanoma B16F10 cells (right panel) that both stably express the globin-SL8-intron construct or (D) MCA205 WT (left panel) or B16F10 WT (right panel) cells that were subcutaneously inoculated in the flank of immunocompetent C57BL/6 mice thereafter injected intraperitoneally with 18mg/kg of isoginkgetin, of M2P2 or of IP2 or 24mg/kg or 36mg/kg of IP2 at day 5, 10 and 15 after inoculation. Tumor size was assessed every 3 to 4 days until reaching the established ethical endpoints. Each line represents the tumor size in area (mm<sup>2</sup>) of at least 6 mice in each group. Data are given as mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, \*\*\*P<0.001 (ANOVA with Tukey's multiple comparison test comparing all groups). Kaplan Meier plots of (E) MCA205 globin-SL8-intron cells inoculated subcutaneously in the flank of immunocompetent C57BL/6 mice thereafter injected intraperitoneally with PBS or

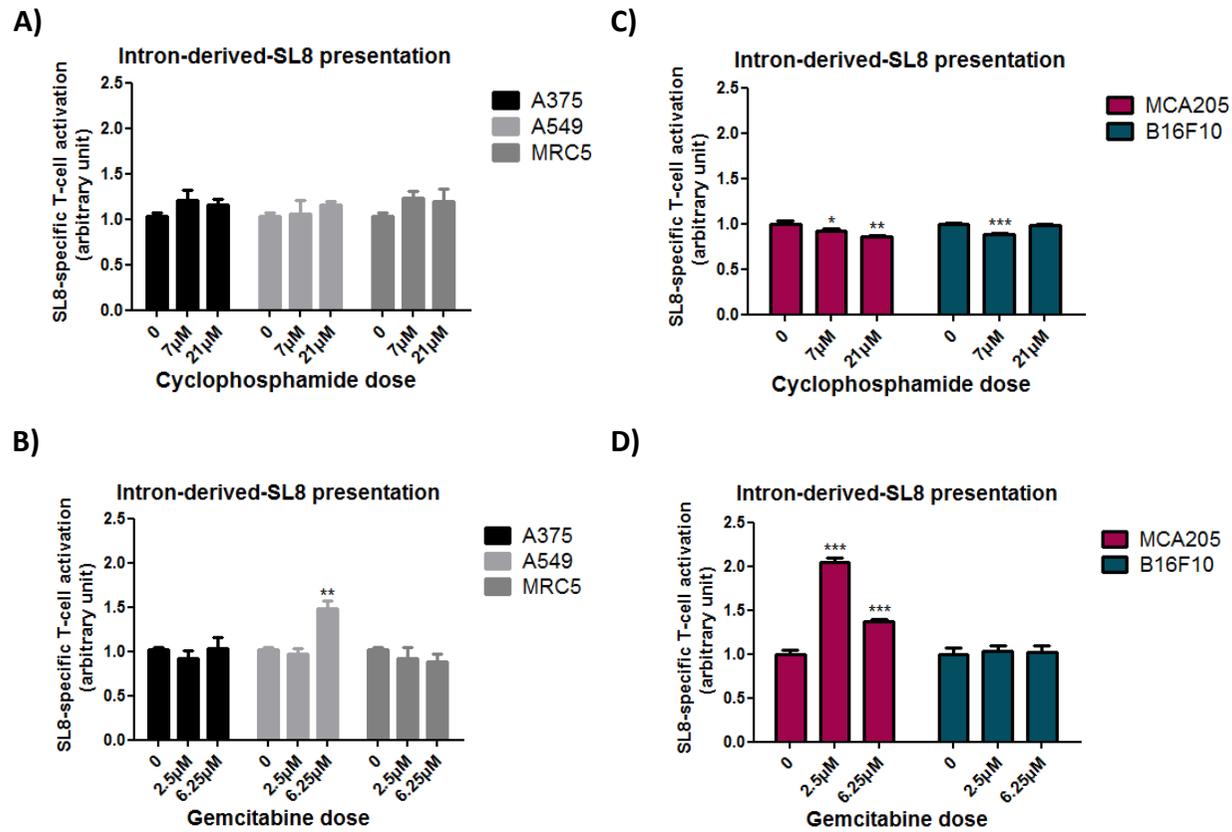
18mg/kg of isoginkgetin, of M2P2 or of IP2. A Log-rank (Mantel-Cox) test was performed.



**Figure 16: IP2 therapeutic effect is dependent on the immune response.**

## Figure 16

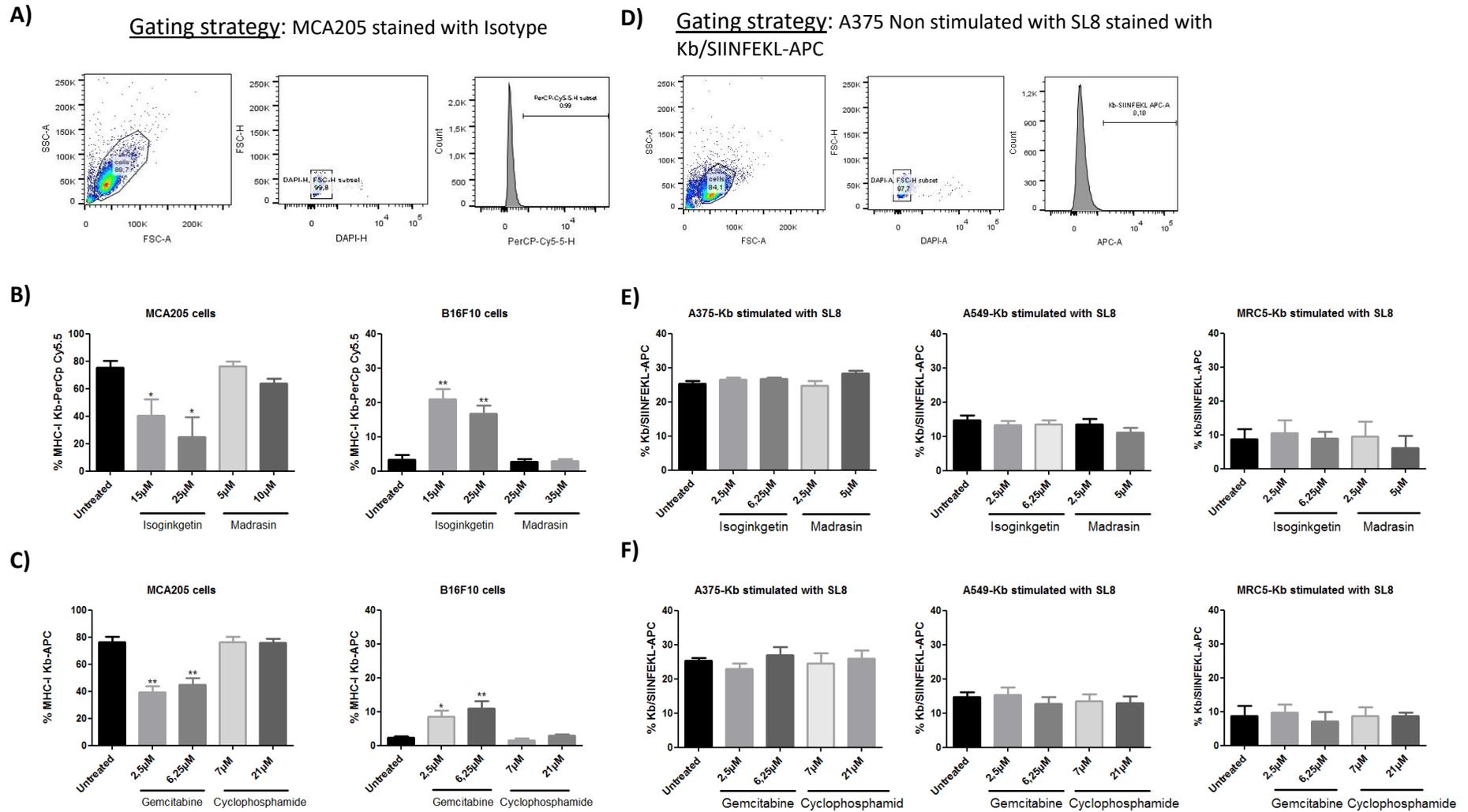
Growth curve of **(A)** MCA205 globin-SL8-intron (left panel), MCA205 WT (right panel), **(B)** B16F10 globin-SL8-intron (left panel) or B16F10 WT (right panel) subcutaneously inoculated in the flank of immunodeficient Nu/Nu Nude mice thereafter intraperitoneally injected at days 5, 10 and 15 with 18, 24 or 36mg/kg of IP2. Growth curve of **(C, left panel)** MCA205 globin-SL8-intron subcutaneously inoculated in the flank of immunocompetent mice thereafter treated with PBS or 24mg/kg of IP2 at day 5, 10 and 15 after inoculation as well as with *in vivo* anti-CD8+ T cell antibody or 2A3 isotype every 3 days. Each line represents the tumor size in area (mm<sup>2</sup>) of at least 6 mice in each group. The C right panel represents the tumor size at day 27. Data are given as mean  $\pm$  SEM. \*p<0.05, \*\*p<0.01, \*\*\*P<0.001 (ANOVA with Tukey's multiple comparison test comparing all groups). **(D)** Growth curve of MCA205 globin-SL8-intron cells or B16F10 WT cells inoculated in 100 day-tumor free C57BL/6 mice previously inoculated with MCA205 globin-SL8-intron and treated with IP2 (left panel) or isoginkgetin (right panel). Each line represents the tumor size in area (mm<sup>2</sup>) of at least 4 mice in each group.



**Figure S1: Impact of cyclophosphamide and gemcitabine treatment on exon- and intron-derived antigenic MHC-I presentation in cancer cells.**

## Figure S1

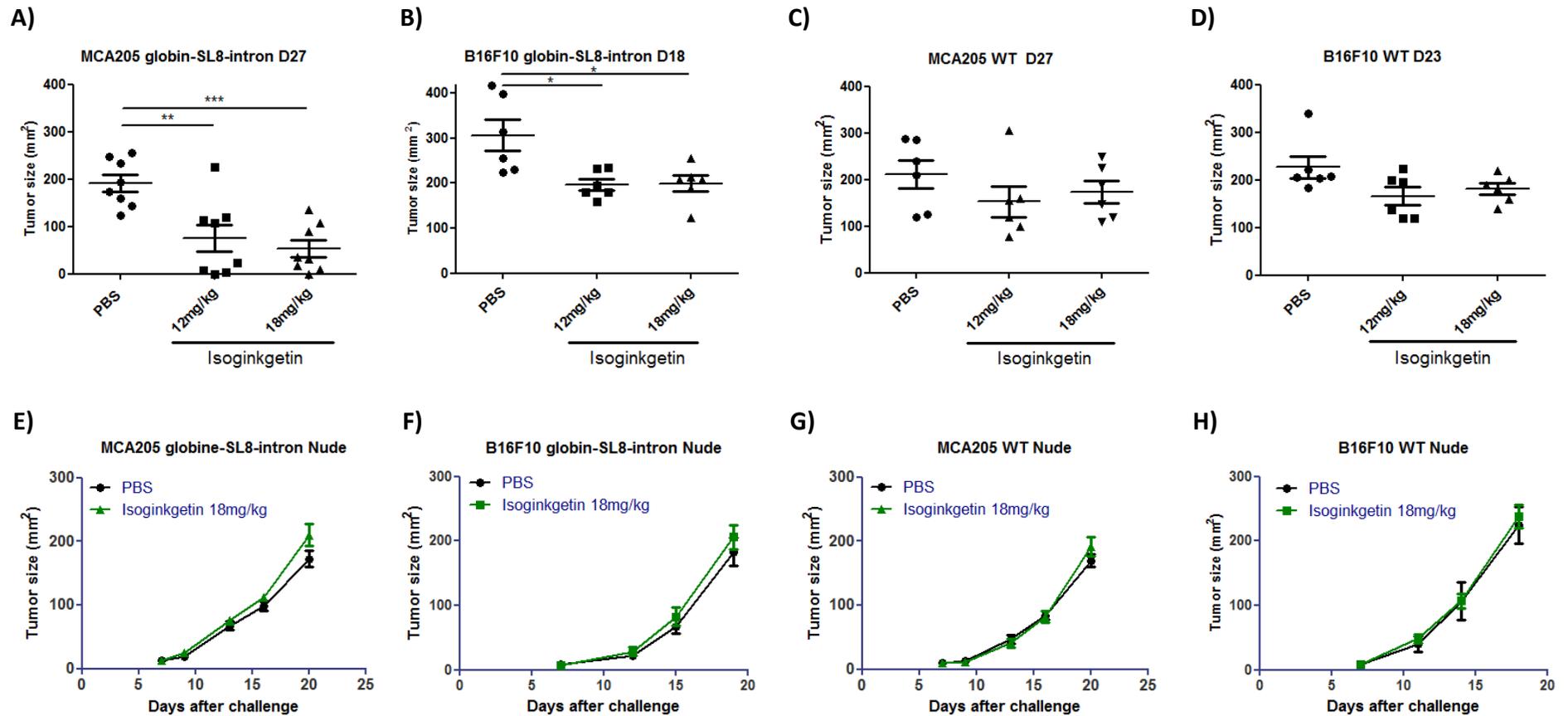
B3Z SL8-specific T-cell activation after co-culture with A375, A549 or MRC5 cell lines, all transiently expressing the intron-derived SL8 peptide and the H2-K<sup>b</sup> molecules and treated upstream with (A) 7μM or 21μM of cyclophosphamide or with (C) 2.5μM or 6.25μM of gemcitabine for 18 hours. B3Z SL8-specific T-cell activation after co-culture with MCA205 or B16F10 cell lines both transiently expressing the intron-derived SL8 peptide and treated upstream with (B) 7μM or 21μM of cyclophosphamide or with (D) 2.5μM or 6.25μM of gemcitabine for 18 hours. Free SL8 peptides were added in each condition to ensure that T-cell assays were carried out at non-saturated conditions and that the expression of MHC-I molecules was taking into account in the results. Each graph is one representative of at least four independent experiments. Data are given as mean ± SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (unpaired student t test).



**Figure S2: Expression of H2-K<sup>b</sup> molecules at the cells surface.**

## Figure S2

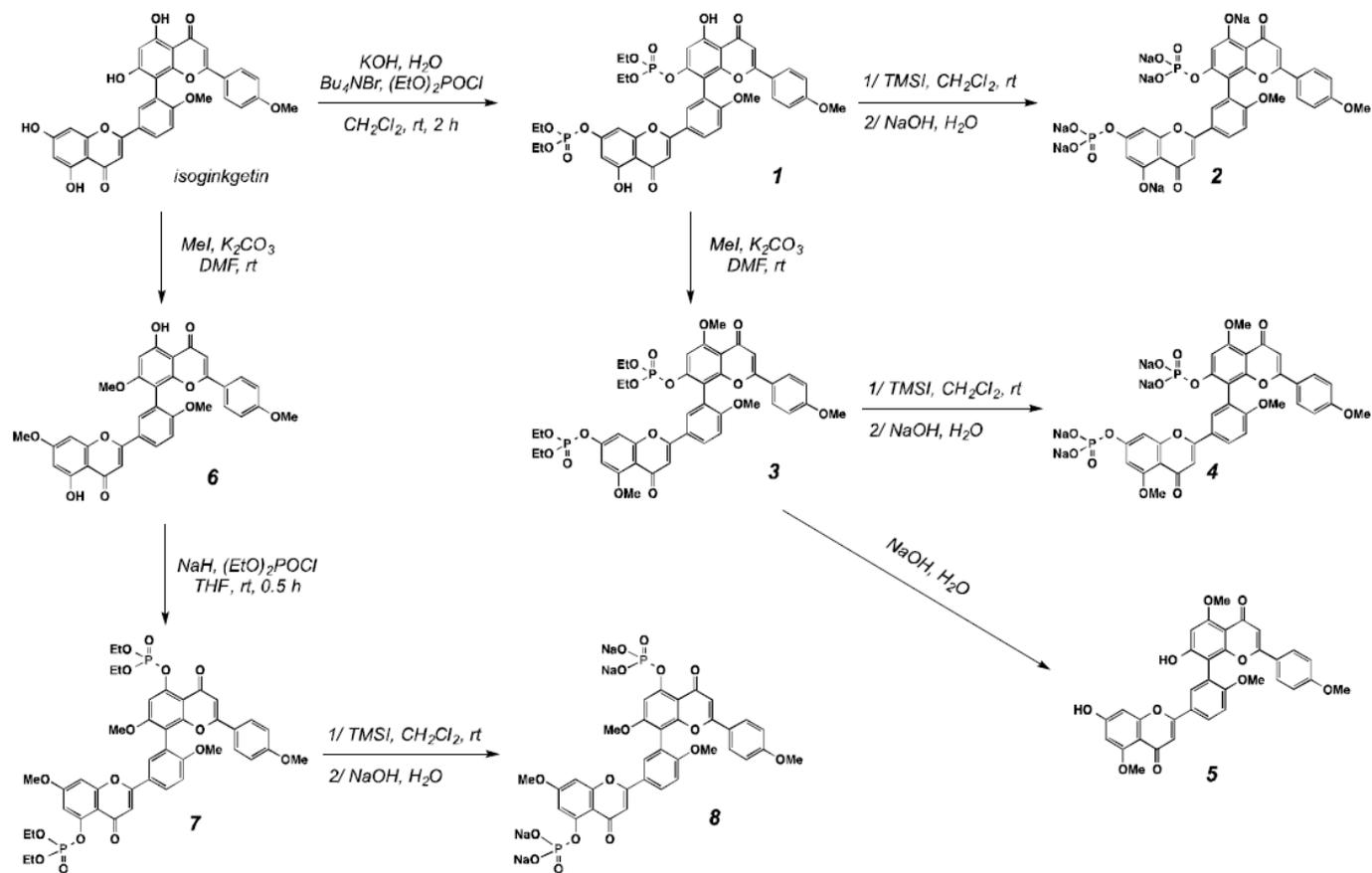
(A-D) Gating strategy. Flow cytometry analyses of H2-K<sup>b</sup> expression on MCA205 and B16F10 cells treated with (B) isoginkgetin, madrasin, (C) gemcitabine or cyclophosphamide. Flow cytometry analyses of transiently expressed H2-K<sup>b</sup> expression on A375, A549 and MRC5 cells treated with (D) isoginkgetin, madrasin, (E) gemcitabine or cyclophosphamide.



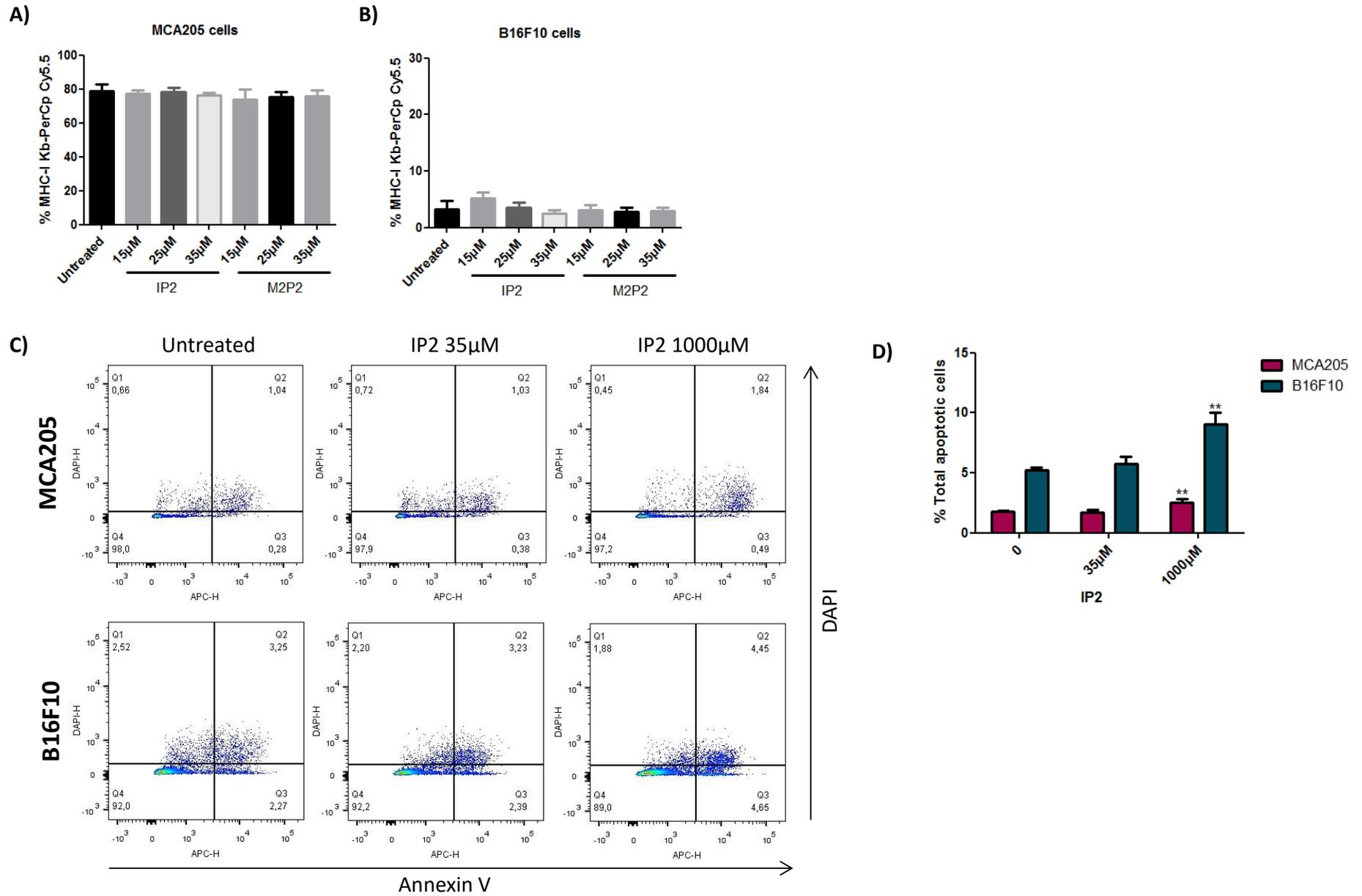
**Figure S3:** Additional data from Figure 2, Isoginkgetin splicing inhibitor reduces the growth of tumor bearing intron-derived-SL8 in an immune-dependent manner.

### Figure S3

Size in area ( $\text{mm}^2$ ) of (A) MCA205 globin-SL8-intron, (B) B16F10 globin-SL8-intron, (C) MCA205 WT or (D) B16F10 WT cells that were subcutaneously inoculated in the flank of immunocompetent C57BL/6 mice thereafter injected intraperitoneally with 12mg/kg or 18mg/kg of isoginkgetin at days 5, 10 and 15 after inoculation. Data are presented at the day before the endpoints are reached. Growth of (E) MCA205 globin-SL8-intron, (F) B16F10 globin-SL8-intron, (G) MCA205 WT or (H) B16F10 WT tumors subcutaneously inoculated in the flank of immunodeficient Nu/Nu Nude mice thereafter injected intraperitoneally with 18mg/kg of isoginkgetin at days 5, 10 and 15 after inoculation. Each line represents the tumor size in area ( $\text{mm}^2$ ) of 6 mice in each group. Data are given as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  (ANOVA with Tukey's multiple comparison test comparing all groups).



**Figure S4:** Scheme of IP2 and M2P2 synthesis.

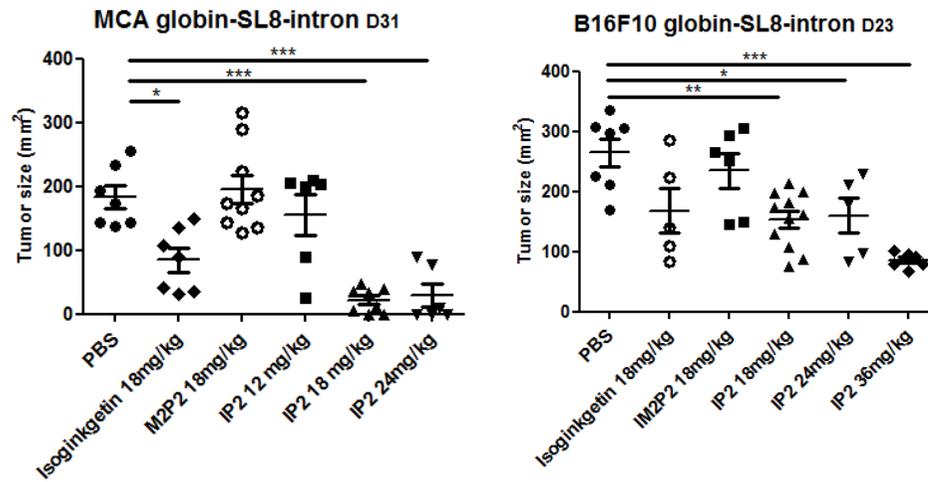


**Figure S5: IP2 does not impact H2-K<sup>b</sup> molecules expression at the cell surface and does not induce apoptosis**

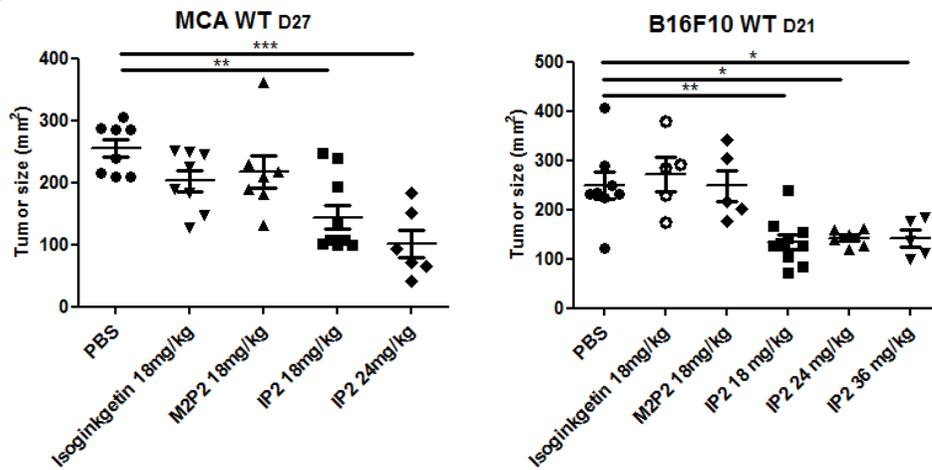
## Figure S5

Flow cytometry analyses of H2-K<sup>b</sup> expression on (A) MCA205 and (B) B16F10 cells treated with IP2 and M2P2. (C-D) Flow cytometry analyses of early, late and total apoptotic MCA205 and B16F10 cells treated with 35 $\mu$ M or 1000 $\mu$ M IP2 for 18 hours.

A)



B)



Size in area (mm<sup>2</sup>) of (A) MCA205 globin-SL8-intron (left panel), B16F10 globin-SL8-intron (right panel), (B) MCA205 WT (left panel) or B16F10 WT (right panel) cells that were subcutaneously inoculated in the flank of immunocompetent C57BL/6 mice thereafter injected intraperitoneally with 18mg/kg of isoginkgetin, M2P2 or IP2 or 12mg/kg, 24mg/kg or 36mg/kg of IP2 at day 5, 10 and 15 after inoculation. Data are presented at the day before the endpoints are reached. Data are given as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  (ANOVA with Tukey's multiple comparison test comparing all groups).

Figure S6: Additional data from Figure 5

	Doses ( $\mu\text{M}$ )	% cell viability ( $\pm$ SEM)		
		A375	A549	MRC5
Isoginkgetin	2,5	100	100	100
	6,25	100	84 ( $\pm 1,86$ )	100
Madrasin	2,5	76,6 ( $\pm 0,6$ )	79,2 ( $\pm 3,2$ )	77 ( $\pm 6,0$ )
	6,25	57,6 ( $\pm 2,8$ )	69 ( $\pm 2,2$ )	49 ( $\pm 2,3$ )

**Table S1: Percentage of human cell line survival after isoginkgetin and madrasin treatment.**

MTT assay on A375, A549 and MRC5 cell lines treated with 2.5 $\mu\text{M}$  or 6.25 $\mu\text{M}$  of isoginkgetin or madrasin. Data are given as the mean of the percentage of cell viability  $\pm$  SEM from at least three independent experiments.

	Doses ( $\mu\text{M}$ )	% cell viability ( $\pm$ SEM)	
		MCA205	B16F10
Isoginkgetin	6,25	84,8 ( $\pm 9,6$ )	86,5 ( $\pm 2,2$ )
	15	56,3 ( $\pm 4,9$ )	58,6 ( $\pm 3,8$ )
	25	51,7 ( $\pm 4,2$ )	51,2 ( $\pm 4,8$ )
Madrasin	5	57,3 ( $\pm 3,2$ )	49,4 ( $\pm 3,3$ )
	10	43,6 ( $\pm 1,6$ )	38,9 ( $\pm 2,9$ )

**Table S2: Percentage of human cell line survival after isoginkgetin and madrasin treatment.**

MTT assay on MCA205 and B16F10 cell lines treated with 6.25 $\mu\text{M}$ , 15 $\mu\text{M}$  or 25 $\mu\text{M}$  of isoginkgetin, or 5 $\mu\text{M}$  or 10 $\mu\text{M}$  madrasin. Data are given as the mean of the percentage of cell viability  $\pm$  SEM from at least three independent experiments.

## Discussion

Splicing abnormalities have emerged as a specific feature of cancer and are studied as predictive markers for patient survival<sup>313,314</sup> as well as targets for cancer treatments with splicing inhibitors<sup>148</sup>. The majority of aberrant splicing functions in cancer is not known, however several known proteins implicated in cancer hallmarks such as proliferation, apoptosis or invasiveness, have been shown to have pro-tumorigenic function as a result of alternative splicing<sup>143</sup>. Here, we highlight splicing inhibitors as modulators of the adaptive anti-cancer immune response and suggest their action on PTPs production and immunopeptidome modulation. This study raises many questions about the mechanism underlying the action of splicing inhibitors and investigations could both serve the understanding of the immunopeptidome dynamic and the tailoring of anti-cancer splicing inhibitor treatments and immunotherapies.

### **The adaptive immune response role in the splicing inhibitor anti-tumoral efficacy**

In our study we observed that treatment with isoginkgetin or IP2 splicing inhibitors reduces the growth of sarcoma MCA205 and melanoma B16F10 tumors expressing the SL8 antigen derived from PTPs and significantly extends mice survival. By performing the same experiment in Nude *nu/nu* mice, which are deficient in T cells but not in B or innate immune cells, we did not observe tumor growth diminution upon treatment. Furthermore, *in vivo* depletion of CD8+ T cells in immunocompetent mice abrogates tumor clearance observed after IP2 treatment. Finally, we observed that mice treated with isoginkgetin or IP2 and which became tumor-free after treatment elicit a long lasting immune response. Therefore we conclude that an efficient adaptive immune response, particularly a CD8+ T cell response, is required for splicing inhibitors to impact tumor growth.

One possible explanation for isoginkgetin or IP2 mechanism of action could be that they induce tumor cell apoptosis leading to enhanced immune recognition. It was indeed observed in many cases that the initiation of cancer cell apoptosis via chemotherapy or radiotherapy can stimulate the priming of CD8+ T cells via professional antigen-presenting dendritic cells by releasing damage associated molecular patterns (DAMPs)<sup>315,316</sup>. However, we showed here that IP2 treatment has no impact on the death of tumors growing in nude mice (Figure 16 A and B) and does not induce apoptosis at the doses used (Figure S5C and S5D), suggesting that it is unlikely to induce a significant immunogenic cell death.

In this study, we observed a difference between the *in vivo* growth rate of cancer cell lines expressing the PTPs-derived SL8 and their wild type (WT) counterparts (data not shown), suggesting that the expression of the SL8 by cancer cells trigger a mild immune response against the tumor. However, the response is not strong enough to prevent cancer growth. The SL8 peptide is highly immunogenic and should be considered as “non-self” by the immune system as it is not subjected to central tolerance. Therefore, it is likely that cancer cells expressing the SL8 develop mechanisms to escape immune recognition or disappear due to immunoediting, promoting the growth of low immunogenic variants. We here observed that the isoginkgetin or IP2 impact on tumor growth is significantly higher in MCA205 or B16F10 expressing the PTPs-derived SL8 than in WT tumors. This first demonstrates that the immunogenic SL8 epitope plays a key role in the immune response induction upon treatment. Besides, we can infer that treatment helps to counteract mechanisms set up by tumor cells to avoid SL8-specific T cell response. An efficient CD8+ T cell response theoretically rely on three main steps: (1) CD8+ T cell activation by pAPCs followed by their proliferation, (2) T cell infiltration in tumor, and (3) T cell recognition of tumor antigens followed by tumor cell lysis. In that study, *in vitro* experiments provide evidence for madrasin, isoginkgetin or IP2 treatment to induce a quantitatively higher number of presented SL8 peptides, driving specific T cell recognition and activation of their cytotoxic functions. Conversely, the isoginkgetin derivative M2P2 does not increase the SL8-derived PTPs presentation *in vitro* and, unlike IP2 or isoginkgetin, does not impact tumor growth *in vivo*, suggesting that both events may be correlated. Hence, we can reasonably hypothesize that the same mechanism is happening *in vivo* wherein isoginkgetin or IP2 enhances direct cancer cell PTPs-derived SL8 presentation, resulting in a higher T cell cytotoxic activity against tumor cells followed by tumor growth delay or tumor eradication.

Interestingly, some researchers found link between the antigen level expressed by tumor cells and antigen loss variants (ALVs) appearance, which fail to be recognized by specific T-cells<sup>317,157,259</sup>. Schreiber et al. exposed this mechanism when studying the MC57 tumor cell lines bearing different SIY-2 or gp33 antigen level. They showed that although cancer cells expressing low antigen level were rejected by transferred specific cytotoxic T cells, ALVs appeared and were not controlled by the T cell response leading to tumor growth. Conversely, they observed that when high level antigen-expressing cells were injected, T cells eliminated them directly and were also able to eliminate the appearing ALVs by an indirect mechanism. In the latter, the stromal cells did cross-present the specific antigens from parental cells and

were subjected to T cell lysis that impacted bystander ALVs<sup>317</sup>. Latter, IFN- $\gamma$  and TNF released from CTL were also described as key effector molecules in that process<sup>318</sup>. It is hence tempting to hypothesize that the isoginkgetin- or IP2-induced increased SL8 level in tumor allows stromal cell PTPs-SL8 cross-presentation followed by the indirect T cell activation and killing of potential ALVs, leading to the complete tumor rejection we observed in some mice. Besides, ALVs often appear to have lost or downregulated their overall MHC-I antigen expression<sup>286,287</sup>, which can be rescued by type I interferon treatment in some cases<sup>289</sup>. A recent study described a mechanism where IFN- $\gamma$  expressed by T cells after their recognition of highly expressing antigen tumor variants leads MHC-I expression rescue in ALVs<sup>292</sup>, allowing new antigen-specific T cell activation. This mechanism was also suggested during the treatment of melanoma patients with therapeutic vaccination in which researchers observed that tumor regression was driven by CTL clones targeting antigens beyond the scope of the initial vaccine target<sup>293</sup>. Hence, enhanced SL8 antigen presentation by isoginkgetin or IP2 treatment followed by T cell recognition and IFN- $\gamma$  expression could drive the non-SL8-specific T cell anti-tumor action.

Cytotoxic T lymphocytes failure to reject tumors can in part be explained by an initial inappropriate CTL activation by pAPCs. A defined subset of dendritic cells (DCs) has been described in the tumor microenvironment (TME) to be able to migrate to the tumor draining lymph nodes, deliver intact antigens encountered in the TME and prime directly or not naïve CD8 + T cells<sup>201,202</sup>. In addition, it was suggested that some DCs are able to directly prime naïve CD8 T cells in the TME<sup>319</sup>. We recently demonstrated that tumor-associated PTPs are a source material for CD8+ T cells cross-priming by DCs and may mainly be transferred from tumor cells to DCs by PTPs-carrying exosomes. Besides, we provided hints that PTPs for endogenous and cross- presentation are produced by the same translation event and that the two pathways then diverge quickly<sup>303</sup>. PTPs are rare products<sup>82</sup> and the efficiency of PTPs vaccines or exosome-containing PTPs vaccines was shown to rely on the previous PTPs enrichment of PTPs proteasome inhibitor<sup>303</sup>. Hence, it would not be surprising that the splicing inhibitor IP2 or isoginkgetin, in addition to provide more source materials for the direct antigen presentation, enriches the pool of SL8-containing PTPs that serve as a source material for intratumoral DC uptake and cross-presentation, inducing an enhanced SL8-specific CD8+ T cell proliferation.

Intriguingly, while isoginkgetin treatment failed to impact MCA205 or B16F10 WT tumor growth, IP2 treatment inhibits both tumors. Hence, we can infer that even if the presence of

an immunodominant epitope emphasizes the IP2 action and can lead to tumor rejection; it is not required to observe an impact on tumor. Based on the previously exposed hypothesis, IP2 could enhance the direct antigen presentation of some naturally presented PTPs-derived antigens leading to a more efficient direct T cell cytotoxicity and to alternative loss variant eradication, or could quantitatively enrich the PTP pool at the source of DCs cross-presentation and CD8+ T cells priming.

Additional to the IP2 quantitative effect on antigen presentation, its activity on WT tumors suggests that qualitative changes might also happen upon treatment. The immunopeptidome plasticity has been highlighted in several studies<sup>320</sup> and has been linked with many parameters such as intracellular metabolism and transcriptional regulations, gene expression and genetic polymorphism<sup>84,98,102</sup>. For example, mouse lymphoma cell treatment with the mTOR inhibitor rapamycin has been demonstrated to greatly impact the immunopeptidome by increasing numerous MHC-I associated peptides (MIPs) abundance at the cell surface and inducing differentially expressed MIP appearance<sup>100</sup>. In addition, the MIP landscape presented by tumors has been proven to change greatly over the course of treatments, often leading to the appearance of the previously introduced ALVs<sup>263,308</sup> that escape tumor recognition. Therefore, IP2 treatment may induce the presentation of new MIPs recognized by CD8+ T cells, delaying the tumor growth. As we will discuss below, the new MIP appearance might result from the translation of pre-spliced mRNAs enriched after IP2 treatment leading to the apparition of more MIPs derived from non-coding regions, which have been suggested to be frequently mutated in cancer<sup>321</sup>. In accordance with this hypothesis our preliminary results show differentially expressed epitope at the cell surface of MCA205 cells upon isoginkgetin treatment.

### **PTPs production and immunopeptidome modulation upon splicing inhibitor treatment**

As mentioned above, we showed that cancer cell lines treated with the splicing inhibitors madrasin, isoginkgetin or IP2 but not M2P2, up-regulate the expression of the SL8 antigen derived from pioneer translation products (PTPs) on MHC-I molecules *in vitro*. We confirmed that splicing inhibition event is required for isoginkgetin to impact SL8 presentation, observing no impact of treatment on cancer cell lines transfected with a cDNA construct expressing the SL8 peptide without previous splicing. Besides, the SL8 expression modulation after splicing inhibition induces CD8+ T cell activation. Therefore, we conclude that splicing alteration provides a mean to modulate the immunopeptidome.

The PTPs model describes the pre-spliced mRNA as the template for PTP by an alternative translational event occurring in the nucleus. In the study describing this alternative translation, Sébastien Apcher *et al* demonstrated that forced nuclear retention of the mRNA encoding the intronic SL8 peptide leads to an increase in the SL8 antigen presentation<sup>82</sup>. Besides, pladienolides and spliceostatin A (SSA) have been shown to inhibit the splicing by targeting the SF3b, a subcomplex of the U2 small nuclear ribonucleoprotein (snRNP) in the spliceosome, and have been described to promote pre-RNA accumulation in the nucleus<sup>145,146</sup>. The isoginkgetin inhibits differently the splicing by preventing stable U4/U6/U5 tri-snRNPs recruitment right after the the U2 snRNP fixation. However, it is likely that it also induces pre-mRNA accumulation in the nucleus, resulting in the increase in antigen presentation. The link between pre-mRNA nuclear accumulation and increased antigen presentation is not known. It is tempting to hypothesize that pre-mRNA accumulation in the nucleus provides more templates for PTP production leading to the enrichment of SL8-containing PTPs, used as a major source for SL8 direct presentation.

As previously said, the immunopeptidome is shaped by the cell metabolism. The rapamycin was shown to greatly impact the quality and the quantity of MIPs, which was then shown to be associated with the mTOR interactome and its signaling network<sup>100</sup>. Interestingly, breast, lung and ovarian cancer gene expression profiling demonstrated an overexpression of some splicing-regulating proteins, which, when depleted, lead to mTOR expression inhibition<sup>322</sup>. Therefore, cell metabolic modulation owing to cancer cell splicing alteration may provide one possible path for isoginkgetin or IP2 to ultimately impact the MIPs.

In addition, supporting the idea that non-canonical translations can give rise to MIPs<sup>114</sup>, S. Apcher *et al*. demonstrated that MIP derived from mRNA carrying PTC and directed to the non-sense mediated decay (NMD) pathway can still be presented at the cell surface<sup>80</sup>. Splicing regulations are closely linked to NMD and both events can regulate gene expression in cells. Aberrant splicing in cancer was in many cases observed to induce intron inclusion leading to the apparition of a premature translation-termination codon (PTC) that steers the mRNA to destruction by the NMD<sup>135</sup>. Correlating with the PTPs model, this observation implies that IP2 disrupted transcripts that acquire PTC are still subjected to translation before being removed by the NMD and that new epitopes from included intron can potentially appear at the cell surface.

The basis for therapeutic targeting of splicing in cancer is the observation of synthetic lethal interactions, i.e. cell death induction after splicing inhibitor treatments only in cells expressing a specific splicing factor mutant<sup>147</sup>, and in the identification of specific pathologic splicing events that drive cell tumorigenicity and that can potentially be reversed by treatments. The latter mechanism was demonstrated on BRAF-mutated melanoma cells, which develop resistance to the BRAF inhibitor vemurafenib owing to an in-frame skipping of exons 3-5 encoding the RAS-binding domain (RBD). In these cells SSA treatment was shown to restore RDB inclusion and therefore prevent resistance<sup>323</sup>. Similarly, considering that aberrant splicing in cancer can lead to the MIPs modulation, splicing related resistance mechanisms to immunotherapeutic treatment may induce immunogenic antigens loss that could be reversed by isoginkgetin or IP2 treatment.

The pladienolide B derivative E7107 is the only splicing inhibitor to date that entered two separate phase I open-label, single-arm, dose-escalation clinical trials in patients with locally advanced or metastatic solid tumors in 2006<sup>324</sup>. While stable disease was observed upon treatment in the best responder patients, trial was stopped due to bilateral optic neuritis appearance in two patients. This disease is characterized by an immune-inflammation of the optic nerve that induces demyelization and is associated with immune disorders such as multiple sclerosis. This toxicity strongly suggests that E7107 can impact immunity by inducing autoimmune response and that splicing inhibitors might have a predominant role in the immune response modulation. Therefore, a better understanding of splicing inhibition related immune mechanism seems to be required for the establishment of efficient therapies with no strong adverse effects. Because pladienolide B and isoginkgetin target different components of the spliceosome, further studies have to be performed to test the potential immunomodulatory effects of molecules targeting SF3b and to assess the toxicity of molecules preventing the recruitment of the U4/U6/U5 tri-snRNPs in humans.

While a deeper understanding of isoginkgetin and IP2 treatments on splicing is needed, their role during immune response opens a large field of study on the link between splicing and immunopeptidome that could lead to the development of innovative anti-cancer therapies. In parallel the spliceosome emerge as a new and promising druggable target and splicing inhibitors are currently tested for their ability to induce tumor cell death in acute myeloid leukemia. Therefore, their role as immunomodulators worth being operated in clinic<sup>147</sup>.



## **Proof of concept: PTPs elicit an anti-tumor immune response *in vivo*.**

### **Context of the study**

Pioneer translation products (PTPs) resulting from alternative translation of pre-mRNA have been demonstrated as a source for the direct MHC-I antigen presentation pathway<sup>82</sup>. Their role in the recognition of cancer cells has been recently demonstrated *in vivo* in mouse sarcoma and melanoma models expressing the SL8 epitope from translation of the pre-mRNA of the  $\beta$ -globin containing the SL8 peptide in its first intron. In fact, tumor cell inoculation in mice followed by the SL8-specific OT1 T cell transfer induced significant tumor growth delay<sup>303</sup>. The recognition of antigens expressed at the tumor cell surface by tumor antigen-specific T lymphocytes requires the previous activation and expansion of the latter through cross-priming. Therefore, the repertoire of antigens presented on MHC-I molecules by cancer cells has to correlate with the one presented by pAPCs in order to trigger an efficient adaptive T cell response<sup>325</sup>. However, the natural peptide material used for cross-priming is unclear. pAPCs have been described to physiologically acquire exogenous elements from different sources including apoptotic, necrotic and live cells, which can transfer their material through different ways like exosome secretion or gap-junction formation<sup>326</sup>. Exosomes are extracellular nanovesicles released upon fusion of multivesicular bodies (MVBs) with the cellular plasma membrane. In tumors, secreted exosomes were shown to contain tumor antigens that can be cross-presented by pAPCs<sup>327-329</sup>. Therefore, following the observation that PTPs can be efficiently presented by tumor cells, E. Duvallat and colleagues investigated the role of PTPs in the cross-presentation pathway<sup>303</sup>. They observed that PTPs are used for CD8+ T cell cross-priming *in vivo*. Moreover, they showed PTPs transfer from tumor cells to bone marrow dendritic cells (BMDCs) *in vitro* in an intercellular manner and mediated by the secretion of PTPs-containing exosomes.

Some current anti-tumoral immunotherapies rely on the activation of the immune system against specific tumor antigens using therapeutic vaccines. Among them are synthetic peptide vaccines. While several phase III clinical trials using short peptides targeting shared tumor antigens failed to prove efficacy, new peptide vaccine generation took advantage of recent knowledge about the immunization process and tumor immune escape mechanisms and

includes, among other things, multi-epitope synthetic long peptides (SLPs) targeting tumor-specific antigens. Advantages of SLPs lie in their ability to be efficiently processed and cross-presented by pAPCs and to include MHC I and MHC II epitopes. To some extent, PTPs resemble SLPs in their size (20 to 45 amino acids) and in their ability and need to be processed and cross-presented by DCs to activate T cell response. In addition, among developed vaccine strategies are exosomes containing tumor antigens. While currently tested exosome-derived vaccines are showing only modest antigen-specific immune response in human, their refinement shows potential for future development<sup>329-332</sup>. Our group recently demonstrated that purified SL8-PTPs from the sarcoma MCA205 cells expressing the  $\beta$ -globin-SL8-intron (MCA205 GI) injected as a prophylactic vaccine significantly reduce the growth of subcutaneously injected MCA205 GI cells<sup>303</sup>. We further observed that purified SL8-PTPs-containing exosomes secreted by MCA205 GI provide the same effect on tumor growth when used as a prophylactic vaccine. Finally, we observed that the combination of PTPs and PTPs-containing exosome vaccine prevents tumor growth in more than 50% of the mouse population<sup>303</sup>. Overall, we showed that prophylactic vaccination with tumor associated (TA)-PTPs alone or in combination with TA-PTPs-containing exosomes can induce an anti-tumor immune response *in vivo* in the MCA205 mouse sarcoma model.

Here we addressed the efficacy of prophylactic TA-PTPs vaccination on tumor growth prevention in the more aggressive B16F10 mouse melanoma model and provided the proof of concept for the use of PTPs-based vaccine against cancer. Moreover, we observed that vesicles bigger than exosomes, i.e. melanosome-enriched vesicles, contain TA-PTPs efficiently cross-presented by DCs and elicit an anti-tumor response when used as vaccine. Therefore, we proposed a role for tumor melanosome secretion in pAPCs cross-priming of CD8<sup>+</sup> T cells in cancer.

## **Material and methods**

### **Purification of PTPs-HIS**

B16F10 tumor cells are transfected with the construct YFP-Globin-SL8-intron-HIS for 48hours. Cells are then harvested and sonicated in 10mL of 6M guanidium-HCl, 15 0.01M Tris/HCl, pH 8.0, 5mM imidazole and 10mM  $\beta$ -mercaptoethanol. The lysate is incubated and rotated with Ni<sup>2+</sup>-NTA agarose beads (Qiagen) for 4h at RT in glass tubes coated in advance

with Sigmacote® (Sigma-Aldrich). The beads are washed successively for 5 min at RT with 8 mL of each of the following buffers: 6M guanidium-HCl, 0.01M Tris/HCl, pH 8.0 and 10mM  $\beta$ -mercaptoethanol; 6M urea, 0.01M Tris/HCl, pH 8.0 and 10mM  $\beta$ -mercaptoethanol; 6M urea, 0.01M Tris/HCl, pH 6.8, 10mM  $\beta$ -mercaptoethanol and 0.2% Triton X-100; 6M urea, 0.01M Tris/HCl, pH 6.8, 10mM  $\beta$ -mercaptoethanol and 0.1% Triton X-100. PTPs are then eluted by incubating the beads for 1 hour at RT in 400mM imidazole, 0.15M Tris/HCl, pH 6.8, 30% glycerol, 0.72M  $\beta$ -mercaptoethanol and 5% SDS. The eluate is dialyzed in PBS using a dialysis tubing Spectra-Por® Float-A-Lyzer® MWCO 0.5-1kD (Dutscher) overnight at RT. Finally, the eluate is quantified by a colorimetric peptide assay (ThermoFisher). PTPs-HIS purification from melanosomes purified from the medium of B16F10 cells transfected with the YFP-Globin-SL8-intron is performed the same way after melanosome sonication.

### **Exosome and melanosome purification, transmission electron microscopy (TEM)**

Exosomes are purified from the medium of B16F10 cells transfected with the YFP-Globin-SL8-intron construct and previously treated with the proteasome inhibitor MG132, by differential ultracentrifugation as described previously<sup>333</sup>. Briefly, the medium is centrifuged at 300g for 10min. The supernatant is then centrifuged at 2,000g for 10min, followed by 10,000g for 30min and exosomes are finally pelleted by a centrifugation at 100,000g for 70min. Melanosomes are purified from the medium of B16F10 cells transfected with the YFP-Globin-SL8-intron construct and then treated with the proteasome inhibitor MG132. The medium is centrifuged at 300g for 10 min. The supernatant is then centrifuged at 1,000g for 30 min, before melanosomes are pelleted by a centrifugation at 20,000g for 60 min. For negative staining of melanosomes, five microliters of melanosome fractions are adsorbed onto a 300 mesh copper grid coated with a collodion film covered by a thin carbon film, activated by glow-discharge. After 1 min, grids are washed with aqueous 2% (w/vol) uranyl acetate (Merck, France) and then dried with ashless filter paper (VWR, France). TEM observations are carried out on a Zeiss 912AB transmission electron microscope in filtered low loss mode. Electron micrographs are obtained using a ProScan 1024 HSC digital camera and Soft Imaging Software system (Eloise).

### **Flow cytometry analysis of exosomes**

Purified exosomes are coated on aldehyde/sulfate latex beads (ThermoFisher Scientific) and are labeled with anti-mouse-CD9-PE or anti-mouse-CD81-PE from BD Biosciences and with

the anti-mouse-MHC-I(H-2K<sup>b</sup>)-FITC or the anti-mouse MHC-I(H-2D<sup>b</sup>)-FITC from ebioscience. The labelling is performed at 4 °C using PBS containing 1% FBS for 30 min. Cells are analyzed using the BD LSRII flow cytometer (BD Biosciences) and data are analyzed with the FlowJow software (V10).

### **BMDCs generation and cross-presentation**

The BMDCs are generated from C57Bl/6 bone marrow precursors. Briefly, mouse femurs are harvested, sterilized with 70% ethanol, and washed in sterile PBS and PBS/ 5% fetal bovine serum (Life Technology) successively, to remove soft tissues; both ends of the femurs are cut off and the bone marrow cells are flushed out with PBS/5% FBS. Red blood cells are removed using the ACK lysis buffer and bone marrow cells are then seeded at  $0.5 \times 10^6$  cells/mL in 145-mm Petri dishes for 6 days in IMDM (Sigma) supplemented with 10% heat-inactivated FCS, 0.5% penicillin/streptomycin, 2mM L-glutamin, 50  $\mu$ M  $\beta$ -mercaptoethanol (Life Technology), and enriched with 50 ng/mL J558 supernatant. After 3 days of culture, the medium is replaced. On day 6 of BMDC differentiation,  $0.3 \times 10^6$  BMDCs are put in contact with 60 $\mu$ g ovalbumin (positive control), 60 $\mu$ g BSA (negative control) or 30 $\mu$ g of exosomes or melanosomes purified from B16F10 cells transfected with the plasmid YFP-Globin-SL8-intron, for 5 hours.  $0.3 \times 10^6$  B3Z are then added in each well and incubated at 37°C with 5% CO<sub>2</sub> overnight. Cells are centrifuged at 1200rpm for 5min, washed twice with PBS 1X and lysed for 5min at 4°C under shaking with the following lysis buffer: 0.2% TritonX-100, 0.2% DTT, 90 mM K<sub>2</sub>HPO<sub>4</sub>, 8.5 mM KH<sub>2</sub>PO<sub>4</sub>. The lysate is centrifuged at 3000rpm for 10min and the supernatant is transferred to a 96-well optiplate (Packard Bioscience, Randburg, SA). The revelation buffer containing 33mM of methylumbellifery  $\beta$ -D-galactopyranoside (MUG) is added and the plate is incubated at room temperature for 3 hours. Finally, the  $\beta$ -galactosidase activity is measured using the FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany). Results are obtained in relative light units (RLU).

### **Vaccination and tumor challenge**

Vaccines are prepared and kept on ice for 2 hours before injection. Seven week old C57Bl/6J female mice obtained from Harlan Laboratories are anesthetized with 3% isoflurane and vaccine is administered intramuscularly (150 $\mu$ L/leg) and in the footpad (50 $\mu$ L/foot). Two weeks later, mice are injected subcutaneously on the right flank with  $5 \times 10^4$  B16F10-Ova cells and on the left flank with  $5 \times 10^4$  B16F10 wild type cells along with matrigel (VWR). Area of

the tumor is recorded every 3 to 4 days until ethical limit points are reached. Results are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (ANOVA with Tukey's multiple comparison test comparing all groups).

## Results

### Melanoma-derived PTPs induce an anti-tumor response *in vivo*

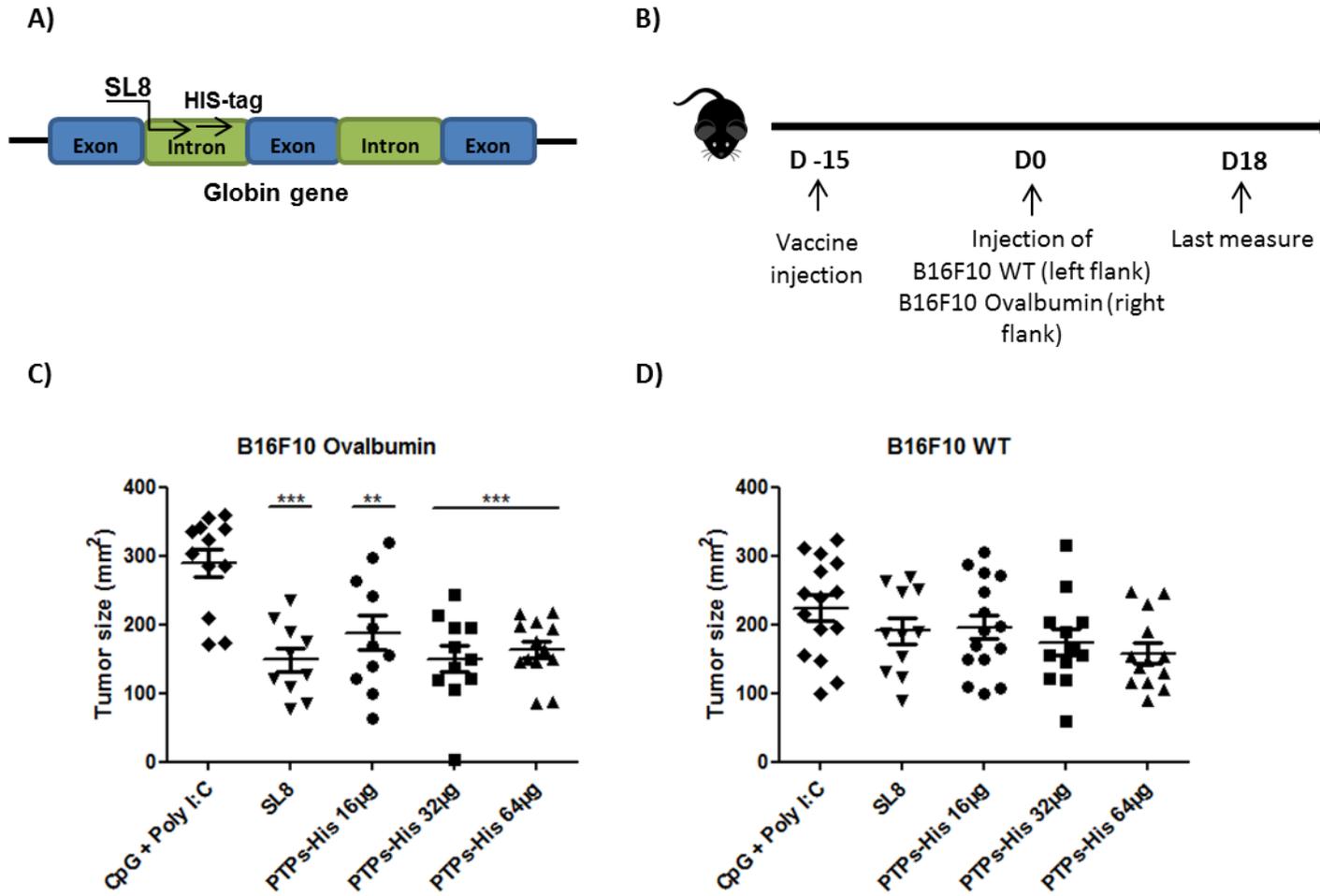
PTPs have been described as 7 to 40 amino acid long peptides potentially deriving from 'non coding' sequences such as intron, intron/exon junctions, 5' and 3' UTRs or alternate translational reading frame<sup>82</sup>. In order to purify PTPs from cancer cells we transfected the B16F10 melanoma cells with the  $\beta$ -globin-SL8-intron-HIS construct in which the SL8 peptide is inserted in the  $\beta$ -globin gene first intron and directly followed by 6xhistidine tag (Figure 17A). PTPs produced from the translation of the  $\beta$ -globin-SL8-intron-HIS construct, referred to as PTPs-HIS hereunder, were isolated and enriched using affinity purification of His-tagged peptides. PTPs-HIS or SL8 synthetic peptides were inoculated intramuscularly in C57BL/6 mice along with CpG and poly I:C adjuvants. Two weeks later mice were re-challenged with the subcutaneous injection of B16F10 cells stably expressing the ovalbumin protein on the right flank and with B16F10 WT cells on the left flank (Figure 17B). We observed that the growth of B16F10 ovalbumin (Ova) tumors was significantly slowed in mice vaccinated with 16 $\mu$ g, 32 $\mu$ g and 64 $\mu$ g PTP-HIS similarly to mice vaccinated with the SL8 synthetic peptide (Figure 17C). In the group of mice treated with 16 $\mu$ g PTPs-HIS few B16F10 Ova tumors were shown to grow at the same pace as the negative control group whereas in groups of mice treated with 32 $\mu$ g and 64 $\mu$ g PTPs-HIS all animal tumors were shown to grow more slowly, showing a dose-dependent effect. Conversely, B16F10 WT tumors grew equally in all groups (Figure 17D), highlighting the role of the dominant SL8 peptide contained in PTPs in tumor control by immune cells. Hence, we confirmed in this mouse melanoma model the results observed in the mouse sarcoma model MCA205, in which tumor-associated PTPs vaccination can efficiently trigger an immune response that is recalled upon tumor re-challenge

## **PTPs-carrying melanosome vaccination but not exosome vaccination prevents subsequent tumor growth.**

We previously observed that the extracellular vesicle exosomes secreted by sarcoma tumor cells contain tumor-associated PTPs that are efficiently cross-presented *in vitro* by BMDCs and constitute an efficient prophylactic anti-cancer vaccine in mice. Secreted vesicles represent an important mode of intercellular communication allowing the transfer of proteins, peptides, lipids and RNAs<sup>334</sup>. Along with exosomes, pigment cells express unique lysosome-related organelles called melanosomes, whose primary role is to transport melanin pigments to keratinocytes. In melanoma, many antigens associated to tumor have been described to originate from proteins with melanosome-related functions<sup>335</sup>. We here tested if vesicles secreted by the melanoma B16F10 model contain PTPs as well. By performing differential ultracentrifugation from the culture supernatant of melanoma B16F10 cells transiently expressing the  $\beta$ -globin-SL8-intron-HIS construct, we obtained one fraction containing 100nm diameter vesicles expressing the CD81 and CD9 surface proteins, i.e. the exosome fraction, (Figure 18A) and one fraction enriched in 200nm diameter melanized vesicles, i.e. the melanosome fraction<sup>336</sup> (Figure 18B). After incubation of the exosome or melanosome fraction with BMDCs *in vitro* we observed the activation of SL8-specific B3Z cells (Figure 18C). These results suggest that the fractions contain SL8-PTPs efficiently cross-presented by BMDCs. In order to confirm it, we purified PTPs-HIS from sonicated melanosome fraction as described above and subjected the eluate to LC-MS/MS mass spectrometry analysis. Table S3 shows different peptide fragments, including the SL8 epitope.

We further tested the ability of the exosome and melanosome fractions to elicit anti-tumor immune response *in vivo* when used as a prophylactic vaccine. Fractions were injected intramuscularly in mice in combination with 32 $\mu$ g PTPs-HIS or alone, along with CpG and poly I:C adjuvants. We first observed that mice treated with exosomes alone do not display reduced B16F10 Ova tumor growth whereas mice treated with PTPs-HIS alone display slowly growing tumors as previously observed (Figure 18D). In addition, when exosomes were injected along with PTPs-HIS, B16F10 Ova tumors grew faster than when PTPs-HIS were injected alone, suggesting that B16F10 exosomes prevent in part the beneficence of PTPs-HIS vaccination for the anti-tumor immune response. Mice injected with B16F10 WT cells did not benefit from any of the vaccines, indicating that the immune response is SL8-specific (Figure 18E). Conversely, we then observed that mice treated with melanosomes significantly display reduced B16F10 Ova tumor growth, similarly to mice treated with PTPs-HIS alone (Figure

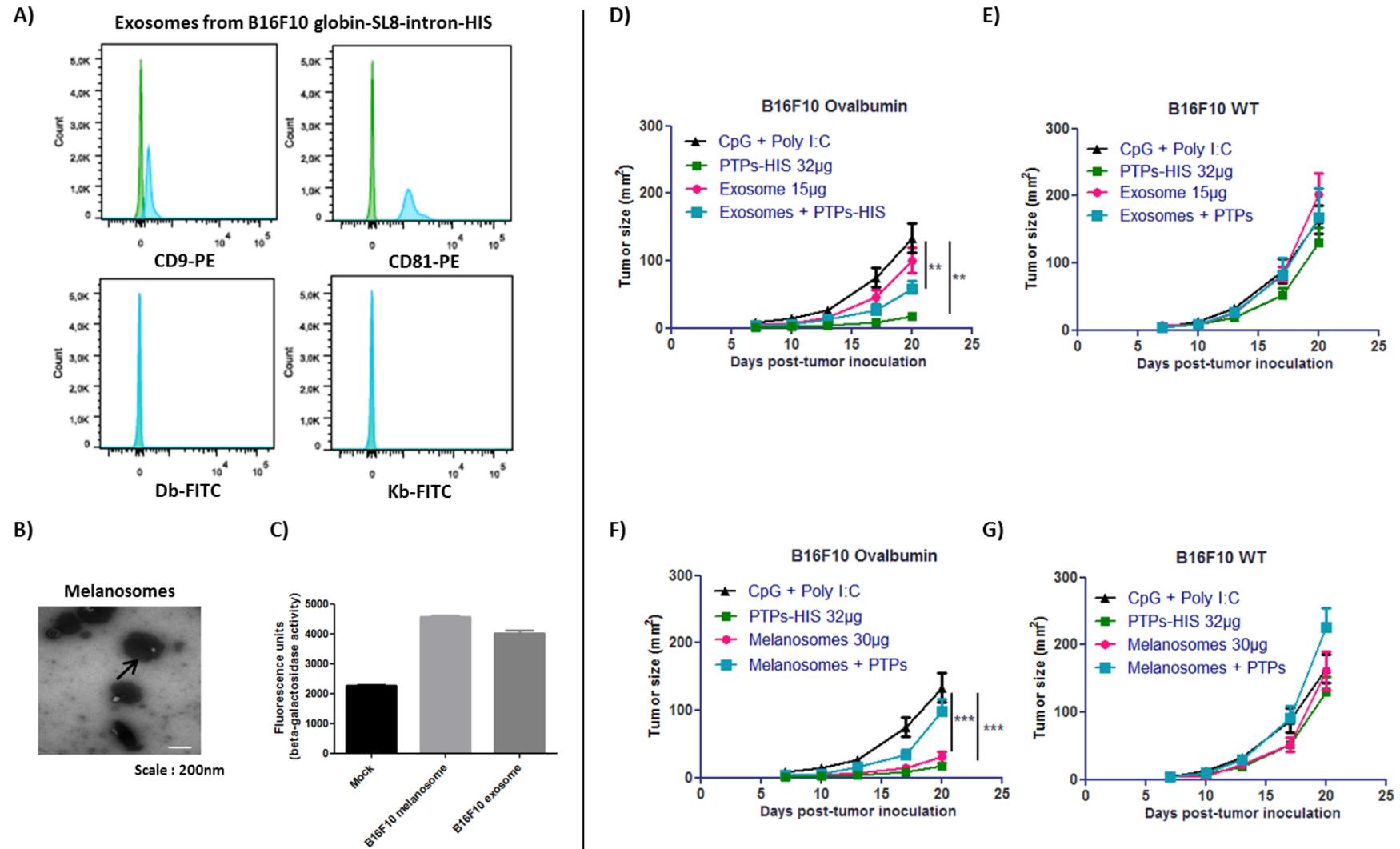
18F). Surprisingly, no significant difference is observed for the B16F10 Ova tumor growth in mice treated with the combination of melanosomes and PTPs-HIS compared to non-treated mice. This result suggests that, in that setting, immunization capacities of melanosomes and PTP-HIS cancel each other out. Again, mice injected with B16F10 WT cells did not benefit from any of the vaccine (Figure 18G). We showed here that, contrary to MCA205 sarcoma tumors, B16F10 melanoma tumors do not benefit from vaccination with exosomes containing tumor associated-PTPs. Intriguingly, we observed that melanosomes secreted by B16F10 cells carry tumor-associated PTPs that can trigger a CD8<sup>+</sup> specific response *in vitro* and an anti-tumor response *in vivo* when used as a prophylactic vaccine. However their mechanism of action *in vivo* is unclear and this finding opens a new research topic on the role of melanosomes in cross-presentation during the anti-cancer immune response.



**Figure 17: PTPs vaccine in melanoma**

## Figure 17

(A) Picture illustrating the position of the SL8 antigenic epitope and the 6xhistidine tag in the sequence of the  $\beta$ -Globin gene. (B) Scheme of the experiment. C57BL/6 mice were vaccinated with 16 $\mu$ g, 32 $\mu$ g or 64 $\mu$ g of PTPs-His or with 8 $\mu$ g of SL8 emulsified in CpG and Poly I:C adjuvants. Fifteen days later mice were rechallenged subcutaneously with (C)  $5 \times 10^4$  B16F10 ovalbumin in the right flank along with matrigel and (D)  $5 \times 10^4$  B16F10 wild type cells in the left flank along with matrigel. Data are presented at the day before the endpoints are reached. Data are given as mean  $\pm$  SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (ANOVA with Tukey's multiple comparison test comparing all groups).



**Figure 18: Exosome- and melanosome-carrying PTPs vaccine in melanoma**

## Figure 18

(A) Analysis of the expression of CD9, CD81, H2-Db and H2-Kb in exosomes purified from B16F10 cells expressing the YFP-Globin-SL8-intron construct by FACS. In green the unstained exosomes, in blue stained exosomes. (B) Negative staining of the melanosome-enriched fraction from B16F10 cells expressing the YFP-Globin-SL8-intron construct by transmission electron microscopy. (C) Activation of SL8-specific B3Z cells after overnight co-culture with BMDCs previously pulsed with exosome or melanosome-enriched fraction purified from B16F10 cells expressing the YFP-Globin-SL8-intron construct. Growth curve of (D) B16F10 ovalbumin cells or (E) B16F10 WT cells in mice vaccinated with 32 $\mu$ g PTPs-HIS, 15 $\mu$ g exosomes purified from B16F10 cells expressing the YFP-Globin-SL8-intron construct, or with a combination of 32 $\mu$ g PTPs-HIS with 15 $\mu$ g exosomes along with CpG and Poly I:C adjuvants two weeks before subcutaneous melanoma cells injection in matrigel. Growth curve of (F) B16F10 ovalbumin cells or (G) B16F10 WT cells in mice vaccinated with 32 $\mu$ g PTPs-HIS, 30 $\mu$ g melanosomes purified from B16F10 cells expressing the YFP-Globin-SL8-intron construct, or with a combination of 32 $\mu$ g PTPs-HIS with 30 $\mu$ g melanosomes along with CpG and Poly I:C adjuvants

two weeks before subcutaneous tumor injection in matrigel. Each line represents the tumor size in area (mm<sup>2</sup>) of at least 5 mice in each group. Data are given as mean  $\pm$  SEM. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $P$ <0.001 (ANOVA with Tukey's multiple comparison test comparing all groups).

Peptide sequence	Peptide length (a.a)
LEYNYNSHNVYIMADK	16
AGYTMVHLTPEEK	13
SAMPEGYVQER	11
SAVTALWGK	9
VNVDEVGGEALGR	13
DHMLLEFVTAAGITLGMDELYK	23
FEGDTLVNR	9
GEELFTGVVPILVELDGDVNGHK	23
AGYTMVHLTPEEK	13
AEVKFEGDTLVNRIELK	17
GIDFKEDGNILGHK	14
TIFFKDDGNYK	11
SIINFEK	7
FSVSGEGEGDATYGK	15
SAVTALWGKVNVDVDEVGGEALGR	22
KAGYTMVHLTPEEK	14
YQTSLYK	7
FSVSGEGEGDATYGKLTLLK	19
FEGDTLVNRIELK	13

**Table S3:** Mass spectrometry analysis of peptides purified from melanosomes produced by B16F10 cells expressing the YFP-Globin-SL8-intron construct

## Discussion

Nowadays, anti-cancer peptide vaccines constitute an extensively investigated immunotherapeutic strategy<sup>337</sup>. Failure of the first therapeutic vaccines in clinical trials encouraged the development of studies that focus on elucidating the key parameters to mount an efficient and durable immune response. Among them are the choice of antigens targeted and the nature of the vaccine<sup>338</sup>. While PTPs were first described in healthy cells, a recent study from our group highlighted their role as a source for the direct MHC-I presentation of tumor antigens (TAs) in cancer cells as well as for TA cross-presentation in mice<sup>303</sup>. Here, we provide evidence for PTPs-based vaccine to trigger an efficient anti-tumor response when used in prophylactic setting in the B16F10 mouse melanoma model, confirming results observed in the mouse sarcoma model MCA205. Therefore, we demonstrate that PTPs-derived antigens play a significant role during the immune response against tumor. Besides, we suggest that antigens derived from alternative translation are pertinent vaccine targets. Supporting the latter idea, some tumor infiltrating lymphocytes (TILs) in human were described to recognize antigens derived from alternative open reading frame<sup>75,339,340</sup>, from non-AUG initiation codons<sup>107,110</sup>, from IRES-dependent translation<sup>121</sup> or from intronic sequences<sup>116,115</sup>. All these antigens can be gathered in the category of antigens derived from defective ribosomal products (DRiPs)<sup>341</sup>, which have been demonstrated to be a source for MHC-I associated peptides (MIPs). DRiPs are described as polypeptides that arise from alternative or defective mRNA, from alternative reading frames or translation initiation codons, and from errors in converting genetic information to proteins<sup>77</sup>. Their large definition hence comprises PTPs, which are produced from an alternative mRNA template by a non-canonical translational mechanism<sup>82</sup>. Along with the example given above in cancer, direct presentation of antigens derived from DRiPs was described during autoimmunity<sup>342</sup> or viral infection<sup>95,111,121</sup>. In addition, a recent large scale study on B-lymphoblastoid cells (B-LCLs) estimated that allegedly noncoding genomic sequences or exonic out-of-frame translation represent around 10% of the MIPs<sup>129</sup> among which intronic source represent 5,2%. Nevertheless, authors argued that they likely have underestimated the proportion of non-conventional antigens present at the cell surface.

Despite these observations, currently targeted antigens in cancer vaccines are derived from canonical translation of mature mRNAs. The first antigens identified and targeted in clinic belonged to the tumor-associated antigen category. The latter includes differentiation,

overexpressed and cancer-testis antigens that share the characteristic of being expressed by some non-tumoral tissues and subject to central tolerance, which eradicates high-avidity T cells<sup>246-248</sup>. While in few cases they proved to be efficient in controlling tumor growth<sup>243</sup>, they were also associated with autoimmune adverse effects<sup>343,344</sup> or clinical trial failures<sup>266</sup>. Evolution of the cancer vaccine field highlighted the potential of neoantigens for eliciting tumor-specific response mediated by high avidity T-cells. Rational for their development lie in the cancer cell expression of epitopes that derive from tumor-specific polypeptides or proteins translated from mutated genes, and that are therefore not subject to central tolerance<sup>280</sup>. An increasing number of cancer patients are identified with neoantigen-specific T cells<sup>345-347</sup> and the therapeutic efficiency of neoantigen vaccines has been proven in few studies<sup>281,348,349</sup>. While mature RNAs are mainly composed of exons, PTPs can result from the translation of intron, exon/intron or exon/UTRs junctions and of 3' or 5' UTRs. Interestingly, the majority of somatic mutations in cancer occurs in non-coding regions<sup>321</sup>, suggesting that PTPs and other non-canonical derived MIPs are likely to constitute a rich source of neoantigens. Moreover, the percentage of MIPs that contains antigens derived from polymorphic regions have been estimated to 12%<sup>102</sup> and the above cited study on B-LCLs found that non-conventional MIPs are enriched in non-synonymous single nucleotide polymorphisms when compared to conventional ones<sup>129</sup>. Therefore, non-conventional antigens are likely to represent a significant part of T-cell targeted antigens in tumor, reinforcing the relevance of their study as vaccine targets.

Furthermore, the MIPs are plastic and reflect the metabolic and the transcriptional states of cells<sup>100</sup>. Cancer cells are strongly exposed to stress and have deregulated metabolism and transcriptional states. The integrative stress response was recently shown to modulate translational events in cells by downregulating the eIF2 expression, inducing a decrease in canonical initiation translation and promoting translation from 5'UTR regions for certain transcripts<sup>350,351</sup>. In addition, aberrant splicing is a feature shared by many cancer types<sup>137</sup> and can lead to increased number of retained introns and RNA containing premature stop codons directed to the non-sense mediated decay (NMD) pathway<sup>135</sup>. As PTPs are produced upstream the NMD pathway<sup>352</sup>, aberrant splicing might impact gene expression and might modulate PTPs production for immunosurveillance. Hence, because non-conventional sources of antigens are likely to be enriched in stressed cells, we can hypothesize that these antigens are preferentially expressed in cancer. This idea reflect our previous work where we showed that cell treatment with the IP2 splicing inhibitor greatly impact the expression of PTPs-derived

antigens. While the current strategy to identify neoantigens relies on whole exome sequencing<sup>281,353,354</sup>, it seems worthwhile to broaden our search of tumor specific antigens to ‘untranslated’ sequences, which are predicted to be particularly enriched in antigenic and immunogenic features<sup>355</sup>. Our work hence provides the first proof that non-conventional antigens are efficiently triggering an anti-cancer immune response when used in vaccine.

Along with the antigen target, the very nature of the vaccine is also important for its success. Short peptides, constituting the exact epitope recognized by CD8 T cells, were the first type of peptide vaccines used in clinical trials. However, they were shown in some cases to trigger exhaustion or deletion of antigen specific T-cells as they do not have to be processed by specific pAPCs to be presented on MHC-I molecules<sup>275,276</sup>. Conversely, previously introduced SLPs comprise several epitopes in one single peptide and need to be uptaken and prepared by pAPCs to prime T-cells<sup>277,278</sup>. Similarly, we showed here that PTPs are efficiently cross-presented by BMDCs and are unlikely to induce T cell exhaustion as they first have to be processed to be presented. The PTP model we are studying contains only one MHC-I associated antigen that activates CD8+ specific T-cells, so we are unable to observe T helper cell activation after vaccination. Nevertheless, as our model PTPs can be longer than 20 amino acids<sup>82</sup> and are transferred from cancer cells to pAPCs<sup>303</sup>, it is possible that naturally expressed PTPs comprise both CD8+ and CD4+ T specific epitopes. Because they are naturally produced by cancer cells and trigger anti-tumor T cell response *in vivo*, one can hypothesized that PTPs have the ‘ideal’ sequence for being correctly processed by pAPCs and presented to T-cells.

The purification and enrichment of PTPs we used in the study were only possible thanks to the expression of the histidine tag by PTPs derived from our artificially expressed construct. In fact, PTPs, such as DRiPs, are characterized by their high instability<sup>356,303</sup> and are hence rapidly degraded in cells. Moreover, the naturally expressed PTPs nature and mechanism of translation and the breath of PTPs-derived MIPs are still unrevealed. Besides, a low proportion of gene products is actually antigenic<sup>84,357</sup> and features of potential immunogenic PTPs are unknown. While there is a big gap between the proof of concept for anti-tumor PTPs-vaccine efficacy and their potential development in clinic, this study provides new hints for alternative antigens to be relevant for the anti-tumor immune response. The identification of potential antigenic and immunogenic cancer specific mutations from RNA sequencing databases is still a challenge and the exploration of mutations that could impact alternative translation products add even more complexity to neoantigens prediction. The development of

computational methods along with new insights on PTPs production mechanism and nature will likely enable personalized PTPs or DRIPs vaccine development.

In addition to PTP vaccine alone, we here provide evidence for melanosome-carrying PTPs but not for exosome-carrying PTPs expressed from melanoma cells to efficiently trigger an anti-tumor immune response. This result contrasts with the previous finding that exosome-carrying PTPs expressed by MCA205 cells constitute an efficient anti-tumor vaccine in mice. Exosomes expressed by tumors were shown to contain tumor-associated antigens<sup>327,328</sup> and were tested in different clinical trials as tumor vaccines. While these trials show only modest effects on the immune response<sup>331</sup>, separate studies demonstrate exosome efficacy in priming T cell response through efficient uptake and delivery of TAs by Dcs<sup>327,329</sup>. Nevertheless, along with tumor antigens, exosomes were shown to contain other elements that can be transferred to dendritic or stromal cells such as proteins, DNA, miRNA or lncRNA. These bioactive molecules can have negative impact on tumor control by promoting tumor development and metastasis, or by suppressing the antigen-specific and non-specific immune cancer response by different means<sup>332</sup>. Therefore, we can hypothesize that B16F10-derived exosome content is enriched in elements suppressing the anti-tumor response and preventing exosome vaccine efficiency, whereas it might not be the case for MCA205-derived exosomes. Moreover, like exosomes, melanosomes are involved in intercellular communication. Melanosome primary function is to transfer the melanin from pigment cells to keratinocytes. Their role in cross-presentation has never been suggested. However, they were recently demonstrated to transfer miRNAs to fibroblasts, inducing the formation of tumor primary niche<sup>358</sup>. Moreover, antigens derived from melanosomal proteins are recurrent targets for CD8+ and CD4+ T response to melanoma cells in humans<sup>335</sup>. Hence, these studies suggest a role, negative and/or positive, for melanosomes in tumor development and in the immune response against melanoma. Here, we provide the first evidence for B16F10 melanosome-enriched fractions to contain tumor associated PTPs that can be cross-presented by BMDCs *in vitro* and that can induce an anti-tumor response *in vivo*. However, melanosome fraction from our study needs to be further characterized and other melanoma models should be used to confirm our observations.

Proteins<sup>359</sup>, proteasome substrates<sup>360</sup>, cytosolic peptides (only in the presence of HSP90)<sup>361,362</sup> or PTPs<sup>303</sup> produced by live tumor cells were shown to be transferred to pAPCs for cross-presentation. In addition to the previously described exosome vesicles, the autophagosomes, which range in size from 300 to 900nm, were shown to carry and transfer DRiPs and short

live proteins (SLiPs) to pAPCs<sup>363</sup>. They were named defective ribosomal products-containing autophagosome-rich blebs (DRibbles). DRibbles anti-cancer vaccine have been tested in few pre-clinical studies and are produced by blocking proteosomal degradation and manipulating the cellular autophagy pathway, leading to DRiPs or SLiPs stabilization and autophagosome microvesicle formation<sup>364-366</sup>. Because they contain DRiPs, DRibbles are likely to contain PTPs. In addition, proteasome inhibition has been shown to stimulate the cross-presentation of PTPs<sup>303</sup> and, before harvesting melanosomes or exosomes for the establishment of vaccine in our study, cells had to be treated with a proteasome inhibitor in order to enrich PTPs in those vesicles. This suggests that a similar peptide material enrichment is needed for DRibbles and exosomes/melanosomes to be used as cancer vaccine. Interestingly, DRibbles were suggested to originate from the fusion of outer autophagosome membranes with multivesicular bodies or the cell plasma membrane<sup>367</sup>, indicating that they might comprise elements present in exosomes such as PTPs. Hence, it would be interesting to look for the presence of PTPs in autophagosomes. In addition, as autophagy is upregulated in tumors, the significant contribution of alternative sources of antigen peptides for cross-presentation in stressed cells is again suggested.

## Résumé de la thèse en Français

Le système immunitaire joue un rôle prépondérant dans le contrôle de la croissance des tumeurs. La compréhension des mécanismes mis en jeu lors de l'élimination des cellules cancéreuses par le système immunitaire est un domaine de la recherche en constante évolution et donne naissance à de nouvelles stratégies thérapeutiques. Toutes les cellules nucléées présentent à leur surface des peptides antigéniques en complexes avec les molécules du complexe majeur d'histocompatibilité de classe I (CMH-I). Ces peptides sont longs de 8 à 12 acides aminés et sont le reflet de l'activité intracellulaire. Leur présentation guide la reconnaissance des éléments potentiellement dangereux par le système immunitaire, principalement par les lymphocytes T cytotoxiques CD8<sup>+</sup> et les lymphocytes T auxiliaires CD4<sup>+</sup>. Les cellules cancéreuses présentent des antigènes associés aux tumeurs (AAT) qui constituent la cible des vaccins thérapeutiques anti-cancéreux actuellement développés. Les premiers vaccins ciblant ces AAT et inclus dans des essais cliniques n'ont cependant pas répondu aux attentes en termes de bénéfices pour la survie des patients. Le développement de mécanismes immunosuppresseurs par les tumeurs et les choix non optimaux des antigènes ciblés ont été décrits comme les principales causes de ces premiers échecs thérapeutiques. Par exemple, la diminution ou la perte de présentation de tous les peptides antigéniques ou de certains peptides antigéniques immunogènes présentés par le CMH-I dans les cellules tumorales est un des événements moteur de l'immuno-sélection des tumeurs au cours des traitements et du développement des tumeurs. Cette diminution ou perte est due à l'apparition de déficiences tout au long de la voie intracellulaire permettant la présentation des peptides antigéniques. En plus de la quantité, la nature des antigènes présentés à la surface cellulaire, aussi appelé l'immunopeptidome du CMH-I, est un élément déterminant pour la réponse immunitaire anti-tumorale. Par exemple, dans les cancers où un AAT est identifié et ciblé par immunothérapie, comme l'antigène Her/neu dans les cancers du sein ou l'antigène CEA dans les cancers du côlon, la perte d'expression de cet AAT à la surface des cellules tumorales conduit à l'échappement de la réponse immunitaire par ces cellules cancéreuses. Afin d'empêcher l'apparition de mécanismes d'échappement immunitaire, les stratégies vaccinales actuelles accroissent le nombre et la qualité des antigènes cibles et recherchent des moyens de restaurer la présentation antigénique dans les cellules tumorales.

Afin d'identifier de nouveaux AAT efficaces en tant que cibles, il est important de comprendre leur origine intracellulaire. L'immunopeptidome est dynamique, flexible et a été montré comme étant le reflet des régulations cellulaires métaboliques et transcriptionnelles

ainsi que de l'expression génique et du polymorphisme génétique. Plusieurs sources de peptides antigéniques pour la voie du CMH de classe I ont été décrites. Les protéines sénescents ont d'abord été identifiées et étudiées comme source, suivie des protéines rapidement dégradées après synthèse. De plus, des produits de traductions alternatives ont été mis en évidence comme une riche source d'AAT et sont généralement regroupés sous le nom de « Defective Ribosomal Products » (DRiPs). Notre groupe a mis en évidence une traduction cellulaire pionnière réalisée à partir d'ARNs précurseurs indépendante de la traduction produisant des protéines fonctionnelles. Cette traduction donne naissance aux « Pioneer Translation Products » (PTPs). Ainsi, les PTPs peuvent dérivés de séquences introniques, de régions 3' ou 5' UTR ou de cadres de lecture alternatifs. Lors de ma thèse l'équipe a montrée que les PTPs jouent un rôle dans le développement des cancers et la réponse immunitaire anticancéreuse. En effet, nous avons observé que les cellules cancéreuses présentant un antigène dérivé de PTPs sont reconnues par des lymphocytes CD8+ spécifiques de cet antigène lorsqu'elles sont inoculées à la souris, conduisant à une diminution de la croissance tumorale dans un modèle de mélanome et un modèle de sarcome murins. De plus, dans ces même modèles, nous avons pu montrer lors de ma thèse que l'injection de PTPs contenant un antigène modèle, purifiés à partir de cellules cancéreuses en culture, promeuvent une réaction contre le développement des cellules cancéreuses chez la souris lorsqu'ils sont injectés en tant que vaccin prophylactique.

La découverte des PTPs met en avant le rôle de l'épissage dans la génération des antigènes associés ou non aux tumeurs (AAT). L'épissage des ARNs précurseurs se passe dans le noyau grâce au protéasome, un complexe multi-protéique dynamique composé de cinq petits ARNs nucléaires interagissant avec plus de 180 protéines. Un nombre croissant d'études rapportent que les dérégulations ou mutations des composants du protéasome, fréquentes dans la plupart des cancers, donnent naissance à des épissages aberrants contribuant à la prolifération des cellules tumorales. Ces dernières années, plusieurs produits microbiens et leurs analogues synthétiques ont été caractérisés comme inhibiteurs du protéasome. Parmi eux on trouve les pladiénolides B et D, la splicéostatine A, le FR901464, l'E7107, l'isoginkgetine ou la madrasine. En plus de leur activité cytotoxique, les inhibiteurs de l'épissage ont été d'un grand intérêt pour l'équipe pour leur impact potentiel sur la production des PTPs et la présentation antigénique.

Ainsi, lors de ma thèse, nous avons montrés que la biflavonoïde isoginkgetine et le dérivé que nous avons synthétisé IP2 augmentent la présentation des antigènes dérivés de PTPs modèles

exprimés dans des lignées cancéreuses humaines de sein, de poumon et de mélanome et murines de sarcome et mélanome. De plus, le traitement chez la souris avec IP2 diminue la croissance tumorale et stimule l'activité anticancéreuse du système immunitaire *in vivo* dans un modèle de sarcome induit. Notre travail propose un nouveau mécanisme d'action des inhibiteurs de l'épissage contre les cancers, agissant en modulant l'immunopeptidome à la surface des cellules cancéreuses par l'augmentation de la présentation des antigènes dérivés des PTPs et en stimulant la réponse immunitaire anti-tumorale spécifique des lymphocytes T CD8+.

## Bibliography

1. Klein, J. George Snell's first foray into the unexplored territory of the major histocompatibility complex. *Genetics* **159**, 435–439 (2001).
2. Breuning, M. H. *et al.* Localization of HLA on the short arm of chromosome 6. *Hum. Genet.* **37**, 131–9 (1977).
3. Benacerraf, B. Role of MHC gene products in immune regulation. *Science* **212**, 1229–1238 (1981).
4. Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat. Rev. Cancer* **14**, 135–46 (2014).
5. McDevitt, H. O. & Chinitz, A. Genetic control of the antibody response: relationship between immune response and histocompatibility (H-2) type. *Science* **163**, 1207–1208 (1969).
6. Zinkernagel, R. M. & Doherty, P. C. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. **141**, 1427–1436 (1975).
7. Shevach, E. M. & Rosenthal, A. S. Function of macrophages in antigen recognition by guinea pig T lymphocytes. **138**, 1194–1212 (1973).
8. Buus, S. *et al.* Interaction between a 'processed' ovalbumin peptide and Ia molecules. *Proc Natl Acad Sci U S A* **83**, 3968–3971 (1986).
9. Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* **317**, 359–361 (1985).
10. Unanue, E. R. From antigen processing to peptide-MHC binding. *Nat. Immunol.* **7**, 1277–9 (2006).

11. Townsend, A. R. M. *et al.* The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959–968 (1986).
12. Bjorkman, P. J. *et al.* Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506–512 (1987).
13. Rammensee, H.-G., Falk, K. & Rotzschke, O. Peptides naturally presented by MHC class I molecules. *Annu. Rev. Immunol.* **11**, 213–244 (1993).
14. Rammensee, H.-G., Bachmann, J., Emmerich, N. P. N., Bachor, O. A. & Stevanović, S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* **50**, 213–219 (1999).
15. Stern, L. J. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* **368**, 19 (1994).
16. Winternitz, J., Abbate, J. L., Huchard, E., Havlíček, J. & Garamszegi, L. Z. Patterns of MHC-dependent mate selection in humans and nonhuman primates: a meta-analysis. *Mol. Ecol.* **26**, 668–688 (2017).
17. Huh, G. S. *et al.* Functional Requirement for Class I MHC in CNS Development and Plasticity. *Science (80-. )*. **290**, 2155–2159 (2000).
18. Nisticò, R. *et al.* Synptoimmunology -roles in health and disease. *Mol. Brain* **10**, 1–12 (2017).
19. Reith, W., LeibundGut-Landmann, S. & Waldburger, J.-M. Regulation of MHC class II gene expression by the class II transactivator. *Nat. Rev. Immunol.* **5**, 793–806 (2005).
20. Neefjes, J., Jongma, M. L., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* **11**, 823–36 (2011).
21. Kurts, C., Robinson, B. W. S. & Knolle, P. A. Cross-priming in health and disease. *Nat. Rev. Immunol.* **10**, 403–14 (2010).
22. Norbury, C. C. Defining cross presentation for a wider audience. *Curr. Opin. Immunol.* **40**, 110–116 (2016).

23. Heath, W. R. & Carbone, F. R. Dendritic cell subsets in primary and secondary T cell responses at body surfaces. *Nat. Immunol.* **10**, 1237–1244 (2009).
24. Vyas, J. M., Van der Veen, A. G. & Ploegh, H. L. The known unknowns of antigen processing and presentation. *Nat. Rev. Immunol.* **8**, 607–18 (2008).
25. Mintern, J. D. *et al.* Differential use of autophagy by primary dendritic cells specialized in cross-presentation. *Autophagy* **11**, 906–917 (2015).
26. Neijssen, J. *et al.* Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* **434**, 83–88 (2005).
27. Paludan, C. *et al.* Endogenous MHC Class II Processing of a Viral Nuclear Antigen After Autophagy. *Science* **307**, 593–596 (2005).
28. Aichinger, M., Wu, C., Nedjic, J. & Klein, L. Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. *J. Exp. Med.* **210**, 287–300 (2013).
29. Coulon, P. *et al.* HIV-Infected Dendritic Cells Present Endogenous MHC Class II – Restricted Antigens to HIV-Specific CD4 + T Cells. *J. Immunol.* **197**, 517–532 (2016).
30. Fonteneau, J. F., Brilot, F., Münz, C. & Gannagé, M. The Tumor Antigen NY-ESO-1 Mediates Direct Recognition of Melanoma Cells by CD4+ T Cells after Intercellular Antigen Transfer. *J. Immunol.* **196**, 64–71 (2015).
31. Sidney, J. *et al.* HLA class I supertypes: a revised and updated classification. *BMC Immunol.* **9**, 1 (2008).
32. Wieczorek, M. *et al.* Major histocompatibility complex (MHC) class I and MHC class II proteins: Conformational plasticity in antigen presentation. *Frontiers in Immunology* **8**, (2017).
33. Abualrous, E. T. *et al.* The carboxy terminus of the ligand peptide determines the stability of the MHC class I molecule H-2Kb: A combined molecular dynamics and experimental study. *PLoS One* **10**, 1–17 (2015).
34. Kim, A. R. & Sadegh-Nasseri, S. Determinants of immunodominance for CD4 T cells. *Current Opinion in Immunology* **34**, 9–15 (2015).

35. Kim, Y. *et al.* Dataset size and composition impact the reliability of performance benchmarks for peptide-MHC binding predictions. *BMC Bioinformatics* **15**, 241 (2014).
36. Rammensee, H.-G., Bachmann, J., Emmerich, N. P. N., Bachor, O. A. & Stevanović, S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* **50**, 213–219 (1999).
37. Christian Probst, H. *et al.* Immunodominance of an Antiviral Cytotoxic T Cell Response Is Shaped by the Kinetics of Viral Protein Expression. *J Immunol Ref.* **171**, 5415–5422 (2003).
38. A M Sijts Parampal Deol, A. J., W Zaiss, D. M., Monaco, J. J. & Deol, P. Immunoproteasome-Generated Epitopes Immunogenicity of Rates of Processing Determine the Rates of Processing Determine the Immunogenicity of Immunoproteasome-Generated Epitopes. *J Immunol Ref.* **178**, 7557–7562 (2007).
39. de Graaf, N. *et al.* PA28 and the proteasome immunosubunits play a central and independent role in the production of MHC class I-binding peptides in vivo. *Eur. J. Immunol.* **41**, 926–935 (2011).
40. Nussbaum, A. K. *et al.* Cleavage motifs of the yeast 20S proteasome subunits deduced from digests of enolase 1. *Immunology* **95**, 12504–12509 (1998).
41. Toes, R. E. M. *et al.* Discrete Cleavage Motifs of Constitutive and Immunoproteasomes Revealed by Quantitative Analysis of Cleavage Products. *J. Exp. Med* **0**, 1–12 (2001).
42. Cascio, P., Call, M., Petre, B. M., Walz, T. & Goldberg, A. L. Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes. *EMBO J.* **21**, 2636–2645 (2002).
43. Groettrup, M., Kirk, C. J. & Basler, M. Proteasomes in immune cells: more than peptide producers? *Nat. Rev. Immunol.* **10**, 73–8 (2010).
44. van Hall, T. *et al.* Differential Influence on Cytotoxic T Lymphocyte Epitope Presentation by Controlled Expression of Either Proteasome Immunosubunits or Pa28. *J. Exp. Med.* **192**, 483–494 (2000).
45. Groettrup, M. *et al.* A role for the proteasome regulator PA28 $\alpha$  in antigen

- presentation. *Nature* **381**, 166–168 (1996).
46. Murata, S. *et al.* Immunoproteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. *EMBO J.* **20**, 5898–5907 (2001).
  47. Dick, T. P. *et al.* Coordinated dual cleavages induced by the proteasome regulator PA28 lead to dominant MHC ligands. *Cell* **86**, 253–262 (1996).
  48. Sun, Y. *et al.* Expression of the proteasome activator PA28 rescues the presentation of a cytotoxic T lymphocyte epitope on melanoma cells. *Cancer Res.* **62**, 2875–2882 (2002).
  49. Whitby, F. G. *et al.* Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* **408**, 115–120 (2000).
  50. Monaco Yunjung Cho, J. J. *et al.* Immune Defects in 28-kDa Proteasome Immune Defects in 28-kDa Proteasome Activator  $\beta$ -Deficient Mice. *J Immunol Ref.* **172**, 3948–3954 (2004).
  51. Li, J. *et al.* Lysine 188 substitutions convert the pattern of proteasome activation by REGy to that of REGs  $\alpha$  and  $\beta$ . *EMBO J.* **20**, 3359–3369 (2001).
  52. Baldin, V. *et al.* A Novel Role for PA28 $\gamma$  -Proteasome in Nuclear Speckle Organization and SR Protein Trafficking. *Mol. Biol. Cell* **19**, 1706–1716 (2008).
  53. Qian, S. B., Princiotta, M. F., Bennink, J. R. & Yewdell, J. W. Characterization of rapidly degraded polypeptides in mammalian cells reveals a novel layer of nascent protein quality control. *J. Biol. Chem.* **281**, 392–400 (2006).
  54. Wei, J. *et al.* Varied Role of Ubiquitylation in Generating MHC Class I Peptide Ligands. *J. Immunol.* **198**, 3835–3845 (2017).
  55. Yewdell, J. W., Reits, E. & Neefjes, J. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat. Rev. Immunol.* **3**, 952–961 (2003).
  56. Oliveira, C. C. & van Hall, T. Alternative antigen processing for MHC class I: Multiple roads lead to Rome. *Front. Immunol.* **6**, 1–10 (2015).
  57. Rock, K. L., York, I. a & Goldberg, A. L. Post-proteasomal antigen processing for major

- histocompatibility complex class I presentation. *Nat. Immunol.* **5**, 670–7 (2004).
58. Rock, K. L., Farfán-Arribas, D. J. & Shen, L. Proteases in MHC class I presentation and cross-presentation. *J. Immunol.* **184**, 9–15 (2010).
  59. York, I. A., Brehm, M. A., Zendzian, S., Towne, C. F. & Rock, K. L. Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims MHC class I-presented peptides in vivo and plays an important role in immunodominance. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 9202–9207 (2006).
  60. Blanchard, N. *et al.* Endoplasmic reticulum aminopeptidase associated with antigen processing defines the composition and structure of MHC class I peptide repertoire in normal and virus-infected cells. *J Immunol* **184**, 3033–3042 (2010).
  61. Reits, E. *et al.* Peptide Diffusion, Protection, and Degradation in Nuclear and Cytoplasmic Compartments before Antigen Presentation by MHC Class I. *Immunity* **18**, 97–108 (2003).
  62. Hulpke, S. & Tampé, R. The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem. Sci.* **38**, 412–420 (2013).
  63. Durgeau, A. *et al.* Different Expression Levels of the TAP Peptide Transporter Lead to Recognition of Different Antigenic Peptides by Tumor-Specific CTL. *J. Immunol.* **187**, 5532–5539 (2011).
  64. Seidel, U. J. E., Oliveira, C. C., Lampen, M. H. & Van Hall, T. A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens. *Cancer Immunology, Immunotherapy* **61**, 119–125 (2012).
  65. Karttunen, J. & Shastri, N. Measurement of ligand-induced activation in single viable T cells using the lacZ reporter gene. *Proc. Natl. Acad. Sci.* **88**, 3972–3976 (1991).
  66. Townsend, A. *et al.* Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. Exp. Med.* **168**, 1211–24 (1988).
  67. Rock, K. L. *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**,

- 761–771 (1994).
68. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* **363**, 552–554 (1993).
  69. Esquivel, F., Yewdell, J. & Bennink, J. RMA/S cells present endogenously synthesized cytosolic proteins to class I-restricted cytotoxic T lymphocytes. *J. Exp. Med.* **175**, 163–168 (1992).
  70. Szikora, J.-P. *et al.* Structure of the gene of tum-transplantation antigen P35B: presence of a point mutation in the antigenic allele. *EMBO J.* **9**, 41–1050 (1990).
  71. De Plaen, E. *et al.* Immunogenic (tum-) variants of mouse tumor P815: Cloning of the gene of tum-antigen P91A and identification of the tum-mutation. *Immunology* **85**, 2274–2278 (1988).
  72. Fetteh, J. V., Roy, N. & Gilboa, E. A frameshift mutation at the NH2 terminus of the nucleoprotein gene does not affect generation of cytotoxic T lymphocyte epitopes. *J. Immunol.* **147**, 2697–2705 (1991).
  73. Malarkannan, S., Horng, T., Shih, P. P., Schwab, S. & Shastri, N. Presentation of out-of-frame peptide/MHC class I complexes by a novel translation initiation mechanism. *Immunity* **10**, 681–690 (1999).
  74. Uenaka, A. *et al.* Identification of a unique antigen peptide pRL1 on BALB/c RL male 1 leukemia recognized by cytotoxic T lymphocytes and its relation to the Akt oncogene. *J. Exp. Med.* **180**, 1599–1607 (1994).
  75. Wang, R.-F. F., Parkhurst, M. R., Kawakami, Y., Robbins, P. F. & Rosenberg, S. A. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J. Exp. Med.* **183**, 1131–1140 (1996).
  76. Yewdell, J. W., Anton, L. C. & Bennink, J. R. Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J Immunol* **157**, 1823–1826 (1996).
  77. Antón, L. C. & Yewdell, J. W. Translating DRiPs: MHC class I immunosurveillance of

- pathogens and tumors. *J. Leukoc. Biol.* **95**, 551–62 (2014).
78. Deslich, J. R. *et al.* Tight Linkage between Translation and MHC Class I Peptide Ligand Generation Implies Specialized Antigen Processing for Defective Ribosomal Products. *J Immunol Ref. J. Immunol. by guest August* **177**, 227–233 (2006).
  79. Apcher, S., Daskalogianni, C., Manoury, B. & Fåhræus, R. Epstein barr virus-encoded EBNA1 interference with MHC class I antigen presentation reveals a close correlation between mRNA translation initiation and antigen presentation. *PLoS Pathog.* **6**, (2010).
  80. Apcher, S. *et al.* Major source of antigenic peptides for the MHC class I pathway is produced during the pioneer round of mRNA translation. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11572–7 (2011).
  81. Maquat, L. E., Tarn, W.-Y. & Olaf, I. The pioneer Round of Translation: Features and Functions. *Cell* **142**, 368–374 (2010).
  82. Apcher, S. *et al.* Translation of pre-spliced RNAs in the nuclear compartment generates peptides for the MHC class I pathway. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 17951–6 (2013).
  83. Granados, D. P., Laumont, C. M., Thibault, P. & Perreault, C. The nature of self for T cells—a systems-level perspective. *Current Opinion in Immunology* **34**, 1–8 (2015).
  84. Pearson, H. *et al.* MHC class I – associated peptides derive from selective regions of the human genome. *J. Clin. Invest.* **126**, 1–12 (2016).
  85. Hassan, C. *et al.* The Human Leukocyte Antigen-presented Ligandome of B Lymphocytes. *Mol. Cell. Proteomics* **12**, 1829–1843 (2013).
  86. Fortier, M.-H. *et al.* The MHC class I peptide repertoire is molded by the transcriptome. *J. Exp. Med.* **205**, 595–610 (2008).
  87. Hickman, H. D. *et al.* Toward a definition of self: proteomic evaluation of the class I peptide repertoire. *J. Immunol.* **172**, 2944–2952 (2004).
  88. Rock, K. L., Farfán-Arribas, D. J., Colbert, J. D. & Goldberg, A. L. Re-examining class-I

- presentation and the DRiP hypothesis. *Trends Immunol.* **35**, 144–152 (2014).
89. Farfán-Arribas, D. J., Stern, L. J. & Rock, K. L. Using intein catalysis to probe the origin of major histocompatibility complex class I-presented peptides. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16998–7003 (2012).
  90. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *Eric. J. Exp. Med.* **203**, 1259–1271 (2006).
  91. Kim, W. *et al.* Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* **44**, 325–340 (2011).
  92. Reits, E. a. J., Vos, J. C., Grommé, M. & Neefjes, J. The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature* **404**, 774–8 (2000).
  93. Milner, E., Barnea, E., Beer, I. & Admon, A. The turnover kinetics of major histocompatibility complex peptides of human cancer cells. *Mol. Cell. Proteomics* **5**, 357–65 (2006).
  94. Milner, E. *et al.* The effect of proteasome inhibition on the generation of the human leukocyte antigen peptidome. *Mol. Cell. Proteomics* **12**, 1–48 (2013).
  95. Croft, N. P. *et al.* Kinetics of Antigen Expression and Epitope Presentation during Virus Infection. *PLoS Pathog.* **9**, (2013).
  96. Bourdetsky, D., Schmelzer, C. E. H. & Admon, A. The nature and extent of contributions by defective ribosome products to the HLA peptidome. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E1591-9 (2014).
  97. Mester, G., Hoffmann, V. & Stevanović, S. Insights into MHC class I antigen processing gained from large-scale analysis of class I ligands. *Cell. Mol. Life Sci.* **68**, 1521–1532 (2011).
  98. Granados, D. P. *et al.* MHC I-associated peptides preferentially derive from transcripts bearing miRNA response elements. *Blood* **119**, (2012).
  99. de Verteuil, D. *et al.* Deletion of immunoproteasome subunits imprints on the transcriptome and has a broad impact on peptides presented by major

- histocompatibility complex I molecules. *Mol. Cell. Proteomics* **9**, 2034–47 (2010).
100. Caron, E. *et al.* The MHC I immunopeptidome conveys to the cell surface an integrative view of cellular regulation. *Mol. Syst. Biol.* **7**, 533–533 (2014).
  101. Hickman, H. D. H. *et al.* Cutting Edge : Class I Presentation of Host Peptides Following HIV Infection. *J. ...* **171**, 22–26 (2003).
  102. Granados, D. P. *et al.* Impact of genomic polymorphisms on the repertoire of human MHC class I-associated peptides. *Nat. Commun.* **5**, 3600 (2014).
  103. Gu, W. *et al.* Both treated and untreated tumors are eliminated by short hairpin RNA-based induction of target-specific immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8314–9 (2009).
  104. Hinnebusch, A. G., Ivanov, I. P. & Sonenberg, N. Translational control by 5'-untranslated regions of eukaryotic mRNAs. *Science (80-. )*. **352**, 1413–1416 (2016).
  105. Leucine-tRNA Initiates at CUG Start Codons for Protein Synthesis and Presentation by MHC Class I. *Science (80-. )*. **336**, 1719–1723 (2012).
  106. Schwab, S. R., Li, K. C., Kang, C. & Shastri, N. Constitutive Display of Cryptic Translation Products by MHC Class I Molecules. *Science (80-. )*. **301**, 1367–71 (2003).
  107. Ronsin, C. *et al.* A non-AUG-defined alternative open reading frame of the intestinal carboxyl esterase mRNA generates an epitope recognized by renal cell carcinoma-reactive tumor-infiltrating lymphocytes in situ. *J. Immunol.* **163**, 483–490 (1999).
  108. Dolstra, H. *et al.* A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. *J. Exp. Med.* **189**, 301–8 (1999).
  109. Berger, C. T. *et al.* Viral adaptation to immune selection pressure by HLA class I-restricted CTL responses targeting epitopes in HIV frameshift sequences. *J. Exp. Med.* **207**, 61–75 (2010).
  110. Weinzierl, A. O. *et al.* A Cryptic Vascular Endothelial Growth Factor T-Cell Epitope: Identification and Characterization by Mass Spectrometry and T-Cell Assays. *Cancer Res* **68**, 2447–54 (2008).

111. Yang, N. *et al.* Defining Viral Defective Ribosomal Products: Standard and Alternative Translation Initiation Events Generate a Common Peptide from Influenza A Virus M2 and M1 mRNAs. *J. Immunol.* **196**, 3608–3617 (2016).
112. Shastri, N., Schwab, S. & Serwold, T. Producing Nature’s Gene-Chips: The Generation of Peptides for Display by MHC Class I Molecules. *Annu. Rev. Immunol.* **20**, 463–493 (2002).
113. Ho, O. & Green, W. R. Alternative translational products and cryptic T cell epitopes: expecting the unexpected. *J. Immunol.* **177**, 8283–8289 (2006).
114. Starck, S. R. & Shastri, N. Non-conventional sources of peptides presented by MHC class I. *Cell. Mol. Life Sci.* **68**, 1471–1479 (2011).
115. Coulie, P. G. *et al.* A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7976–80 (1995).
116. Guilloux, Y. *et al.* A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. *J. Exp. Med.* **183**, 1173–1183 (1996).
117. Van Den Eynde, B. J. *et al.* A New Antigen Recognized by Cytolytic T Lymphocytes on a Human Kidney Tumor Results from Reverse Strand Transcription. *J. Exp. Med* **190**, 1793–1799 (1999).
118. Lupetti, R. *et al.* Translation of a retained intron in tyrosinase-related protein (TRP) 2 mRNA generates a new cytotoxic T lymphocyte (CTL)-defined and shared human melanoma antigen not expressed in normal cells of the melanocytic lineage. *J. Exp. Med.* **188**, 1005–16 (1998).
119. Probst-Kepper, M. *et al.* An Alternative Open Reading Frame of the Human Macrophage Colony-stimulating Factor Gene Is Independently Translated and Codes for an Antigenic Peptide of 14 Amino Acids Recognized by Tumor-infiltrating CD8 T Lymphocytes. *J. Exp. Med* **51000**, 1189–1198 (2001).
120. Van Bergen, C. A. M. *et al.* Selective graft-versus-leukemia depends on magnitude and

- diversity of the alloreactive T cell response. *J. Clin. Invest.* **127**, 517–529 (2017).
121. Carbonnelle, D. *et al.* The Melanoma Antigens MELOE-1 and MELOE-2 Are Translated from a Bona Fide Polycistronic mRNA Containing Functional IRES Sequences. *PLoS One* **8**, (2013).
  122. Kracht, M. J. L. *et al.* Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes. *Nat. Med.* **23**, 501–507 (2017).
  123. Andreev, D. E. *et al.* Insights into the mechanisms of eukaryotic translation gained with ribosome profiling. *Nucleic Acids Research* **45**, 513–526 (2017).
  124. Vanderperre, B. *et al.* Direct Detection of Alternative Open Reading Frames Translation Products in Human Significantly Expands the Proteome. *PLoS One* **8**, (2013).
  125. Wilhelm, M. *et al.* Mass-spectrometry-based draft of the human proteome. *Nature* **509**, (2014).
  126. Menschaert, G. *et al.* Deep Proteome Coverage Based on Ribosome Profiling Aids Mass Spectrometry-based Protein and Peptide Discovery and Provides Evidence of Alternative Translation Products and Near-cognate Translation Initiation Events. *Mol. Cell. Proteomics* **12**, 1780–1790 (2013).
  127. Ingolia, N. T. *et al.* Ribosome Profiling Reveals Pervasive Translation Outside of Annotated Protein-Coding Genes. **8**, 1365–1379 (2014).
  128. Michel, A. M. *et al.* Observation of dually decoded regions of the human genome using ribosome profiling data. *Genome Res.* **22**, 2219–2229 (2012).
  129. Laumont, C. M. *et al.* Global proteogenomic analysis of human MHC class I-associated peptides derived from non-canonical reading frames. *Nat. Commun.* **7**, 10238 (2016).
  130. Dolan, B. P., Knowlton, J. J., David, A., Bennink, J. R. & Yewdell, J. W. RNA polymerase II inhibitors dissociate antigenic peptide generation from normal viral protein synthesis: a role for nuclear translation in defective ribosomal product synthesis? *J. Immunol.* **185**, 6728–33 (2010).
  131. David, A., Bennink, J. R. & Yewdell, J. W. Emetine optimally facilitates nascent chain

- puromylation and potentiates the ribopuromylation method (RPM) applied to inert cells. *Histochem. Cell Biol.* **139**, 501–504 (2013).
132. Pederson, T. The persistent plausibility of protein synthesis in the nucleus: Process, palimpsest or pitfall? *Curr. Opin. Cell Biol.* **25**, 520–521 (2013).
  133. David, A. *et al.* Nuclear translation visualized by ribosome-bound nascent chain puromylation. *J. Cell Biol.* **197**, 45–57 (2012).
  134. Lev, A. *et al.* Compartmentalized MHC class I antigen processing enhances immunosurveillance by circumventing the law of mass action. *Proc Natl Acad Sci U S A* **107**, 6964–6969 (2010).
  135. da Costa, P. J., Menezes, J. & Romão, L. The role of alternative splicing coupled to nonsense-mediated mRNA decay in human disease. *Int. J. Biochem. Cell Biol.* (2017). doi:10.1016/j.biocel.2017.07.013
  136. Matera, A. G. & Wang, Z. A day in the life of the spliceosome. *Nat. Rev. Mol. Cell Biol.* **15**, 108–21 (2014).
  137. Song, X., Zeng, Z., Wei, H. & Wang, Z. Alternative splicing in cancers: From aberrant regulation to new therapeutics. *Semin. Cell Dev. Biol.* (2017). doi:10.1016/j.semcdb.2017.09.018
  138. Wang, Y., Ma, M., Xiao, X. & Wang, Z. Intronic splicing enhancers, cognate splicing factors and context-dependent regulation rules. *Nat. Struct. Mol. Biol.* **19**, 1044–1052 (2012).
  139. Wang, Y. *et al.* A complex network of factors with overlapping affinities represses splicing through intronic elements. *Nat. Struct. Mol. Biol.* **20**, 36–45 (2012).
  140. Miura, K., Fujibuchi, W. & Unno, M. Splice variants in apoptotic pathway. *Experimental Oncology* **34**, 212–217 (2012).
  141. Bechara, E. G., Sebestyén, E., Bernardis, I., Eyras, E. & Valcárcel, J. RBM5, 6, and 10 differentially regulate NUMB alternative splicing to control cancer cell proliferation. *Mol. Cell* **52**, 720–733 (2013).
  142. Moorhead, G., Trinkle-Mulcahy, L. & Ulke-Lemee, A. Emerging roles of nuclear protein

- phosphatases. *Nat. Rev. Mol. Cell Biol.* **8**, 234–244 (2007).
143. Sveen, A., Kilpinen, S., Ruusulehto, A., Lothe, R. A. & Skotheim, R. I. Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. *Oncogene* **35**, 2413–2427 (2016).
  144. Kaida, D., Schneider-Poetsch, T. & Yoshida, M. Splicing in oncogenesis and tumor suppression. *Cancer Sci.* **103**, 1611–1616 (2012).
  145. Kaida, D. *et al.* Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. *Nat. Chem. Biol.* **3**, 576–583 (2007).
  146. Kotake, Y. *et al.* Splicing factor SF3b as a target of the antitumor natural product pladienolide. *Nat. Chem. Biol.* **3**, 570–575 (2007).
  147. Lee, S. C.-W. *et al.* Modulation of splicing catalysis for therapeutic targeting of leukemia with mutations in genes encoding spliceosomal proteins. *Nat. Med.* **22**, 672–678 (2016).
  148. Lee, S. C.-W. & Abdel-Wahab, O. Therapeutic targeting of splicing in cancer. *Nat. Med.* **22**, 976–986 (2016).
  149. Pawellek, A. *et al.* Identification of small molecule inhibitors of pre-mRNA splicing. *J. Biol. Chem.* **289**, 34683–34698 (2014).
  150. O’Brien, K., Matlin, A. J., Lowell, A. M. & Moore, M. J. The biflavonoid isoginkgetin is a general inhibitor of Pre-mRNA splicing. *J. Biol. Chem.* **283**, 33147–54 (2008).
  151. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
  152. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 (2011).
  153. Klein, G. Tumor Antigens. *Annu. Rev. Microbiol.* **20**, 223–252 (1966).
  154. Ribatti, D. The concept of immune surveillance against tumors. The first theories. *Oncotarget* **8**, 7175–7180 (2015).
  155. Boon, T. & van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* **183**, 725–9 (1996).

156. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* **3**, 991–998 (2002).
157. Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural Innate and Adaptive Immunity to Cancer. *Annu. Rev. Immunol.* **29**, 235–271 (2011).
158. Koebel, C. M. *et al.* Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* **450**, 903–907 (2007).
159. Strauss, D. C. & Thomas, J. M. Transmission of donor melanoma by organ transplantation. *The Lancet Oncology* **11**, 790–796 (2010).
160. Braumüller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, (2013).
161. Müller-Hermelink, N. *et al.* TNFR1 Signaling and IFN- $\gamma$  Signaling Determine whether T Cells Induce Tumor Dormancy or Promote Multistage Carcinogenesis. *Cancer Cell* **13**, 507–518 (2008).
162. Teng, M. W. L. *et al.* Opposing roles for IL-23 and IL-12 in maintaining occult cancer in an equilibrium state. *Cancer Res.* **72**, 3987–3996 (2012).
163. Romero, I. *et al.* T lymphocytes restrain spontaneous metastases in permanent dormancy. *Cancer Res.* **74**, 1958–1968 (2014).
164. Teng, M. W. L., Galon, J., Fridman, W. H. & Smyth, M. J. From mice to humans: Developments in cancer immunoediting. *J. Clin. Invest.* **125**, 3338–3346 (2015).
165. Cavallo, F., De Giovanni, C., Nanni, P., Forni, G. & Lollini, P. L. 2011: The immune hallmarks of cancer. in *Cancer Immunology, Immunotherapy* **60**, 319–326 (2011).
166. Gajewski, T. F., Schreiber, H. & Fu, Y.-X. Innate and adaptive immune cells in the tumor microenvironment. *Nat. Immunol.* **14**, 1014–1022 (2013).
167. Harlin, H. *et al.* Chemokine expression in melanoma metastases associated with CD8 + T-Cell recruitment. *Cancer Res.* **69**, 3077–3085 (2009).
168. Gajewski, T. F., Louahed, J. & Brichard, V. G. Gene Signature in Melanoma Associated With Clinical Activity. *Cancer J.* **16**, 399–403 (2010).

169. Gajewski, T. F. Next Hurdle in Cancer Immunotherapy: Overcoming Non-T-Cell-Inflamed Tumor Microenvironment. *Semin. Oncol.* **42**, 663–671 (2015).
170. Galon, J. *et al.* Towards the introduction of the ‘Immunoscore’ in the classification of malignant tumours. *J. Pathol.* **232**, 199–209 (2014).
171. Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* **19**, 1423–1437 (2013).
172. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer Immunoediting: Integrating Immunity’s Roles in Cancer Suppression and Promotion. *Science (80-. )*. **331**, 1565–1570 (2011).
173. Schäfer, C. *et al.* Innate immune cells for immunotherapy of autoimmune and cancer disorders. *Int. Rev. Immunol.* **0**, 1–23 (2017).
174. Morvan, M. G. & Lanier, L. L. NK cells and cancer: you can teach innate cells new tricks. *Nat. Rev. Cancer* **16**, 7–19 (2015).
175. Coca, S. *et al.* The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* **79**, 2320–2328 (1997).
176. Ishigami, S. *et al.* Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* **88**, 577–583 (2000).
177. Villegas, F. R. *et al.* Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer* **35**, 23–28 (2002).
178. Woo, S.-R., Corrales, L. & Gajewski, T. F. Innate Immune Recognition of Cancer. *Annu. Rev. Immunol* **33**, 445–74 (2015).
179. Paul, S. & Lal, G. Regulatory and effector functions of gamma-delta ( $\gamma\delta$ ) T cells and their therapeutic potential in adoptive cellular therapy for cancer. *Int. J. Cancer* **139**, 976–985 (2016).
180. Smyth, M. J. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* **191**, 661–668 (2000).

181. Crowe, N. Y., Smyth, M. J. & Godfrey, D. I. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* **196**, 119–127 (2002).
182. Terabe, M. *et al.* NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol.* **1**, 515–20 (2000).
183. Galdiero, M. R. *et al.* Tumor associated macrophages and neutrophils in cancer. *Immunobiology* **218**, 1402–1410 (2013).
184. Zhang, Q. *et al.* Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature. *PLoS One* **7**, e50946 (2012).
185. Medrek, C., Pontén, F., Jirström, K. & Leandersson, K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer* **12**, 306 (2012).
186. Ryder, M., Ghossein, R. A., Ricarte-Filho, J. C. M., Knauf, J. A. & Fagin, J. A. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. *Endocr. Relat. Cancer* **15**, 1069–1074 (2008).
187. Duluc, D. *et al.* Interferon- $\gamma$  reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int. J. Cancer* **125**, 367–373 (2009).
188. Guiducci, C., Vicari, A. P., Sangaletti, S., Trinchieri, G. & Colombo, M. P. Redirecting In vivo Elicited Tumor Infiltrating Macrophages and Dendritic Cells towards Tumor Rejection. *Cancer Res* **65**, 3437–46 (2005).
189. Huang, Y. *et al.* Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc. Natl. Acad. Sci.* **109**, 17561–17566 (2012).
190. Chesney, J. A., Mitchell, R. A. & Yaddanapudi, K. Myeloid-derived suppressor cells—a new therapeutic target to overcome resistance to cancer immunotherapy. *J. Leukoc. Biol.* **102**, 727–740 (2017).
191. Varricchi, G. *et al.* Are mast cells MASTers in cancer? *Front. Immunol.* **8**, 1–13 (2017).

192. Labidi-Galy, S. I. *et al.* Plasmacytoid dendritic cells infiltrating ovarian cancer are associated with poor prognosis. *Oncoimmunology* **1**, 380–382 (2012).
193. Treilleux, I. *et al.* Dendritic cell infiltration and prognosis of early stage breast cancer. *Clin. Cancer Res.* **10**, 7466–7474 (2004).
194. Labidi-Galy, S. I. *et al.* Quantitative and functional alterations of plasmacytoid dendritic cells contribute to immune tolerance in ovarian cancer. *Cancer Res.* **71**, 5423–5434 (2011).
195. Swiecki, M. & Colonna, M. The multifaceted biology of plasmacytoid dendritic cells. *Nat. Rev. Immunol.* **15**, 471–485 (2015).
196. Tel, J. *et al.* Natural Human Plasmacytoid Dendritic Cells Induce Antigen-Specific T-Cell Responses in Melanoma Patients. *Cancer Res.* **73**, 1063–1075 (2013).
197. Liu, C. *et al.* Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. *J. Clin. Invest.* **118**, 1165–1175 (2008).
198. Laoui, D. *et al.* The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nat. Commun.* **7**, 13720 (2016).
199. Veglia, F. & Gabrilovich, D. I. Dendritic cells in cancer: the role revisited. *Current Opinion in Immunology* **45**, 43–51 (2017).
200. Gardner, A. & Ruffell, B. Dendritic Cells and Cancer Immunity. *Trends in Immunology* **37**, 855–865 (2016).
201. Roberts, E. W. *et al.* Critical Role for CD103 + /CD141 + Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma HHS Public Access. *Cancer Cell* **30**, 324–336 (2016).
202. Salmon, H. *et al.* Expansion and Activation of CD103+ Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* **44**, 924–938 (2016).
203. Sluijter, B. J. R. *et al.* Arming the Melanoma Sentinel Lymph Node through Local

- Administration of CpG-B and GM-CSF: Recruitment and Activation of BDCA3/CD141+ Dendritic Cells and Enhanced Cross-Presentation. *Cancer Immunol. Res.* **3**, 495–505 (2015).
204. Scarlett, U. K. *et al.* Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. *J. Exp. Med.* **209**, 495–506 (2012).
205. Shurin, G. V., Ma, Y. & Shurin, M. R. Immunosuppressive mechanisms of regulatory dendritic cells in cancer. *Cancer Microenviron.* **6**, 159–167 (2013).
206. Conejo-Garcia, J. R., Rutkowski, M. R. & Cubillos-Ruiz, J. R. State-of-the-art of regulatory dendritic cells in cancer. *Pharmacol. Ther.* **164**, 97–104 (2016).
207. Adams, J. L., Smothers, J., Srinivasan, R. & Hoos, A. Big opportunities for small molecules in immuno-oncology. *Nat. Rev. Drug Discov.* **14**, 603–622 (2015).
208. Rosser, E. C. & Mauri, C. Regulatory B Cells: Origin, Phenotype, and Function. *Immunity* **42**, 607–612 (2015).
209. Rosser, E. C., Blair, P. A. & Mauri, C. Cellular targets of regulatory B cell-mediated suppression. *Molecular Immunology* **62**, 296–304 (2014).
210. Nielsen, J. S. *et al.* CD20+ tumor-infiltrating lymphocytes have an atypical CD27 - memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clin. Cancer Res.* **18**, 3281–3292 (2012).
211. Kemp, T. J., Moore, J. M. & Griffith, T. S. Human B cells express functional TRAIL/Apo-2 ligand after CpG-containing oligodeoxynucleotide stimulation. *J. Immunol.* **173**, 892–9 (2004).
212. Sarvaria, A., Madrigal, J. A. & Saudemont, A. B cell regulation in cancer and anti-tumor immunity. *Cell. Mol. Immunol.* **1435**, 662–674 (2017).
213. Tsou, P., Katayama, H., Ostrin, E. J. & Hanash, S. M. The emerging role of b cells in tumor immunity. *Cancer Research* **76**, 5591–5601 (2016).
214. Senovilla, L. *et al.* Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology* **1**, 1323–1343 (2012).

215. Ivanova, E. A. & Orekhov, A. N. T Helper lymphocyte subsets and plasticity in autoimmunity and cancer: An overview. *Biomed Res. Int.* **2015**, (2015).
216. Kalinski, P. & Moser, M. Consensual immunity: success driven development of T-helper-1 and T-helper-2 responses. *Nat. Rev.* **5**, 251–60 (2005).
217. Ellyard, J. I., Simson, L. & Parish, C. R. Th2-mediated anti-tumour immunity: Friend or foe? *Tissue Antigens* **70**, 1–11 (2007).
218. Guéry, L., Hugues, S., Hugues, S. & phanie. Th17 Cell Plasticity and Functions in Cancer Immunity. *Biomed Res. Int.* **2015**, 1–11 (2015).
219. Nishikawa, H. & Sakaguchi, S. Regulatory T cells in cancer immunotherapy. *Current Opinion in Immunology* **27**, 1–7 (2014).
220. Mucida, D. *et al.* Transcriptional reprogramming of mature CD4<sup>+</sup> helper T cells generates distinct MHC class II–restricted cytotoxic T lymphocytes. *Nat. Immunol.* **14**, 281–289 (2013).
221. Xie, Y. *et al.* Naive tumor-specific CD4<sup>+</sup> T cells differentiated in vivo eradicate established melanoma. *J. Exp. Med.* **207**, 651–667 (2010).
222. Quezada, S. A. *et al.* Tumor-reactive CD4<sup>+</sup> T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J. Exp. Med.* **207**, 637–650 (2010).
223. Park, I. A. *et al.* Expression of the MHC class II in triple-negative breast cancer is associated with tumor-infiltrating lymphocytes and interferon signaling. *PLoS One* **12**, e0182786 (2017).
224. Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A. & Pagès, F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science (80-. )*. **313**, 1960–1963 (2006).
225. Djenidi, F. *et al.* CD8<sup>+</sup> + CD103<sup>+</sup> Tumor–Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients. *J. Immunol.* **194**, 3475–3486 (2015).
226. Kmiecik, J. *et al.* Elevated CD3<sup>+</sup> and CD8<sup>+</sup> tumor-infiltrating immune cells correlate

- with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level. *J. Neuroimmunol.* **264**, 71–83 (2013).
227. Mahmoud, S. M. A. *et al.* Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J. Clin. Oncol.* **29**, 1949–1955 (2011).
228. Perret, R. & Ronchese, F. Effector CD8+ T cells activated in vitro confer immediate and long-term tumor protection in vivo. *Eur. J. Immunol.* **38**, 2886–2895 (2008).
229. Palmer, D. C. *et al.* Vaccine-stimulated, adoptively transferred CD8+ T cells traffic indiscriminately and ubiquitously while mediating specific tumor destruction. *J. Immunol.* **173**, 7209–16 (2004).
230. Egelston, C., Simons, D., Miyahira, A. & Lee, P. Effector memory CD8 T cells and central memory CD4 T cells dominate a proliferation deficient T cell population in the primary tumor of breast cancer patients (P2207). *J. Immunol.* **190**, (2016).
231. Beckhove, P. *et al.* Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors. *J. Clin. Invest.* **114**, 67–76 (2004).
232. Reiser, J. & Banerjee, A. Effector, Memory, and Dysfunctional CD8+ T Cell Fates in the Antitumor Immune Response. *J. Immunol. Res.* **2016**, (2016).
233. Jiang, Y., Li, Y. & Zhu, B. T-cell exhaustion in the tumor microenvironment. *Cell Death Dis.* **6**, e1792 (2015).
234. Burugu, S., Dancsok, A. R. & Nielsen, T. O. Emerging targets in cancer immunotherapy. *Semin. Cancer Biol.* (2017). doi:10.1016/j.semcancer.2017.10.001
235. Filaci, G. *et al.* CD8+ CD28- T regulatory lymphocytes inhibiting T cell proliferative and cytotoxic functions infiltrate human cancers. *J. Immunol.* **179**, 4323–34 (2007).
236. Montes, C. L. *et al.* Tumor-induced senescent T cells with suppressor function: A potential form of tumor immune evasion. *Cancer Res.* **68**, 870–879 (2008).
237. Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat. Rev. Cancer*

- 14**, 135–146 (2014).
238. Brichard, V. *et al.* The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **178**, 489–95 (1993).
239. Wölfel, T. *et al.* Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur. J. Immunol.* **24**, 759–764 (1994).
240. Kawakami, Y. *et al.* Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3515–9 (1994).
241. Coulie, P. G. *et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **180**, 35–42 (1994).
242. Bakker, A. B. H. *et al.* Identification of a novel peptide derived from the melanocyte-specific gp100 antigen as the dominant epitope recognized by an HLA-A2.1-restricted anti-melanoma CTL line. *Int. J. Cancer* **62**, 97–102 (1995).
243. Kantoff, P. W. *et al.* Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. *N. Engl. J. Med.* **363**, 411–422 (2010).
244. Bright, R. K., Bright, J. D. & Byrne, J. A. Overexpressed oncogenic tumor-self antigens. *Hum. Vaccines Immunother.* **10**, 3297–3305 (2014).
245. Gjerstorff, M. F., Kock, K., Nielsen, O. & Ditzel, H. J. MAGE-A1, GAGE and NY-ESO-1 cancer/testis antigen expression during human gonadal development. *Hum. Reprod.* **22**, 953–960 (2007).
246. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* **9**, 833–844 (2009).
247. Pedersen, S. R., Sorensen, M. R., Buus, S., Christensen, J. P. & Thomsen, A. R. Comparison of Vaccine-Induced Effector CD8 T Cell Responses Directed against Self- and Non-Self-Tumor Antigens: Implications for Cancer Immunotherapy. *J. Immunol.* **191**, 3955–3967 (2013).

248. Bos, R. *et al.* Expression of a natural tumor antigen by thymic epithelial cells impairs the tumor-protective CD4+ T-cell repertoire. *Cancer Res.* **65**, 6443–6449 (2005).
249. Pinto, S. *et al.* Misinitiation of intrathymic MART-1 transcription and biased TCR usage explain the high frequency of MART-1-specific T cells. *Eur. J. Immunol.* **44**, 2811–2821 (2014).
250. Engelhard, V. H., Bullock, T. N. J., Colella, T. a, Sheasley, S. L. & Mullins, D. W. Antigens derived from melanocyte differentiation proteins: self-tolerance, autoimmunity, and use for cancer immunotherapy. *Immunol. Rev.* **188**, 136–146 (2002).
251. Hui, E. P. *et al.* Phase I trial of recombinant modified vaccinia ankara encoding Epstein-Barr viral tumor antigens in nasopharyngeal carcinoma patients. *Cancer Res.* **73**, 1676–1688 (2013).
252. Kenter, G. G. *et al.* Vaccination against HPV-16 Oncoproteins for Vulvar Intraepithelial Neoplasia. *N. Engl. J. Med.* **361**, 1838–1847 (2009).
253. Van Rooij, N. *et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J. Clin. Oncol.* **31**, e439-42 (2013).
254. Parkhurst, M. *et al.* Isolation of T-Cell Receptors Specifically Reactive with Mutated Tumor-Associated Antigens from Tumor-Infiltrating Lymphocytes Based on CD137 Expression. *Clin. Cancer Res.* 1–16 (2016). doi:10.1158/1078-0432.CCR-16-2680
255. Robbins, P. F. *et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat. Med.* **19**, 747–752 (2013).
256. Johanns, T. M. *et al.* Endogenous Neoantigen-Specific CD8 T Cells Identified in Two Glioblastoma Models Using a Cancer Immunogenomics Approach. *Cancer Immunol. Res.* **4**, 1007–1015 (2016).
257. Tran, E. *et al.* Cancer immunotherapy based on mutation-Specific CD4+ T cells in a patient with epithelial cancer. *Science (80-. ).* **9**, 641–645 (2014).
258. Türeci, Ö. *et al.* Targeting the heterogeneity of cancer with individualized neoepitope vaccines. *Clin. Cancer Res.* **22**, 1885–1896 (2016).

259. Matsushita, H. *et al.* Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* **482**, 400–404 (2012).
260. Dupage, M., Mazumdar, C., Schmidt, L. M., Cheung, A. & Jacks, T. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature* **482**, 405–409 (2012).
261. von Boehmer, L. *et al.* NY-ESO-1-specific immunological pressure and escape in a patient with metastatic melanoma. *Cancer Immun.* **13**, (2013).
262. Verdegaaal, E. M. E. *et al.* Neoantigen landscape dynamics during human melanoma–T cell interactions. *Nature* **536**, 91–95 (2016).
263. Anagnostou, V. *et al.* Evolution of neoantigen landscape during immune checkpoint blockade in non-small cell lung cancer. *Cancer Discov.* **7**, 264–276 (2017).
264. Schneble, E., Clifton, T. G., Hale, D. F. & Peoples, G. E. Peptide-Based Cancer Vaccine Strategies and Clinical Results. *Methods Mol. Biol.* **1**, (2016).
265. US National Library of Medicine. ClinicalTrials.gov [online]. <https://clinicaltrials.gov/ct2/show/NCT00796445>. (2014). at <<https://clinicaltrials.gov/ct2/show/NCT00796445?term=NCT00796445&rank=1>>
266. Vansteenkiste, J. F. *et al.* Efficacy of the MAGE-A3 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small-cell lung cancer (MAGRIT): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol.* **17**, 822–835 (2016).
267. [online], U. N. L. of M. C. go. <https://clinicaltrials.gov/ct2/show/NCT00358566>. at <<https://clinicaltrials.gov/ct2/show/NCT00358566?term=NCT00358566&rank=1>>
268. Yewdell, J. W. Designing CD8+ T cell vaccines: It’s not rocket science (yet). *Current Opinion in Immunology* **22**, 402–410 (2010).
269. Hale, D. F. *et al.* Cancer vaccines: should we be targeting patients with less aggressive disease? *Expert Rev. Vaccines* **11**, 721–31 (2012).
270. Schuster, S. J. *et al.* Vaccination with patient-specific tumor-derived antigen in first remission improves disease-free survival in follicular lymphoma. *J. Clin. Oncol.* **29**,

- 2787–2794 (2011).
271. Arens, R., van Hall, T., van der Burg, S. H., Ossendorp, F. & Melief, C. J. M. Prospects of combinatorial synthetic peptide vaccine-based immunotherapy against cancer. *Semin. Immunol.* **25**, 182–190 (2013).
272. Van Hall, T. & Van der Burg, S. H. *Mechanisms of Peptide Vaccination in Mouse Models. Tolerance, Immunity, and Hyperreactivity. Advances in Immunology* **114**, (Elsevier Inc., 2012).
273. Van Der Sluis, T. C. *et al.* Vaccine-Induced tumor necrosis factor- Producing T cells synergize with cisplatin to promote tumor cell death. *Clin. Cancer Res.* **21**, 781–794 (2015).
274. Khong, H. & Overwijk, W. W. Adjuvants for peptide-based cancer vaccines. *J. Immunother. Cancer* **4**, 56 (2016).
275. Bijker, M. S. *et al.* Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation. *Eur. J. Immunol.* **38**, 1033–1042 (2008).
276. Gnjatic, S. & Bhardwaj, N. Antigen depots: T cell traps? *Nat. Med.* **19**, 397–398 (2013).
277. Rosalia, R. A. *et al.* Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *Eur. J. Immunol.* **43**, 2554–2565 (2013).
278. Ménager, J. *et al.* Cross-presentation of synthetic long peptides by human dendritic cells: A process dependent on ERAD component p97/VCP but not sec61 and/or Derlin-1. *PLoS One* **9**, (2014).
279. Aspord, C., Leloup, C., Reche, S. & Plumas, J. pDCs efficiently process synthetic long peptides to induce functional virus- and tumour-specific T-cell responses. *Eur. J. Immunol.* **44**, 2880–2892 (2014).
280. Verdegaal, E. M. E. & van der Burg, S. H. The Potential and Challenges of Exploiting the Vast But Dynamic Neoepitope Landscape for Immunotherapy. *Front. Immunol.* **8**, 1113 (2017).

281. Ott, P. A. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547**, 217–221 (2017).
282. Kmiecik, M., Knutson, K. L., Dumur, C. I. & Manjili, M. H. HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. *Eur. J. Immunol.* **37**, 675–685 (2007).
283. Schrörs, B. *et al.* HLA class I loss in metachronous metastases prevents continuous T cell recognition of mutated neoantigens in a human melanoma model. *Oncotarget* **8**, 28312–28327 (2017).
284. Del Campo, A. B. *et al.* Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma. *Int. J. Cancer* **134**, 102–113 (2014).
285. Carretero, R. *et al.* Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. *Immunogenetics* **60**, 439–447 (2008).
286. Leone, P. *et al.* MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. *J. Natl. Cancer Inst.* **105**, 1172–87 (2013).
287. Garcia-Lora, A., Martinez, M., Algarra, I., Gaforio, J. J. & Garrido, F. MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. *Int. J. Cancer* **106**, 521–527 (2003).
288. Garrido, F., Cabrera, T. & Aptsiauri, N. ‘Hard’ and ‘soft’ lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. *International Journal of Cancer* **127**, 249–256 (2010).
289. Seliger, B., Ruiz-Cabello, F. & Garrido, F. IFN Inducibility of Major Histocompatibility Antigens in Tumors. *Advances in Cancer Research* **101**, 249–276 (2008).
290. Wang, W. *et al.* Modulation of signal transducers and activators of transcription 1 and 3 signaling in melanoma by high-dose IFN $\alpha$ 2b. *Clin. Cancer Res.* **13**, 1523–1531 (2007).
291. Mocellin, S., Pasquali, S., Rossi, C. R. & Nitti, D. Interferon alpha adjuvant therapy in

- patients with high-risk melanoma: A systematic review and meta-analysis. *J. Natl. Cancer Inst.* **102**, 493–501 (2010).
292. Buferne, M. *et al.* IFN $\gamma$  producing CD8 + T cells modified to resist major immune checkpoints induce regression of MHC class I-deficient melanomas. *Oncoimmunology* **4**, e974959 (2015).
293. Corbière, V. *et al.* Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res.* **71**, 1253–1262 (2011).
294. Rodríguez, T. *et al.* Distinct mechanisms of loss of IFN-gamma mediated HLA class I inducibility in two melanoma cell lines. *BMC Cancer* **7**, 34 (2007).
295. Komyod, W., Bohm, M., Metze, D., Heinrich, P. C. & Behrmann, I. Constitutive Suppressor of Cytokine Signaling 3 Expression Confers a Growth Advantage to a Human Melanoma Cell Line. *Mol. Cancer Res.* **5**, 271–281 (2007).
296. Lampen, M. H. & van Hall, T. Strategies to counteract MHC-I defects in tumors. *Current Opinion in Immunology* **23**, 293–298 (2011).
297. Garrido, F., Aptsiauri, N., Doorduijn, E. M., Garcia Lora, A. M. & van Hall, T. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Current Opinion in Immunology* **39**, 44–51 (2016).
298. Liu, W. M., Fowler, D. W., Smith, P. & Dalglish, a G. Pre-treatment with chemotherapy can enhance the antigenicity and immunogenicity of tumours by promoting adaptive immune responses. *Br. J. Cancer* **102**, 115–23 (2010).
299. Takahara, A. *et al.* Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response. *Cancer Immunol. Immunother.* **60**, 1289–97 (2011).
300. van Hall, T. *et al.* Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Med.* **12**, 417–424 (2006).
301. Oliveira, C. C. *et al.* New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. *J. Immunol.* **191**, 4020–8 (2013).
302. Doorduijn, E. M. *et al.* TAP-independent self-peptides enhance T cell recognition of

- immune-escaped tumors. *J. Clin. Invest.* **126**, 784–794 (2016).
303. Duvallet, E. *et al.* Exosome-driven transfer of tumor-associated Pioneer Translation Products (TA-PTPs) for the MHC class I cross-presentation pathway. *Oncoimmunology* **5**, (2016).
304. Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480**, 480–489 (2014).
305. Burg, S. H. Van Der, Arens, R., Ossendorp, F., Hall, T. Van & Melief, C. J. M. Vaccines for established cancer : overcoming the challenges posed by immune evasion. *Nat. Publ. Gr.* **16**, 219–233 (2016).
306. Watson, N. F. S. *et al.* Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int. J. Cancer* **118**, 6–10 (2006).
307. Liu, Y. *et al.* Expression of antigen processing and presenting molecules in brain metastasis of breast cancer. *Cancer Immunol. Immunother.* **61**, 789–801 (2012).
308. Verdegaal, E. M. E. *et al.* Neoantigen landscape dynamics during human melanoma-T cell interactions. *Nature* **536**, 91–5 (2016).
309. Lee, S. & Sin, J. MC32 tumor cells acquire Ag-specific CTL resistance through the loss of CEA in a colon cancer model. *Hum. Vaccines Immunother.* **11**, 2012–2020 (2015).
310. Dolan, B. P. *et al.* Distinct pathways generate peptides from defective ribosomal products for CD8+ T cell immunosurveillance. *J. Immunol.* **186**, 2065–72 (2011).
311. Starck, S. R. *et al.* Leucine-tRNA Initiates at CUG Start Codons for Protein Synthesis and Presentation by MHC Class I. *Science (80-. ).* **336**, 1719–1723 (2012).
312. Doherty, S. J. *et al.* Immunoproteasome Subunit Deficiencies Impact Differentially on Two Immunodominant Influenza Virus-Specific CD8+ T Cell Responses. *J Immunol Ref.* **177**, 7680–7688 (2006).
313. Shen, S., Wang, Y., Wang, C., Wu, Y. N. & Xing, Y. SURVIV for survival analysis of mRNA isoform variation. *Nat. Commun.* **7**, 1–11 (2016).

314. Tsai, Y. S., Dominguez, D., Gomez, S. M. & Wang, Z. Transcriptome-wide identification and study of cancer-specific splicing events across multiple tumors. *Oncotarget* **6**, 6825–6839 (2015).
315. Yatim, N., Cullen, S. & Albert, M. L. Dying cells actively regulate adaptive immune responses. *Nat. Rev. Immunol.* **17**, 262–275 (2017).
316. Casares, N. *et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J. Exp. Med.* **202**, 1691–1701 (2005).
317. Spiotto, M. T., Rowley, D. A. & Schreiber, H. Bystander elimination of antigen loss variants in established tumors. *Nat. Med.* **10**, 294–298 (2004).
318. Zhang, B., Karrison, T., Rowley, D. & Schreiber, H. IFN- $\gamma$ - and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J. Clin. Invest.* **118**, 1398–1404 (2008).
319. Thompson, E. D., Enriquez, H. L., Fu, Y.-X. & Engelhard, V. H. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J. Exp. Med.* **207**, 1791–1804 (2010).
320. de Verteuil, D., Granados, D. P., Thibault, P. & Perreault, C. Origin and plasticity of MHC I-associated self peptides. *Autoimmunity Reviews* **11**, 627–635 (2012).
321. Khurana, E. *et al.* Role of non-coding sequence variants in cancer. *Nat. Rev. Genet.* **17**, 93–108 (2016).
322. Quidville, V. *et al.* Targeting the deregulated spliceosome core machinery in cancer cells triggers mTOR blockade and autophagy. *Cancer Res.* **73**, 2247–2258 (2013).
323. Inhibition of vemurafenib-resistant melanoma by interference with pre-mRNA splicing. *Nature* **20**, 1–26 (2015).
324. Hong, D. S. *et al.* A phase I, open-label, single-arm, dose-escalation study of E7107, a precursor messenger ribonucleic acid (pre-mRNA) spliceosome inhibitor administered intravenously on days 1 and 8 every 21 days to patients with solid tumors. *Invest. New Drugs* **32**, 436–444 (2014).
325. Apcher, S., Prado Martins, R. & Fåhræus, R. The source of MHC class I presented

- peptides and its implications. *Current Opinion in Immunology* **40**, 117–122 (2016).
326. Cruz, F. M., Colbert, J. D., Merino, E., Kriegsman, B. A. & Rock, K. L. The Biology and Underlying Mechanisms of Cross-Presentation of Exogenous Antigens on MHC-I Molecules. *Annu. Rev. Immunol* **35**, 403–39 (2017).
327. Wolfers, J. *et al.* Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* **7**, 297–303 (2001).
328. Andre, F. *et al.* Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* **360**, 295–305 (2002).
329. Menay, F. *et al.* Exosomes isolated from ascites of T-cell lymphoma-bearing mice expressing surface CD24 and HSP-90 induce a tumor-specific immune response. *Front. Immunol.* **8**, 1–14 (2017).
330. Morishita, M., Takahashi, Y., Matsumoto, A., Nishikawa, M. & Takakura, Y. Exosome-based tumor antigens–adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials* **111**, 55–65 (2016).
331. Syn, N. L., Wang, L., Chow, E. K. H., Lim, C. T. & Goh, B. C. Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges. *Trends in Biotechnology* **35**, 665–676 (2017).
332. Yu, S., Cao, H., Shen, B. & Feng, J. Tumor-derived exosomes in cancer progression and treatment failure. *Oncotarget* **6**, 37151–37168 (2015).
333. Théry, C., Amigorena, S., Raposo, G. & Clayton, A. in *Current Protocols in Cell Biology* **Chapter 3**, Unit 3.22 (John Wiley & Sons, Inc., 2006).
334. Kalra, H., Drummen, G. P. C. & Mathivanan, S. Focus on extracellular vesicles: Introducing the next small big thing. *International Journal of Molecular Sciences* **17**, (2016).
335. Robila, V. *et al.* MHC class II presentation of gp100 epitopes in melanoma cells requires the function of conventional endosomes and is influenced by melanosomes. *J. Immunol.* **181**, 7843–52 (2008).

336. Breathnach, A. S. & Wyllie, L. M. Electron microscopy of melanocytes and melanosomes in freckled human epidermis. *J. Invest. Dermatol.* **42**, 389–394 (1964).
337. Hirayama, M. & Nishimura, Y. The present status and future prospects of peptide-based cancer vaccines. *Int. Immunol.* **28**, 319–328 (2016).
338. Melief, C. J. M., Van Hall, T., Arens, R., Ossendorp, F. & Van Der Burg, S. H. Therapeutic cancer vaccines. *J. Clin. Invest.* **125**, 3401–3412 (2015).
339. Wang, R. F. *et al.* A Breast and Melanoma-Shared Tumor Antigen: T Cell Responses to Antigenic Peptides Translated from Different Open Reading Frames. *J. Immunol.* **161**, 3598–606 (1998).
340. Rosenberg, S. A. *et al.* Identification of BING-4 Cancer Antigen Translated From an Alternative Open Reading Frame of a Gene in the Extended MHC Class II Region Using Lymphocytes From a Patient With a Durable Complete Regression Following Immunotherapy. *J. Immunol.* **168**, 2402–2407 (2002).
341. Yewdell, J. W. DRIPs Solidify: Progress in understanding endogenous MHC class I antigen processing. **32**, 548–558 (2012).
342. Wei, J. & Yewdell, J. W. Autoimmune T cell recognition of alternative-reading-frame-encoded peptides. *Nat. Med.* **23**, 409–410 (2017).
343. Morgan, R. A. *et al.* Cancer Regression and Neurological Toxicity Following Anti-MAGE-A3 TCR Gene Therapy. *J. Immunother.* **36**, 133–151 (2013).
344. Parkhurst, M. R. *et al.* T Cells Targeting Carcinoembryonic Antigen Can Mediate Regression of Metastatic Colorectal Cancer but Induce Severe Transient Colitis. *Mol. Ther.* **19**, 620–626 (2011).
345. Wick, D. A. *et al.* Surveillance of the tumor mutanome by T cells during progression from primary to recurrent ovarian cancer. *Clin. Cancer Res.* **20**, 1125–1134 (2014).
346. Cohen, C. J. *et al.* Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. *J. Clin. Invest.* **125**, 3981–3991 (2015).
347. Tran, E. *et al.* Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science (80-. ).* **350**, 1387–1391 (2015).

348. Yadav, M. *et al.* Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature* **515**, 572–576 (2014).
349. Carreno, B. M. *et al.* A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science (80-. )*. **348**, 803–808 (2015).
350. Andreev, D. E. *et al.* Translation of 5' leaders is pervasive in genes resistant to eIF2 repression. *Elife* **4**, e03971 (2015).
351. Starck, S. R. *et al.* Translation from the 5' untranslated region shapes the integrated stress response. *Science (80-. )*. **351**, aad3867-aad3867 (2016).
352. Apcher, S. *et al.* Major source of antigenic peptides for the MHC class I pathway is produced during the pioneer round of mRNA translation. *Proc. Natl. Acad. Sci.* **108**, 11572–11577 (2011).
353. Bassani-Sternberg, M. *et al.* Direct identification of clinically relevant neoepitopes presented on native human melanoma tissue by mass spectrometry. *Nat. Commun.* **7**, 13404 (2016).
354. Wang, R.-F. & Wang, H. Y. Immune targets and neoantigens for cancer immunotherapy and precision medicine. *Cell Res.* **27**, 11–37 (2016).
355. Laumont, C. M. & Perreault, C. Exploiting non-canonical translation to identify new targets for T cell-based cancer immunotherapy. *Cellular and Molecular Life Sciences* **1**, 1–15 (2017).
356. Dersh, D. & Yewdell, J. W. I've got algorithm: Predicting tumor and autoimmune peptide targets for CD8+ T cells. *J. Clin. Invest.* **126**, 4399–4401 (2016).
357. Granados, D. P., Laumont, C. M., Thibault, P. & Perreault, C. The nature of self for T cells-a systems-level perspective. *Current Opinion in Immunology* **34**, 1–8 (2015).
358. Dror, S. *et al.* Melanoma miRNA trafficking controls tumour primary niche formation. *Nat. Cell Biol.* **18**, 1006–1017 (2016).
359. Shen, L. & Rock, K. L. Cellular protein is the source of cross-priming antigen in vivo. *Proc. Natl. Acad. Sci.* **101**, 3035–3040 (2004).

360. Norbury, C. C. *et al.* CD8(+)T cell cross-priming via transfer of proteasome substrates. *Science (80-. ).* **304**, 1318–1321 (2004).
361. Lev, A. *et al.* The Exception that Reinforces the Rule: Crosspriming by Cytosolic Peptides that Escape Degradation. *Immunity* **28**, 787–798 (2008).
362. Kunisawa, J. & Shastri, N. Hsp90alpha Chaperones Large C-Terminally Extended Proteolytic Intermediates in the MHC Class I Antigen Processing Pathway. *Immunity* **24**, 523–534 (2006).
363. Li, Y. *et al.* Cross-presentation of tumor associated antigens through tumor- derived autophagosomes. *Autophagy* **5**, 576–577 (2009).
364. Page, D. B. *et al.* Glimpse into the future: harnessing autophagy to promote anti-tumor immunity with the DRibbles vaccine. *J. Immunother. Cancer* **4**, 25 (2016).
365. Ren, H. *et al.* Therapeutic antitumor efficacy of B cells loaded with tumor-derived autophagosomes vaccine (DRibbles). *J. Immunother.* **37**, 383–93 (2014).
366. Li, Y. *et al.* Tumor-Derived Autophagosome Vaccine: Mechanism of Cross-Presentation and Therapeutic Efficacy. *Clin Cancer Res* **17**, 7047–57 (2011).
367. Münz, C. Autophagy Beyond Intracellular MHC Class II Antigen Presentation. *Trends in Immunology* **37**, 755–763 (2016).

## Annexes

1. Article: “Tumors escape immunosurveillance by overexpressing a regulator of the proteasome: REGy”

# Tumors escape immunosurveillance by overexpressing a regulator of the proteasome: REG $\gamma$

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## Summary

With the clear success of CD8+ T cell based immunotherapy it is critical to understand i) how tumor cells generate MHC class I peptide antigens? and ii) the various mechanisms used to evade immunosurveillance. One of them is to up-regulate the REG $\gamma$  proteasome regulator which results in an increase destruction of MHC class I peptides in the nucleus thus subverting immunosurveillance.

## Abstract

The success of CD8+ T cell based cancer immunotherapy emphasizes the importance of understanding the generation of MHC class I peptide ligands and possible pathways of tumor cell escape from immunosurveillance. Recently, we showed that peptides generated in the nucleus during the pioneer round of mRNA translation (pioneer translation products, or PTPs) can be a potentially important source of tumor specific peptides, given the presence of aberrant splicing and transcription associated with oncogenesis. Here we show that cancer cells up-regulation of the REG $\gamma$  proteasome regulator results in increased destruction of PTP-derived peptides in the nucleus thus subverting immunosurveillance. These findings add to understanding of the role of REG $\gamma$  in antigen processing and identify it as a druggable target for improving the efficacy of cancer immunotherapy.

## **Introduction**

Cellular immune reactions against cancer cells expressing non-self epitopes require activation of CD8<sup>+</sup> T cells by professional antigen presenting cells (pAPCs), which take up external peptide material and present this on their Major Histocompatibility Complexes class I (MHC-I) molecules through a process called cross-presentation (1). The MHC-I direct and cross presentation pathways are fundamental processes for the detection and elimination of cells that pose a threat to the host. Some years ago, it was suggested that the source of peptides for direct presentation to the MHC class I restricted pathway are not derived from the degradation of full length proteins but from so-called defective ribosomal products, or DRiPs (2). In addition, some MHC-I-bound peptides have been described as being generated from cryptic translation, which refers to polypeptides synthesized in the cell from non-conventional translational mechanisms. These can either be peptides encoded by intron, intron/exon junctions, 5' and 3' untranslated regions, alternate translational reading frame, or even fusion peptides generated by the proteasome (3-6). All these observations led to a shift of focus towards the notion that protein degradation of full-length proteins is not the only critical process for antigenic production. More recently, we have shown that antigenic presentation is equivalent whether the peptide is expressed intronically or exonically, and could be supported by the so called Pioneer Translation Products (PTPs) (7), which are produced by a translation event distinct from the canonical one and that occurs before mRNA splicing. The model of PTPs is appealing as it offers an explanation to how the immune system can “tolerate” tissue-dependent alternative splicing products. Working at first on direct presentation applies to viral mechanisms of immune evasion, we have demonstrated very recently that PTPs can also be a source of peptides for the exogenous MHC-I pathway. In fact, they can be cross presented by pAPCs to specifically activate naïve CD8<sup>+</sup>T cells (8). Moreover, PTPs were found present in exosomes that were engulfed by BMDCs to cross present them. Finally, PTPs purified from

tumor cells (tumor-associated PTPs or TA-PTPs) can be used in combination with exosomes as a potent immune cancer vaccine (8).

Polypeptides such as PTPs that enter either the endogenous or the exogenous MHC-I pathway have to be processed in order to fit the MHC-I molecule binding groove to have a chance to elicit an immune response. Playing a key role in that process is the key proteolytic system of the eukaryotic cell, the ubiquitin-proteasome system (9). Central to this system is the proteasome, a multicatalytic complex consisting of a 20S proteolytic core controlled by regulatory complexes that bind to it (10, 11). One of these regulatory complexes is the 19S particle, which along with the 20S proteolytic core forms the 26S proteasome that degrades ubiquitylated proteins in an ATP-dependent manner. Other regulatory complexes, among which is the REG/PA28 family, have been shown to associate with the 20S proteasome (12). The REG family is formed by three related subunits, which together form two proteasome regulatory complexes: (i) REG $\alpha\beta$ , a heteroheptamer formed by REG $\alpha$  and REG $\beta$  subunits, located mainly in the cytoplasm; (ii) REG $\gamma$ , a homoheptamer formed by the REG $\gamma$  subunit, located in the nucleus (13-15). Exact functions and mechanisms of action of REG $\gamma$  remain elusive, as only a limited number of proteins whose degradation is mediated or controlled by this regulator have been described. Among those are cell-cycle regulators, including the Cyclin-Dependent Kinases Inhibitors p21 or p16, the oncogene SRC-3 or the tumor suppressor p53 (16-19). This is in line with the proliferation-promoting and anti-apoptotic properties of REG $\gamma$  deduced from the analysis of KO mice and from the fact that it is found overexpressed in many cancers and is often associated with poor prognosis. Moreover, other observations; point to a central role of REG $\gamma$  in intranuclear dynamics through the regulation of (i) nuclear bodies (including nuclear speckles, Cajal and PML bodies), and (ii) nuclear trafficking of splicing factors (20-22). Evolutionary analyses showed that REG $\alpha$  and REG $\beta$  appeared much later in evolution than REG $\gamma$ , and diverged from it concomitantly with the

emergence of MHC (23). REG $\gamma$  is important for the responses to genotoxic and oxidative stress and upon the impairment of proteasome function such as in neurodegenerative diseases (24). Interestingly, it has been shown recently that in cells, the REG $\gamma$  is massively recruited on proteasomes after a non-toxic treatment with proteasome inhibitors (25). We have also recently reported that by blocking the proteasomal system, using the highly specific proteasome inhibitor epoxomicin, the amount of PTPs inside the nuclear compartment increases (7).

Although many results demonstrated a strong link between REG $\alpha\beta$  and antigen production for the immune system (26), no data exist at the moment that would suggest a negative or positive role of REG $\gamma$  in this process even if REG-knockout MEF cells and REG-deficient animal models have been produced and challenged for specific immune responses (27, 28). Here, we show that i) there is an inverse correlation between the expression of the regulator REG $\gamma$  and the presentation of MHC class I antigenic peptides in different cancer cell lines, ii) REG $\gamma$  is involved in the nuclear processing of PTPs and doing so regulates the CD8<sup>+</sup> T cell responses against cancer negatively, iii) downregulation of REG $\gamma$  by either cisplatin treatment or miRNA-7 surexpression decreases the presentation of MHC class I PTPs-derived antigens. These findings provide a mechanism in which REG $\gamma$  influences the cancer immune responses negatively at the opposite effect of the other members of the REG family. All these results might potentially serve as a starting point for developing new chemotherapies aimed at decreasing the intracellular level of REG $\gamma$ , and in this way enhance the production of tumor-associated antigens and so stimulate specific immune responses against cancer.

## **RESULTS**

**Inverse correlation between expression of REG $\gamma$  and antigen presentation in multiple cancer cell lines.**

A series of studies have reported the overexpression of REG $\gamma$  in different cancer types (29-31). In order to clarify the specific role of REG $\gamma$  in MHC class I antigen presentation, we took different cancer cell lines and looked at the REG $\gamma$  mRNA level and protein expression. We first analyzed the REG $\gamma$  mRNA level by real-time qRT-PCR in three human melanoma cell lines (A375, MW3526 and MW3682), one human lung cancer cell line (A549) and two colon cancer cell lines (HT29 and T84), and observed that REG $\gamma$  is greatly upregulated in each compared to the control normal lung cell line MRC5 (Figure 1A). Then, we analyzed the REG $\gamma$  protein expression and found that it is overexpressed in all cancer cell lines compare to MRC5 normal cells. Nevertheless, we found that REG $\gamma$  is overexpressed at different level. While in melanoma and colon cell lines REG $\gamma$  is highly overexpressed, it seems mildly expressed in the lung cancer cell line A549 and very lowly expressed in the normal cell line (Figure 1B). Furthermore we checked the cellular distribution of REG $\gamma$  in these different cancer cell lines. Figure 1C shows that localization of the endogenous REG $\gamma$  is nuclear, independent of the level of expression and of the cell line used.

To determine to what extent the difference in expression of REG $\gamma$  contributes to antigen production and presentation in cancer cell lines, we looked at the presentation of a PTP-derived-antigens at the cell surface of the different tumor cell lines. To this aim, the cells were expressing respectively the mouse MHC class I K<sup>b</sup> molecule and the Globin-intron-SL8 constructs that enabled to look at the production of the specifics SL8-comprising-PTPs as previously shown (6, 7). Using the B3Z T-cell hybridoma (32) that specifically detects the SL8 epitope presented on K<sup>b</sup> molecules, we first found that the antigenic presentation in all the cancer cell lines tested was lower than in the normal cell line MRC5 (Figure 1D). This result is not due to a difference of expression of our  $\beta$ -globin construct since we could see an equal expression of the Glob-intron-SL8 protein in the cell lines tested (Supplementary Figure1A). Even more interestingly, we could see a complete inverse correlation between the

PTPs-derived-SL8 presentation and the REG $\gamma$  expression in all cell lines tested. This results suggest that the overexpression of REG $\gamma$  could affect the PTP-derived-antigens presentation negatively (compare Figures 1B and 1D). As control, we observed by FACS analysis that the overall H2-K<sup>b</sup> expression is different among the cancer cell lines but is not correlated to the SL8-expression we could observed with the B3Z assay. In fact, we observed that MHC class I molecules are more abundant at the cell surface of tumor cell lines that overexpressed REG $\gamma$  and exhibited a decrease in the PTPs-dependent antigen presentation (Supplementary Figure 1B). These results confirm the negative role of REG $\gamma$  in the MHC class I antigen presentation pathway since in cell lines that overexpressed it, the MHC class I molecules are more abundant and the antigenic presentation is lower.

We then determined whether the previously observed differences of SL8 antigenic presentation is restricted to the intron-derived tumor-associated antigens (ITAAAs) or if they could also be observed from an exon-derived tumor-associated antigen (ETAAs). For that purpose the cells were expressing respectively the mouse MHC class I K<sup>b</sup> molecule and the Globin-exon-SL8 construct or the Ovalbumin cDNA, where the SL8 epitope is found in its right setting. Figure 1E shows that independently of the setting of the antigenic epitope in exon sequence (left panel) or in cDNA constructs (right panel), the antigen presentation was in inverse correlation with REG $\gamma$ 's expression in all the cell lines tested.

Furthermore, we made the different cell lines expressed respectively the mouse MHC class I K<sup>b</sup> molecule and the SL8-minigene construct, which only contains the 8 amino acid sequence of the SL8, and observed that the difference of expression of REG $\gamma$  in the different cancer cell lines is not correlated to the SL8 antigenic presentation (Supplementary Figure 1C). Hence, the effect of REG $\gamma$  on MHC class I antigen presentation only happen on polypeptides longer than 8 amino acids that contain MHC class I epitope, suggesting that the effect of REG $\gamma$  could be specific to PTPs.

All these results support the hypothesis that the proteasome regulator REG $\gamma$  is involved in the inhibition of the presentation of MHC class I antigens derived from PTPs, a mechanism that could constitute a way for tumor cells to avoid immune responses.

### **Exogenous REG $\gamma$ overexpression decreases MHC class I antigens presentation**

To investigate the specific effect of the regulator REG $\gamma$  on the PTP-dependent MHC class I antigen presentation, the MRC5 cells that have been shown to have a very low expression of REG $\gamma$  and the lung cancer cells A549 that have been shown to mildly express the REG $\gamma$  regulator (see Figures 1A and 1B) have been respectively co-transfected with the mouse MHC class I K<sup>b</sup> molecule, the Globin-intron-SL8 and an exogenous Flag- REG $\gamma$  construct. Using either, western-blot (Figure 2A), immunofluorescence (Figure 2B) or qRT-PCR (Supplementary Figure 2A), we monitored the expression of the exogenous REG $\gamma$  construct comparing with the endogenous one. The exogenous REG $\gamma$  has the same expression pattern and localization when it is overexpressed compare to the endogenous one. Then we looked at the antigenic presentation of the SL8 epitope in these cell lines. The introduction of increasing amounts of REG $\gamma$  in both A549 and MRC5 cell lines, which express a fixed amount of the Glob-intron-SL8 and MHC class I K<sup>b</sup> molecules, results in a dose-dependent decrease of the activation of B3Z hybridoma (Figure 2C). This observation supports the hypothesis that overexpression of REG $\gamma$  has a net negative effect on PTP-dependent antigen presentation.

As mentioned earlier, REG $\gamma$  is not the sole regulator in the proteasomal pathway, as the 20S proteasome can also bind to the REG $\alpha/\beta$  regulator, which has been shown to be implicated in the right processing of the MHC class I antigenic epitopes. To investigate at which level in the proteasomal pathway the regulator REG $\gamma$  contributes to the PTP-dependent antigen presentation regulation, we determined to which extent an overexpression of the

regulator REG $\gamma$  can influence the expression of the regulator REG $\alpha$ . Figure 2D shows that increasing the amounts of exogenous REG $\gamma$  do not affect the expression of the regulator REG $\alpha$ . This result demonstrates that a decrease in the PTP-dependent antigen presentation in the different cancer cell lines is not due to a decrease of expression of the regulator REG $\alpha$ . This result supports the idea of a direct effect of the regulator REG $\gamma$  on the 20S proteasome. The presentation of antigenic peptides depends on proteolytic processing by the 20S proteasome complex. To ensure that the difference of production of the SL8 peptides by the proteasome could be due to the overexpression of REG $\gamma$  we treated the MRC5 cell that express increasing amounts of REG $\gamma$  with a fixed amount of the Glob-intron-SL8 and MHC class I K<sup>b</sup> molecules, with the 20S proteasome specific inhibitor epoxomicin and looked at the SL8 presentation. While non-treated cells show a decrease in antigen presentation with REG $\gamma$  overexpression, a partial increase in the MHC class I antigen presentation was observed when cells were treated with epoxomicin (Figure 2E). This observation support the idea that the effect of REG $\gamma$  on MHC class I antigen presentation is not due to an unspecific processing effect on cell lines that overexpressed it but to a real and specific effect on the 20S core proteasome. Moreover, as control of efficacy of the proteasomal inhibitor in the cell lines treated, we looked at the level of p21 protein. The p21 protein has been reported to be regulated by the REG $\gamma$ -20S proteasome complex. The REG $\gamma$  has been shown to be involved in an ubiquitin and ATP-independent degradation of p21 by the proteasomal pathway(17). We observed that the p21 protein level increases following 18h of epoxomicin treatment despite the fact that the treated cells overexpressed the exogenous REG $\gamma$  regulator (Figure 2F).

Hence, all these results confirmed that overexpression of REG $\gamma$  is not linked to (i) a decrease of MHC class I expression or export, (ii) a decrease of the expression of the other member of the REG family, but is directly due to an effect of REG $\gamma$  on the proteasomal activities.

### **Knockdown and knock-out of the regulator REG $\gamma$ promotes antigen presentation.**

In view of our findings that endogenous overexpression of REG $\gamma$  regulator affects the cancer cells MHC class I antigen presentation negatively, we wondered whether a knockdown of REG $\gamma$  could restore the PTPs-dependent antigen presentation. To test this hypothesis, we silenced REG $\gamma$  with transient siRNA treatment in human melanoma and colon cancer cell lines. Knockdown of REG $\gamma$  (Figure 3A) enhanced the level of the p21 protein as expected in all cancer cell lines as compared to the scramble siRNA (Figure 3B). In term of PTP-dependent antigen presentation we also observed a close correlation between the knockdown of REG $\gamma$  and the increase of the SL8 epitope at the cells surface (Figure 3C).

The expression of REG $\gamma$  has been shown to be controlled by several mechanisms inside the cell. The miRNA-7, which has been reported to be a tumor suppressor (33, 34) and to be downregulated (35, 36) in several human cancers, has also been found to control the expression of the regulator REG $\gamma$  in lung cancer negatively (37). The A375 melanoma cancer cell line that, in our hand is the cell line that express the most REG $\gamma$ , has been transfected with increasing amounts of miRNA-7. We observed that miRNA-7 inhibits the REG $\gamma$  expression (Supplementary Figure 3A) and leads to an increase in PTP-SL8-dependent antigen presentation (Supplementary Figure 3B). (33, 34)

In addition, treatment of cancer cells with the chemotherapeutic molecule cisplatin has been shown to induce a decrease in the amount of the mRNA and protein levels of the REG $\chi$  regulator (38). By treating A375 cells with cisplatin we were able to restore the expression of the PTPs-dependent antigens at the cell surface (Supplementary Figure 3C). These results support the idea that REG $\gamma$  downregulation restores PTPs-derived antigen presentation and provide way to do so by chemotherapeutic treatment. Furthermore

To clarify the functional importance of REG $\gamma$  on the cancer immune escape, we utilized the CRISPR/Cas9 system to create a melanoma A375 REG $\gamma$ -knock-out cell line (A375 $\Delta$ REG $\gamma$ ). Heterozygous knock-out A375 $\Delta$ REG $\gamma$  was confirmed by Sanger sequencing, qRT-PCR and western-blot analysis. We confirmed that A375 $\Delta$ REG $\gamma$  cell line does not express anymore the REG $\gamma$  mRNA (Figure 3D) and the corresponding protein (Figure 3E). As a control of loss of expression of REG $\gamma$ , the p21 protein has been found to be accumulated in this cell line compare to the A375 WT cell line or cell line that were knockdown using specific REG $\gamma$  siRNAs (Figure 3E). In term of antigen presentation and processing we were able to show that contrary to the A375 WT, the A375 $\Delta$ REG $\gamma$  cells were now able to activate the PTP-dependent immune response. Moreover, this antigen presentation was even better than the antigen presentation seen with the control MRC5 cell line that has been reported to express the regulator REG $\gamma$  weakly (Figure 3F). As control, we showed that both cell lines tested expressed at their cell surface an equal amount of the mouse MHC class I molecule and an equal amount of exogenous Glob-intron-SL8 protein (Figure 3G, left and right panel).

This rescue experiment corroborates the different results reported above and finally proves that there is an inverse correlation between the expression of REG $\gamma$  and the PTPs-dependent antigen presentation in cancer cell lines.

### **REG $\gamma$ regulates the nuclear proteasomal pathway.**

In our previous studies, we have demonstrated that PTPs are produced by a translation event distinct from the canonical one, and that they are generated before mRNAs splicing occur, supporting the idea that PTPs are generated by a nuclear translation event. Furthermore, we have also reported that proteasome inhibitor epoxomicin treatment increases the amount of PTPs inside the nuclear compartment, which supports the idea that the nuclear proteasome could be involved in the processing and or the degradation of the PTP-antigenic

epitopes (7). In line with these studies and with all the results shown previously, the next evident question was to understand and clarify in which cell compartment and how the REG $\gamma$  contributes to specifically inhibit PTP-dependent cancer immune responses. Antigenic epitopes derived from PTPs processing can be generated either by a cytosolic or nuclear proteasomal complex, where the REG family plays an essential role. As a proteasomal regulator, REG $\gamma$  is obliged to bind to the 20S proteasome to promote protein or polypeptide degradation. This interaction is supposed to happen in the nucleus. REG $\gamma$  gene sequence mutation that leads to the replacement of Asn 151 to Tyr (N151Y) has been reported to impair the ability of the regulator to activate the trypsin-like activity of the 20S proteasome (39). A375 $\Delta$ REG $\gamma$  cells were transfected with increasing amounts of Flag-REG $\gamma$  WT or the mutated Flag-REG $\gamma$ N151Y. First we observed by western blot that the mutated regulator is not able anymore to activate the trypsin-like activity of the 20S proteasome to degrade the p21 protein compare to the WT REG $\gamma$  and that in all conditions the expression of the other member of the REG family is not impacted (Figure 4A). More importantly, we have shown that Flag-REG $\gamma$ N151Y is less efficient of inhibiting the SL8 antigenic production and presentation at the cell surface compare to Flag-REG $\gamma$  WT (Figure 4B). This inhibition of SL8 presentation is again not due to a decrease in expression of the other REG family members shown to be involved in the antigen presentation. Two explanations can explain this result. One could be due to the fact that the exogenous mutated Flag-REG $\gamma$ N151Y might not be localized anymore in the nucleus. The other explanation could be due to the fact that the exogenous mutated Flag-REG $\gamma$ N151Y might has lost its capacity to bind to the 20S proteasome, and by this, might not be able anymore to inhibit the proteasome activity as compare to Flag-REG $\gamma$  WT. To discriminate these hypotheses, we first looked at the distribution of the mutated Flag-REG $\gamma$ N151Y and then at this capacity to bind to the 20S proteasome *in cellulo*. Figure 4C shows that the mutated and the WT exogenous

REG $\gamma$  regulators are together localized in the nucleus. Since we could not see any difference of localisation that can explain the difference of production of MHC class I epitopes between the cell lines that expressed the mutated and the WT regulators, we determined whether the mutated Flag-REG $\gamma$ N151Y still has the capacity to bind to the nuclear 20S proteasome *in cellulo*. For that purpose we used the proximal ligation assay (PLA)-labelled secondary antibodies, which allow the detection of two primary antibodies in close proximity. The combination of PLA-labelled anti-Flag and anti-proteasomal  $\alpha$ 4 antibodies, we observed a specific signal in the nuclear compartment of cells expressing respectively the mutated and the WT REG $\gamma$  constructs revealing for both their co-localisation with the 20S proteasome (Figure 4D). Furthermore as control, the anti-Flag alone under these conditions produced no PLA reaction, and no positive signals were detected in non-transfected cells (Figure 4D, top panel). We confirmed the interaction between respectively the WT Flag-REG $\gamma$  and the mutated Flag-REG $\gamma$ N151Y with the 20S core proteasome by coimmunoprecipitation, using an antibody directed against the REG $\gamma$  regulator (Figure 4E). Both regulators bind to the 20S core proteasome independently or not of the mutation at the amino acid 151. The fact that the localisation and the capacity to bind to the 20S core are equal between the exogenous Flag-REG $\gamma$  WT and the mutated Flag-REG $\gamma$ N151Y demonstrates again that the REG $\gamma$ -20S proteasome complex is involved in the processing/degradation process of the PTPs-derived antigenic peptides.

To go further about the idea of defining the compartment of the REG $\gamma$ -dependent PTPs degradation, we generated an exogenous Flag-REG $\gamma$  construct that does not contain anymore its nuclear localisation signal (NLS). Unlike Flag-REG $\gamma$  WT, the Flag-REG $\gamma$  $\Delta$ NLS is expressed throughout the cell and not only in the nucleus (Figure 4C). The fact that our Flag-REG $\gamma$  $\Delta$ NLS is not only expressed in the cytoplasm suggests that REG $\gamma$  might contain more than one nuclear localization signal. The combination of PLA-labelled anti-Flag and

anti-proteasomal  $\alpha 4$  antibodies produced a specific signal in the cytoplasmic compartment of cells expressing respectively the Flag-REG $\gamma$  $\Delta$ NLS and Flag-REG $\gamma$  WT constructs. Nevertheless, we could also observe some staining corresponding to an interaction between the Flag-REG $\gamma$  $\Delta$ NLS and the 20S core in the nucleus. This result supports the idea that the Flag-REG $\gamma$  $\Delta$ NLS, that is found localized throughout the cell, has still the capacity to bind to the 20S proteasome in both the cytoplasm and the nucleus (Figure 4D). We also confirmed the interaction between respectively the Flag-REG $\gamma$  WT and the Flag-REG $\gamma$  $\Delta$ NLS with the 20S core proteasome by coimmunoprecipitation, using an antibody directed against the REG $\gamma$  regulator (Figure 4E). Moreover, A375 $\Delta$ REG $\gamma$  cells transfected with Flag-REG $\gamma$  $\Delta$ NLS slightly prevent the nuclear 20S proteasome to degrade de p21 protein in a REG $\gamma$  dependent fashion when compare to cells transfected with the Flag- REG $\gamma$  WT (Figure 4F). Hence, this result supports the fact that the weak amount of REG $\gamma$  $\Delta$ NLS, in the nucleus, still has the capacity to activate partially the nuclear 20S proteasome and furthermore demonstrate that the degradation of the p21 protein seems to be a nuclear and not a cytoplasmic event (Figure 4fF). In parallel, when we looked at the effect of the partial relocalization of Flag-REG $\gamma$  $\Delta$ NLS in the cytoplasm on the antigenic presentation pathway, we observed that SL8-PTP-dependent antigen presentation is increased compared to the loss of antigen presentation in cells expressing the exogenous nuclear Flag-REG $\gamma$  WT (Figure 4G).

All these results demonstrate that REG $\gamma$  is involved in the processing of PTP-dependent antigen presentation by binding with the 20S proteasome in the nucleus.

### **REG $\gamma$ promotes the degradation of MHC class I PTP-derived antigenic epitopes.**

The above results have raised at least two hypotheses on the role of REG $\gamma$  on the PTP-dependent proteasomal degradation pathway: i) REG $\gamma$  could inhibit the 20S proteasomal

activities, which are responsible for producing antigenic peptides at the right length for the MHC class I pathway with the consequence of decrease in the production of the right PTP-dependent antigenic epitopes or ii) REG $\gamma$  could stimulate a specific 20S proteasomal activity that will induce a complete degradation of the right MHC class I antigenic epitopes. Recently, we have shown that the inhibition of the proteasomal pathway with epoxomicin leads to an increased amount of unprocessed PTPs in the cells and promotes the cross presentation of longer polypeptides compare to untreated cells (8). In order to test the first hypothesis, we have transfected respectively the different cancer cell lines, the normal fibroblast MRC5 and the A375 $\Delta$ REG $\gamma$  cell line with the Globin-intron-SL8 construct and incubated them with mouse bone marrow dendritic cells (BMDCs) for 24h. We observed a reduction of B3Z activation when the tumor cell lines are incubated with BMDCs as compared to the A375 $\Delta$ REG $\gamma$  and the normal fibroblast MRC5 cells (Figure 5A). Since we see a decrease of cross presentation in the different cell lines that overexpressed REG $\gamma$  suggests that REG $\gamma$  is not inhibiting the processing of PTPs leading to an accumulation of unprocessed polypeptides, source of material for the exogenous MHC class I pathway.

To confirm that REG $\gamma$  does not inhibit the 20S peptidase activities, we assessed the effect of the regulator on the 20S core particle by means of an *in vitro* degradation assay. For that purpose, a 45-mer (MP-45) and a 52-mer (KH-52) precursor peptide containing the SIINFEKL epitope in their sequences were synthesized (Figure 5B). The 45-mer precursor peptide corresponds to the amino acid sequence of the  $\beta$ -Globin exon region where the SL8 epitope has been introduced in the construct used above (Figure 1E) and the 52-mer precursor peptide is equal to the amino acid sequence of the  $\beta$ -Globin intron region where the SL8 epitope has been introduced in the construct used above (Figure 1D). Importantly, preliminary studies demonstrated that the commercial enzymes used for this assays were active and free of contaminant proteases, as shown by the ability of epoxomicin to completely inhibit the

chymotrypsin- and trypsin-like activities of these preparations (Supplementary Table 1). KH-52 was then incubated *in vitro* with h20Sc at 37° C for several hours and the rates of peptide bonds hydrolysis were assessed by measuring the generation of new amino groups with fluorescamine (40). Under these conditions KH-52 was clearly degraded by h20Sc at linear rates over the entire time course of incubation. Moreover, the rates of peptide bonds hydrolysis were not appreciably enhanced in presence of REG $\gamma$  and they appear just slightly lower of the rates measured when proteasome associates with REG $\alpha\beta$  (Figure 5C, compare blue and red curves). Furthermore, indicative of absolutely proteasome-dependent degradation, peptide hydrolysis was totally prevented in presence of 20  $\mu$ M epoxomicin (Figure 5C, green curve). Similar results were also obtained when MP-45 precursor peptide was incubated in the same experimental conditions (Figure 5d).

We, then, assessed the second hypothesis, i.e. that REG $\gamma$  could stimulate the 20S proteasome in a way that leads to a complete degradation of MHC class I epitopes. In fact, although binding of REG $\gamma$  does not result in increased rates of substrate hydrolysis, however it might modify proteasome cleavage specificities so that a different pattern of peptide products is generated. Such a change in enzymatic properties potentially can strongly affect formation of specific antigenic peptides like SIINFEKL. To directly test this possibility, peptides released during hydrolysis of KH-52 by equimolar amounts of h20Sc with and without REG $\gamma$  were analyzed by tandem mass spectrometry (MS/MS). Importantly, in this mass spectrometry study shorter peptides (that represent the great majority of proteasomal products (41-43)) were excluded in order to minimize false positive identifications and signals originating from small chemical compounds, and therefore could not be identified neither quantified. On the contrary, peptides with the correct size to bind to MHC class I heterodimers (i.e. 8-10 mers) could be accurately analyzed. By this approach, 76 different peptides from KH-52 were identified; those ranged in length from 8 to 16 residues and were

derived from the entire length of the substrate. Importantly, some of them were generated exclusively by one form of proteasome (i.e. h20Sc or h20Sc-REG $\gamma$ ) (supplementary Table 2), while several others were released by both proteasomal forms. Although MS/MS does not provide quantitative information about the absolute abundance of the peptides detected, however, their relative amounts can be assessed by comparing the corresponding ion intensities measured in sequential MS/MS analyses. Therefore, we used ion intensities to quantify the relative amount of single fragments generated from KH-52 in both degradation reactions. Remarkably, this analysis demonstrated that peptides products are released by h20Sc and h20Sc-REG $\gamma$  in very different amounts (Figure 5E). Most importantly, we could demonstrate that the generation of SIINFEKL is strikingly suppressed in presence of REG $\gamma$ , as inferred by a two log values reduction of its ion intensity (Figure 5F).

All these results demonstrate that the regulator REG $\gamma$  has the capacity to modify the cleavage properties of the 20S proteasome in the nucleus compartment in such a way that MHC class I antigenic peptides present in PTPs are destroyed rather than correctly processed.

## **Discussion.**

This study describes a distinctive and unexpected role of REG $\gamma$ -proteasome complex that is responsible for the MHC class I antigen degradation in cancer. In this study we demonstrate an inverse correlation between REG $\gamma$  expression and MHC class I antigen presentation in cancers, thus giving rise to a new role of REG $\gamma$  in the MHC class I antigen processing pathway.

Surprisingly, around twenty years ago, REG $\gamma$ <sup>-/-</sup> mice have been reported to have any positive or negative effects on antigen presentation. Barton et al, have demonstrated that REG $\gamma$ <sup>-/-</sup> mice did not shown an important impairment in antigen presentation after viral infection (27). The only observed phenotypes reported of REG $\gamma$ <sup>-/-</sup> mice are to date a small

reduction in the numbers of specific CD8+ T lymphocytes and growth retardation (27, 28). In this study, we demonstrate clearly for the first time a specific role of REG $\gamma$  in MHC class I antigen processing and presentation. We show that REG $\gamma$  is a negative regulator of the MHC class I antigen presentation in cancer. We prove that REG $\gamma$  degrades *in vitro* and *in cellulo* MHC class I peptides derived from the production of pioneer translation products. The fact that Barton, et al have not seen any effect on antigen presentation in mice lacking REG $\gamma$  is not surprising in view of our results. We were only able to see a defect in MHC class I antigen presentation in cancer cell lines when REG $\gamma$  was naturally overexpressed. Indeed, when REG $\gamma$  was knocked down in the different cancer cell lines using either siRNA, overexpression of miRNA-7, cisplatin treatment, or the CRISPR technology, we observed an important benefit in CD8+ T cells activation *in cellulo*. In contrast, in the normal cell line where the REG $\gamma$  was not overexpressed or in a rescue experiment in the melanoma CRISPR cell line, we managed to inhibit the MHC class I antigen presentation at the same level of what we saw in cancer cell lines overexpressing naturally the REG $\gamma$ . It is generally believed that, only the REG $\alpha/\beta$  associated with the immunoproteasome can have a positive effect on the production of MHC class I antigenic epitopes. Strikingly, these results clearly demonstrate a close correlation between the expression of REG $\gamma$  and the tumor immune evasion. They support the idea that not only the heterodimer REG $\alpha/\beta$  has a role in antigen presentation but also REG $\gamma$ , in an opposite fashion than the former complex.

As mentioned above, REG $\gamma$  has been reported by several studies to be overexpressed in several cancers (29-31). This overexpression has been seen as a beneficial setting for the tumor to proliferate and become metastatic. In fact, recently, REG $\gamma$  has been shown to be involved in the degradation of some regulatory proteins. For example, the cyclin-dependent kinase inhibitor p21 has been demonstrated to be specifically degraded in an ATP and

ubiquitin independent manner via the REG $\gamma$ -proteasomal pathway (17). Furthermore, two other cyclin-dependent kinases inhibitors, p16 and p14 have also been reported to be degraded by the same pathway (16). Even more importantly, REG $\gamma$  has been shown to be involved in the MDM2-degradation process of p53 (19). These findings demonstrate the important role of REG $\gamma$  on the regulation of cell proliferation and in apoptosis. With this study, we demonstrate a new role for the proteasome REG $\gamma$  regulator that is beneficial for cancers. Indeed, REG $\gamma$  overexpression is not only beneficial for cancer progression but, as it is proven by our data, it is also-involved in the reduction of the numbers of polypeptides containing MHC class I epitope thus giving rise to an inefficient immune response against tumor cell lines leading to an invisibility of the cancer cells to the host immune system.

If the REG $\alpha/\beta$  complex is mostly reported to be located in the cytoplasm, the REG $\gamma$  is mostly a nuclear proteasomal regulator (14). Furthermore, REG $\gamma$  has been shown to be associated with the 20S proteasome in a subnuclear part of the nucleus, in nuclear speckles (20). Moreover, many reports demonstrate that 20S proteasome and immunoproteasome are present in the nucleus and more specifically in clastosome structures (44). Taking into account the fact that PTPs are produced by a nuclear translation event, it will be more beneficial for the cells to process the different PTPs in the same compartment where they are produced. In terms of cancer invisibility by the host's immune system, it would be more advantageous also if the cancer cells degrade as soon as possible the tumor-associated PTPs (TA-PTPs) at the place where they are produced than to let them to have the chance to be properly processed in the cytoplasm and induce specific cancer immune responses. By overexpressing REG $\gamma$  in the nucleus where PTPs are located, the cancer cell lines are adding another step of regulation in the immune response. In fact, we show that by manipulating the localization of REG $\gamma$ , with an overexpression of the  $\Delta$ NLS form of the regulator, we have been able to restore a proper immune response against TA-PTPs. This finding of a specific

site of degradation of TA-PTPs reinforced our recent idea that, in normal conditions, the MHC class I antigen epitopes are produced and processed in the nucleus and that in abnormal conditions, such as during cancer, the TA-PTPs are still produced in the nucleus, but instead, to be properly processed by different types of proteasomes such as the standard 20S proteasome, the immunoproteasome or a REG $\alpha$ / $\beta$ -proteasomal complex (45-48), they are destroyed by the REG $\gamma$ -proteasomal complex.

The eukaryotic proteasome exhibits at least three different activities. The  $\beta$ 1 subunits show a peptidyl-glutamyl peptide hydrolysing (PGPH) activity, while the  $\beta$ 2 subunit exhibits a trypsin-like activity and the  $\beta$ 5 subunit shows a chymotrypsin-like activity (49). When cells are treated with interferon gamma, the expression of three additional  $\beta$ -type subunits is stimulated. All the three active  $\beta$ -subunits are replaced by a further set of three active  $\beta$ -subunits: LMP2 ( $\beta$ 1i), LMP7 ( $\beta$ 5i) and LMP10 ( $\beta$ 2i) (Mec11), named the immuno-subunits, producing the so called immunoproteasome (26). Although the heptamer REG $\alpha$ / $\beta$  has been reported to stimulate all three proteasomal activities (12), REG $\gamma$  has been shown to stimulate only the trypsin-like activity (18). The 20S proteasome and immunoproteasome have been reported to bind to the heptamer REG $\alpha$ / $\beta$  regulator to process correctly some but not all MHC class I epitopes. Two different reasons can explain the fact that REG $\gamma$  decreases the PTP-dependent antigen presentation, either REG $\gamma$  could inhibit the processing of the PTPs by the proteasome complex or it could enhance the destruction of MHC class I epitopes embedded in PTPs by the proteasomal complex. Here we speculate that the REG $\gamma$  would bind to the nuclear 20S proteasome to stimulate the degradation and not the proper processing of MHC class I antigenic peptides, produced by the tumor cells, by changing the activity of the 20S core. Accordingly, when the precursor peptide KH-52 was degraded by 20S constitutive proteasome activated by REG $\gamma$ , generation of the immunodominant epitope SIINFEKL was completely abolished. Although the mass spectrometry analysis employed herein precludes

recognition of the small peptides generated following fragmentation of SIINFEKL, stimulation of proteasomal peptidase activities, resulting in the destruction of the antigenic peptide, appears as the more likely explanation for the impossibility to detect the immunodominant epitope in presence of REG $\gamma$ . This hypothesis is further supported by the fact that the mutated REG $\gamma$  (N151Y), that is still capable to bind the 20S core, do not anymore stimulate the trypsin-like activity and so the degradation of MHC class I PTPs-derived peptides, leading to an increase of the antigen presentation.

We have recently reported that the PTPs are one of the major sources of MHC class I antigenic peptides for the endogenous and exogenous pathways. We show that by inhibiting the degradation of PTPs by the proteasome in tumor cell lines, we were able to increase the amount of unprocessed PTPs to enter the exogenous pathway with the consequence of an increase in the activation of naïve CD8<sup>+</sup> T cells (7, 8). The fact that we show that REG $\gamma$  is responsible for the degradation of PTPs, and by doing so, inhibit the endogenous MHC class I antigen presentation from TA-PTPs in tumor makes us to hypothesize that this phenomenon might also affect the PTP-dependent exogenous MHC class I presentation. Indeed, by overexpressing REG $\gamma$  in a CRISPR REG $\gamma$  knockout cell line, we show that this cell line is not able to induce the proliferation of CD8<sup>+</sup> T cells via the exogenous pathway anymore. Moreover, when we show that the mutated and the  $\Delta$ NLS-REG $\gamma$  constructs are less active to degrade the PTPs, these prove that the sole role of REG $\gamma$  is to stimulate the nuclear proteasomal complex, not only to inhibit the MHC class I endogenous antigen presentation but also the MHC class I exogenous pathway. Thereby, we speculate that the cancer cell lines are inducing the overexpression of REG $\gamma$  in purpose, not only to inhibit the MHC class I endogenous pathway, but also to make sure that none of the TA-PTPs would have the chance to enter the MHC class I exogenous pathway inducing the proliferation of naïve CD8<sup>+</sup> T cells.

In conclusion, all the results from this study and from the previous studies on REG $\gamma$  support the idea that the tumor cell lines have established a system to positively regulate REG $\gamma$  expression for specific purposes, in order to i) control cell cycle arrest, ii) control apoptosis, iii) control cell proliferation, and most importantly iv) to inhibit endogenous and exogenous MHC class I antigen presentation, by degrading specifically the TA-PTPs encoding MHC class I epitopes, leading the cancer cell lines to become invisible to the host's immune pathway.

### **Materials and methods**

More detailed descriptions of experimental procedures used are provided in Supplementary information.

#### **T-cell hybridoma, cell culture and transfection**

The SIINFEKL:K<sup>b</sup>-specific (B3Z) T-cell reporter hybridoma has been described previously (32). Human A375, WM3526 and WM3682 melanoma cell lines, HT29 and T84 colon tumor cell lines, A549 adenocarcinoma lung cells, MRC5 “normal” lung cells were cultivated in medium recommended by ATCC.

Cells were transfected with different quantities of expression plasmids along with 2  $\mu$ L of JetPrime according to the manufacturer's protocol (Ozyme). Each plasmid vector contained a flag tag.

#### **CRISPR/Cas9 transfection and selection**

CRISPR/Cas9 system was performed on A375 cell line. After transfection of 1 $\mu$ g of CRISPR plasmid vector, cells were sorted 2 days later (1 cell / well). PCR and Western Blot were performed on clones; selected ones were sent to sequencing. A TOPO TA cloning (Life technologies) was carried out on selected clones. A375 clone number 11 was generated. It was called A375c.XI.

#### **In vitro peptides degradation**

Reconstitution of 20S-PA28 complexes and degradation of short fluorogenic substrates, of MP-45 polypeptide and of KH-52 polypeptide were performed according methods already described (40, 41, 50). Briefly, REG $\gamma$ - and REG $\alpha\beta$ -20S proteasomes were reconstituted by preincubating human 20S constitutive particles (BostonBiochem, USA) with a 6-fold molar excess of REG $\gamma$  (BostonBiochem, USA) or REG $\alpha\beta$  (41) at 37° C for 30 min in 20 mM HEPES, pH 7.6, and 2 mM NaCl and were used immediately for degradation experiments. For kinetics analysis and generation of peptide products for MS/MS studies, KH-52 (50  $\mu$ M) was incubated with h20Sc, REG $\gamma$ -h20Sc or REG $\alpha\beta$ -h20Sc (20 nM), for 8 hr at 37° C in 20 mM HEPES, pH 7.6, 2 mM NaCl. To assay peptides generated during protein degradation, we measured the appearance of new amino groups using fluorescamine as described (40). At the end of the incubation, peptide products were separated from none degraded polypeptide by ultrafiltration through a membrane with a 3 kDa cutoff (Nanosep, Pall, USA), and these samples were used for MS/MS analysis.

### **Liquid chromatography–tandem MS (LC–MS/MS) analysis**

5 $\mu$ l of samples from experiments h20Sc +/- REG $\gamma$  containing approximately 130 pmoles NH<sub>2</sub>/ $\mu$ l were loaded onto StageTips $\mu$ C18 [1,2]; peptides were eluted in 40  $\mu$ l 80% acetonitrile in 0.1% formic acid. The acetonitrile was allowed to evaporate in a Speed-Vac and then the samples were resuspended in 6 $\mu$ l eluent A (see the composition below) for nLC-MS/MS analysis. 2 $\mu$ l of each sample were injected as technical replicate on a nLC–ESI–MS/MS quadrupole Orbitrap QExactive-HF mass spectrometer (Thermo Fisher Scientific). Peptides separation was achieved on a linear gradient from 95% solvent A (2% ACN, 0.1% formic acid) to 50% solvent B (80% acetonitrile, 0.1% formic acid) over 23 min and from 60 to 100% solvent B in 2 min at a constant flow rate of 0.25  $\mu$ l/min on UHPLC Easy-nLC 1000 (Thermo Scientific) connected to a 25-cm fused-silica emitter of 75  $\mu$ m inner diameter (New Objective, Inc. Woburn, MA, USA), packed in-house with ReproSil-Pur C18-AQ 1.9  $\mu$ m

beads (Dr Maisch GmbH, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark). MS data were acquired using a data-dependent top 15 method for HCD fragmentation. Survey full scan MS spectra (300–1750 Th) were acquired in the Orbitrap with 60000 resolution, AGC target  $1^{e6}$ , IT 120 ms. For HCD spectra, resolution was set to 15000 at  $m/z$  200, AGC target  $1^{e5}$ , IT 120 ms; NCE 28% and isolation width 3.0  $m/z$ .

### **Data processing and analysis**

For quantitative proteomic Raw data were processed with MaxQuant (ver. 1.5.2.8) searching against a database containing only the sequence of KH-52 Intron SIINFEKL, no enzyme specificity was selected and no difference between I and L. Mass deviation for MS-MS peaks was set at 20 ppm and peptides false discovery rates (FDR) at 0.01; the minimal length required for a peptide identification was eight amino acids. The list of identified peptides was filtered to eliminate reverse hits. Statistical analysis was done with Perseus (ver. 1.5.1.6) considering peptides intensity; normalization with Z-score and imputation were applied. Significant peptides were calculated with t-test, Benjamini Hochberg correction and  $FDR < 0.05$  (more stringent assignments) and t-test with p-value 0.05 (less stringent assignments). Only significant peptides were used for supervised Hierarchical Clustering analysis.

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## References

1. Joffre OP, Segura E, Savina A, & Amigorena S (2012) Cross-presentation by dendritic cells. *Nature reviews. Immunology* 12(8):557-569.
2. Yewdell JW, Anton LC, & Bennink JR (1996) Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J Immunol* 157(5):1823-1826.
3. Coulie PG, *et al.* (1995) A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proceedings of the National Academy of Sciences of the United States of America* 92(17):7976-7980.
4. Guilloux Y, *et al.* (1996) A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. *The Journal of experimental medicine* 183(3):1173-1183.
5. Shastri N, Cardinaud S, Schwab SR, Serwold T, & Kunisawa J (2005) All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunological reviews* 207:31-41.
6. Apcher S, *et al.* (2011) Major source of antigenic peptides for the MHC class I pathway is produced during the pioneer round of mRNA translation. *Proceedings of the National Academy of Sciences of the United States of America* 108(28):11572-11577.
7. Apcher S, *et al.* (2013) Translation of pre-spliced RNAs in the nuclear compartment generates peptides for the MHC class I pathway. *Proceedings of the National Academy of Sciences of the United States of America* 110(44):17951-17956.
8. Duvallat E, *et al.* (2016) Exosome-driven transfer of tumor-associated Pioneer Translation Products (TA-PTPs) for the MHC class I cross-presentation pathway. *Oncoimmunology* 5(9):e1198865.
9. Collins GA & Goldberg AL (2017) The Logic of the 26S Proteasome. *Cell* 169(5):792-806.
10. Kish-Trier E & Hill CP (2013) Structural biology of the proteasome. *Annu Rev Biophys* 42:29-49.
11. Leone P, *et al.* (2013) MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. *J Natl Cancer Inst* 105(16):1172-1187.
12. Whitby FG, *et al.* (2000) Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* 408(6808):115-120.
13. Cascio P (2014) PA28alpha: the enigmatic magic ring of the proteasome? *Biomolecules* 4(2):566-584.
14. Soza A, *et al.* (1997) Expression and subcellular localization of mouse 20S proteasome activator complex PA28. *FEBS letters* 413(1):27-34.
15. Wojcik C, Tanaka K, Paweletz N, Naab U, & Wilk S (1998) Proteasome activator (PA28) subunits, alpha, beta and gamma (Ki antigen) in NT2 neuronal precursor cells and HeLa S3 cells. *Eur J Cell Biol* 77(2):151-160.
16. Chen X, Barton LF, Chi Y, Clurman BE, & Roberts JM (2007) Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. *Molecular cell* 26(6):843-852.
17. Li X, *et al.* (2007) Ubiquitin- and ATP-independent proteolytic turnover of p21 by the REGgamma-proteasome pathway. *Molecular cell* 26(6):831-842.
18. Li X, *et al.* (2006) The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. *Cell* 124(2):381-392.
19. Zhang Z & Zhang R (2008) Proteasome activator PA28 gamma regulates p53 by enhancing its MDM2-mediated degradation. *The EMBO journal* 27(6):852-864.

20. Baldin V, *et al.* (2008) A novel role for PA28gamma-proteasome in nuclear speckle organization and SR protein trafficking. *Molecular biology of the cell* 19(4):1706-1716.
21. Cioce M, Boulon S, Matera AG, & Lamond AI (2006) UV-induced fragmentation of Cajal bodies. *The Journal of cell biology* 175(3):401-413.
22. Zannini L, Buscemi G, Fontanella E, Lisanti S, & Delia D (2009) REGgamma/PA28gamma proteasome activator interacts with PML and Chk2 and affects PML nuclear bodies number. *Cell Cycle* 8(15):2399-2407.
23. Fort P, Kajava AV, Delsuc F, & Coux O (2015) Evolution of proteasome regulators in eukaryotes. *Genome Biol Evol* 7(5):1363-1379.
24. Boulon S, Westman BJ, Hutten S, Boisvert FM, & Lamond AI (2010) The nucleolus under stress. *Molecular cell* 40(2):216-227.
25. Welk V, *et al.* (2016) Inhibition of Proteasome Activity Induces Formation of Alternative Proteasome Complexes. *The Journal of biological chemistry* 291(25):13147-13159.
26. Groettrup M, Kirk CJ, & Basler M (2010) Proteasomes in immune cells: more than peptide producers? *Nature reviews. Immunology* 10(1):73-78.
27. Barton LF, *et al.* (2004) Immune defects in 28-kDa proteasome activator gamma-deficient mice. *J Immunol* 172(6):3948-3954.
28. Murata S, *et al.* (1999) Growth retardation in mice lacking the proteasome activator PA28gamma. *The Journal of biological chemistry* 274(53):38211-38215.
29. Wang X, *et al.* (2011) REG gamma: a potential marker in breast cancer and effect on cell cycle and proliferation of breast cancer cell. *Medical oncology* 28(1):31-41.
30. Chai F, *et al.* (2014) High expression of REGgamma is associated with metastasis and poor prognosis of patients with breast cancer. *Int J Clin Exp Pathol* 7(11):7834-7843.
31. He J, *et al.* (2012) REGgamma is associated with multiple oncogenic pathways in human cancers. *BMC Cancer* 12:75.
32. Shastri N & Gonzalez F (1993) Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. *J Immunol* 150(7):2724-2736.
33. Giles KM, *et al.* (2016) microRNA-7-5p inhibits melanoma cell proliferation and metastasis by suppressing RelA/NF-kappaB. *Oncotarget* 7(22):31663-31680.
34. Xiong S, *et al.* (2011) MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2. *Int J Biol Sci* 7(6):805-814.
35. Kefas B, *et al.* (2008) microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res* 68(10):3566-3572.
36. Saydam O, *et al.* (2011) miRNA-7 attenuation in Schwannoma tumors stimulates growth by upregulating three oncogenic signaling pathways. *Cancer Res* 71(3):852-861.
37. Xiong S, *et al.* (2014) PA28gamma emerges as a novel functional target of tumour suppressor microRNA-7 in non-small-cell lung cancer. *British journal of cancer* 110(2):353-362.
38. Ali A, *et al.* (2013) Differential regulation of the REGgamma-proteasome pathway by p53/TGF-beta signalling and mutant p53 in cancer cells. *Nature communications* 4:2667.
39. Kanai K, Aramata S, Katakami S, Yasuda K, & Kataoka K (2011) Proteasome activator PA28gamma stimulates degradation of GSK3-phosphorylated insulin transcription activator MAFA. *J Mol Endocrinol* 47(1):119-127.
40. Raule M, Cerruti F, & Cascio P (2014) Enhanced rate of degradation of basic proteins by 26S immunoproteasomes. *Biochim Biophys Acta* 1843(9):1942-1947.
41. Raule M, *et al.* (2014) PA28alpha/beta reduces size and increases hydrophilicity of 20S immunoproteasome peptide products. *Chem Biol* 21(4):470-480.
42. Kisselev AF, Akopian TN, Woo KM, & Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *The Journal of biological chemistry* 274(6):3363-3371.

43. Nussbaum AK, *et al.* (1998) Cleavage motifs of the yeast 20S proteasome beta subunits deduced from digests of enolase 1. *Proceedings of the National Academy of Sciences of the United States of America* 95(21):12504-12509.
44. Lafarga M, *et al.* (2002) Clastosome: a subtype of nuclear body enriched in 19S and 20S proteasomes, ubiquitin, and protein substrates of proteasome. *Molecular biology of the cell* 13(8):2771-2782.
45. Guillaume B, *et al.* (2010) Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proceedings of the National Academy of Sciences of the United States of America* 107(43):18599-18604.
46. Morel S, *et al.* (2000) Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity* 12(1):107-117.
47. Van den Eynde BJ & Morel S (2001) Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. *Current opinion in immunology* 13(2):147-153.
48. Vigneron N & Van den Eynde BJ (2012) Proteasome subtypes and the processing of tumor antigens: increasing antigenic diversity. *Current opinion in immunology* 24(1):84-91.
49. Livneh I, Cohen-Kaplan V, Cohen-Rosenzweig C, Avni N, & Ciechanover A (2016) The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. *Cell Res* 26(8):869-885.
50. Cascio P & Goldberg AL (2005) Preparation of hybrid (19S-20S-PA28) proteasome complexes and analysis of peptides generated during protein degradation. *Methods in enzymology* 398:336-352.

## Figure Legends

**Figure 1 – Inverse correlation between expression of REG $\gamma$  and antigen presentation in multiple cancer cell lines.** **A)** REG $\gamma$  mRNA level was analyzed by qPCR in different cell lines and was normalized to  $\beta$ -actin mRNA level. The MRC5 cell line was used as reference. Experiments were performed in triplicate. Data are expressed as mean  $\pm$  SEM from three technical replicates. **B)** Western blot analysis and quantification (relative to the housekeeping protein  $\beta$ -actin) of REG $\gamma$  expression. The protein level is expressed below each gel. **C)** REG $\gamma$  protein localization was determined by immunofluorescence in the several tumor cell lines and in the MRC5 normal cell line. REG $\gamma$  was stained with Alexa Fluor 488 and nuclei were counterstained with DAPI. Cells were analysed by confocal microscopy. As expected the regulator REG $\gamma$  is localized in the nucleus in all cell lines tested. **D,E)** All cell types were *in vitro* transfected with respectively  $\beta$ -glob-intron-SL8 (**D**),  $\beta$ -glob-exon-SL8 (**E, left panel**) and OVA cDNA (**E, right panel**) expressing constructs. The cells were incubated with the

SL8-specific CD8<sup>+</sup> T-cell hybridoma (B3Z) for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. Free SL8 peptides were added to the cells to ensure that T-cell assays were carried out at non-saturated conditions and that the expression of MHC class I molecules was not affected.

**Figure 2 – Exogenous REG $\gamma$  overexpression decreases antigens presentation.** **A)** The MRC5 and A549 were transfected with a construct expressing REG $\gamma$  WT from 0.0625 to 0.5 $\mu$ g or a corresponding empty construct for 48h. REG $\gamma$  protein level was examined by western blot using  $\beta$ -actin as a loading control. The protein level is expressed below each gel. **B)** The MRC5 and A549 were transfected with a construct expressing Flag-REG $\gamma$  WT (0.25  $\mu$ g). The flag tag was stained with Alexa Fluor 488 and nuclei with DAPI. Cells were analysed by confocal microscopy. As expected the exogenous transfected REG $\gamma$  WT is localized in nuclei. **C)** The MRC5 cells (left panel) and A549 cells (right panel) were transfected with glob-intron-SL8 construct (0.5 $\mu$ g). The cells were incubated with the SL8-specific CD8<sup>+</sup> T-cell hybridoma (B3Z) for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. **D)** MRC5 cells were transfected with a REG $\gamma$  WT construct for 48h. The increase of REG $\gamma$  protein expression has no effect on the expression of REG $\alpha$  protein. **E)** MRC5 cells were transfected with a Glob-intron-SL8 construct (0.5 $\mu$ g) for 48h. At 36h post-transfection, MRC5 cells were overnight treated with epoxomicin (300 nM). Then the cells were incubated with B3Z T-cell hybridoma for 16h. The data show the average of at least three independent experiments  $\pm$  SD. \*\*\*  $p < 0.001$ , \*  $p < 0.05$  (unpaired t test). **F)** Western blot analysis and quantification (relative to the housekeeping protein  $\beta$ -actin) of p21 expression in MRC5 cells treated overnight with 300nM of epoxomicin. The protein level is expressed below each gel. As

expected, the p21 protein level increases following 12h of epoxomicin treatment, despite the fact that the treated cells overexpressed the exogenous regulator REG $\gamma$ .

**Figure 3 – Knockdown and knock-out expression of the regulator REG $\gamma$  promotes antigen presentation.** The A375, WM3526, WM3682, HT29 and T84 cell lines were transfected with siRNA specific for REG $\gamma$  (50 and 100 nM) or siRNA negative control for 48h and then were analyzed by RT-qPCR analysis (**A**), by western-blot (**B**) and by T cell assay (**C**). **A**) Experiments were performed in triplicate. Data are expressed as mean  $\pm$  SEM from three technical replicates. **B**) Western blot was performed to analyze and quantify REG $\gamma$  and p21 expressions,  $\beta$ -actin was used as reference protein. The protein level is expressed below each gel. **C**) Cell lines were transfected with glob-intron-SL8 construct at different concentration (0.5 $\mu$ g). Then 48h later the different cell lines were incubated with B3Z T-cell hybridoma for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. **D**) qPCR analysis of A375 cells transfected with siRNA specific for REG $\gamma$  (50 and 100 nM) was assessed. Inhibition of the REG $\gamma$  mRNA level was quantified and was normalized to  $\beta$ -actin mRNA level. Experiments were performed in triplicate. Data are expressed as mean  $\pm$  SEM from three technical replicates. **E**) Western-blot analysis and quantification (relative to the housekeeping protein  $\beta$ -actin) of REG $\gamma$  and p21 expression. The protein level is expressed below each gel. **F**) A375cXI and A375 cell lines were both transfected with glob-intron-SL8 construct (0.5  $\mu$ g) for 48h and REG $\gamma$ -siRNA (50 or 100 nM) was only added on A375 cells. The cells were incubated with B3Z T-cell hybridoma for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$  (unpaired t test). **G**) A375 and A375cXI cells were transfected with Glob-intron-SL8 construct vector for 48h. The transfection efficacy was analyzed by FACS (Top panel).

Human tumor cell lines were transfected with a construct expressing the H-2K<sup>b</sup> gene and stimulated with extracellular SIINFEKL synthetic peptide for 15 min. The transfection efficacy of the MHC class I kb molecules was analysis by FACS (bottom panels). Controls were stained with mouse IgG1 K isotype control APC. Data are given as percentage.

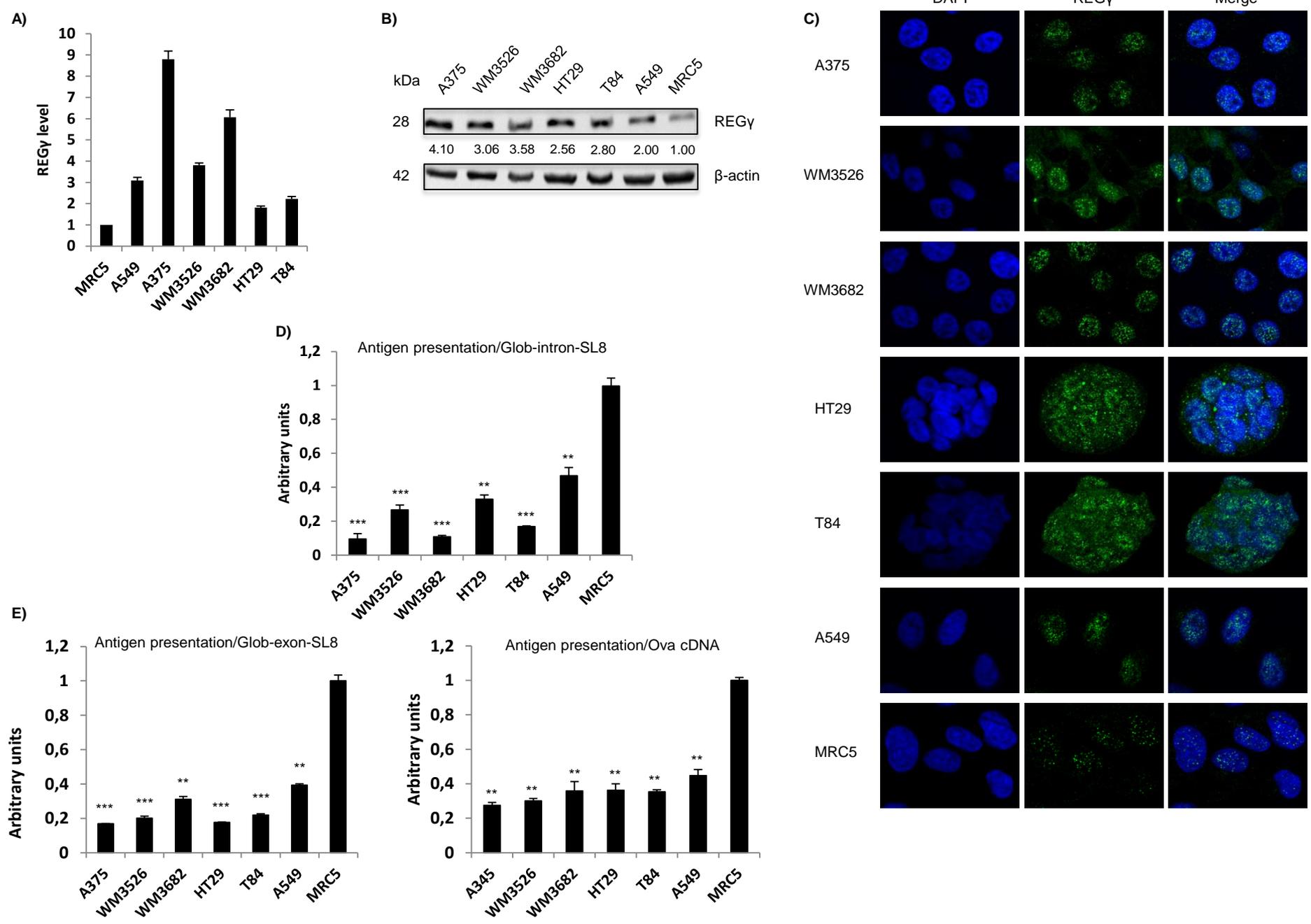
**Figure 4 – REG $\gamma$  regulates the nuclear proteasomal pathway.** **A)** Western-blot was performed to analyze and quantify REG $\gamma$ , REG $\alpha$  and p21 proteins expression in A375c.XI cells (relative to the housekeeping protein  $\beta$ -actin). They were transfected with a construct expressing REG $\gamma$  WT or the mutated REG $\gamma$  N151Y at different concentrations or with a corresponding empty construct for 48h. The protein level is expressed below each gel. **B)** Cells were transfected with glob-intron-SL8 construct (0.5 $\mu$ g). The cells were then incubated with B3Z T-cell hybridoma for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. **C)** A375c.XI cells were transfected with a construct expressing a flag tag REG $\gamma$  WT or a flag tag REG $\gamma$  N151Y. The flag tag was stained with Alexa Fluor 488 and nuclei with DAPI. Cells were analyzed by confocal microscopy. As expected the exogenous REG $\gamma$  WT and REG $\gamma$  N151Y proteins are localized in nuclei. **D)** The interaction of 20S proteasome and exogenous REG $\gamma$  WT and REG $\gamma$ N151Y constructs were analyzed by duolink. The PLA was performed using an antibody against the flag tag and the alpha subunit 4 of 20S proteasome. The staining was analyzed by confocal microscopy. **E)** The immunoprecipitation was assessed using the REG $\gamma$  antibody and the western blot was carried out using an antibody against 20S $\alpha$ 6 subunit. This experiment confirms the interaction of the 20S proteasome and the exogenous REG $\gamma$  WT or REG $\gamma$ N151Y. **F)** Western-blot analysis and quantification (relative to the housekeeping protein  $\beta$ -actin) of REG $\gamma$ , REG $\alpha$  and p21 proteins level in A375c.XI cells transfected with a construct expressing REG $\gamma$  WT or REG $\gamma$   $\Delta$ NLS at different concentrations or with a

corresponding empty construct for 48h. The protein level is expressed below each gel. **G)** Cells were transfected with glob-intron-SL8 construct (0.5 $\mu$ g). The cells were incubated with B3Z T-cell hybridoma for 16h. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells.

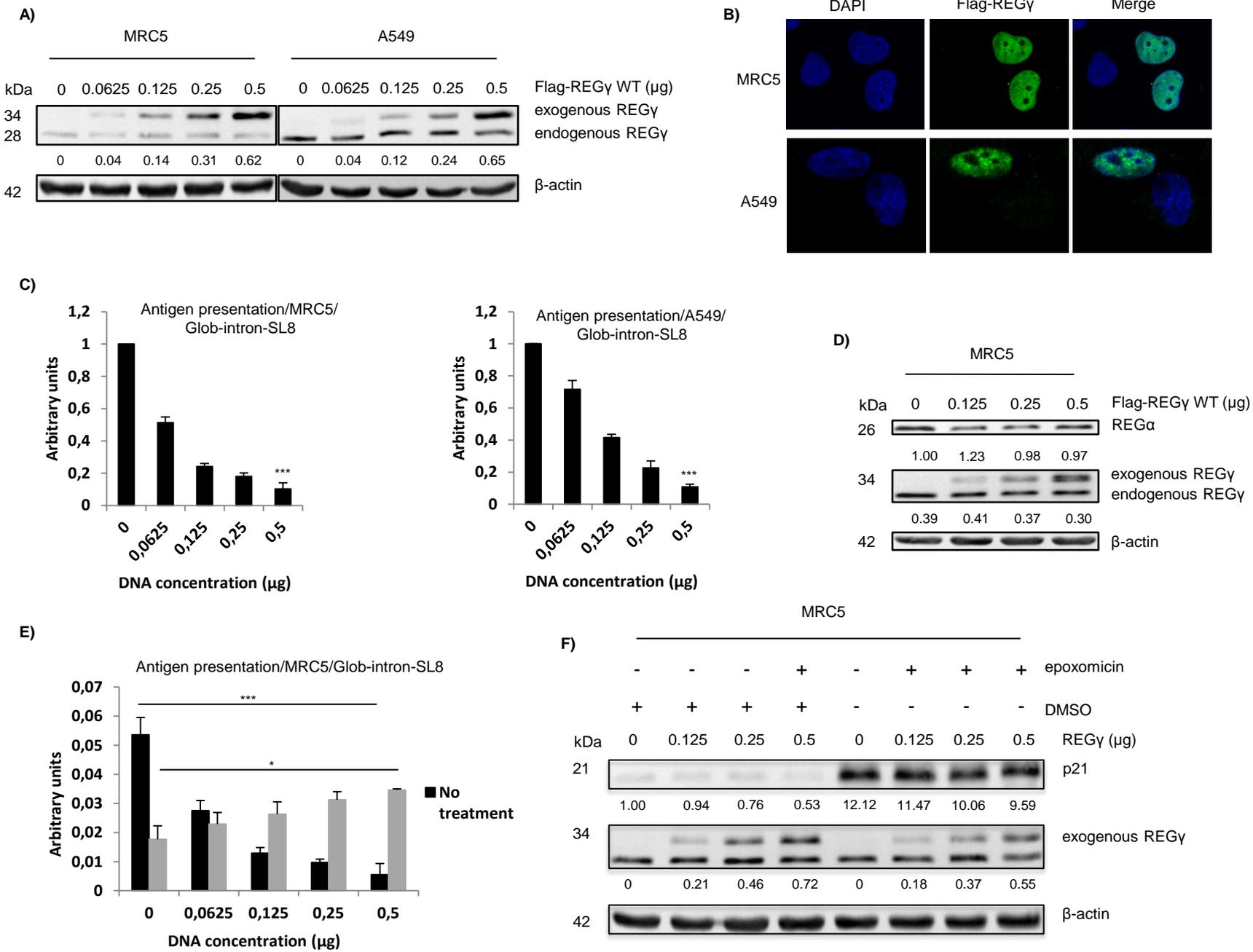
**Figure 5 – REG $\gamma$  promotes the degradation of MHC class I PTP-derived antigenic epitopes.** **A)** A375, WM3526, WM3682, HT29, T84, A549, MRC5 and the A375 $\Delta$ REG $\gamma$  cell lines expressing the glob-intron-SL8 construct (0.5 $\mu$ g) were cultured with BMDCs for 24h. The BMDCs were then co-culture with the SL8-specific CD8<sup>+</sup> T-cell hybridoma (B3Z) for 16h and T-cell activation was estimated by measuring  $\beta$ -galactosidase. The data show the average of at least three independent experiments  $\pm$  SD minus the values from mock-transfected cells. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$  (unpaired t test). **B)** Amino acids sequences of both polypeptides used for the *in vitro* degradation, with the SIINFEKL in red in middle of each precursor peptides. Hydrolysis rates of KH-52 (**C**) and MP-45 (**D**) peptides. Precursor substrates were incubated with human 20S constitutive proteasomes alone or activated by REG $\gamma$  and REG $\alpha/\beta$ , and the amino groups released were measured with fluorescamine at the indicated time points. Data are representative of three independent experiments. **E)** Box plot of SIINFKEL peptide intensity calculated by MaxQuant, in samples h20Sc- (in green) and +REG $\gamma$  (in blue). **F)** HeatMap comparison of abundance levels for significant peptides in samples h20Sc- (biological and technical replicate of samples named A and C respectively) and +REG $\gamma$  (biological and technical replicate of samples named B and D respectively). Difference and similarities between intensity of peptides (normalized by Z-score) are shown; green indicates decreased levels, and red increased levels. Data obtained from supervised Hierarchical Clustering analysis applying t-test and p-value 0.05.

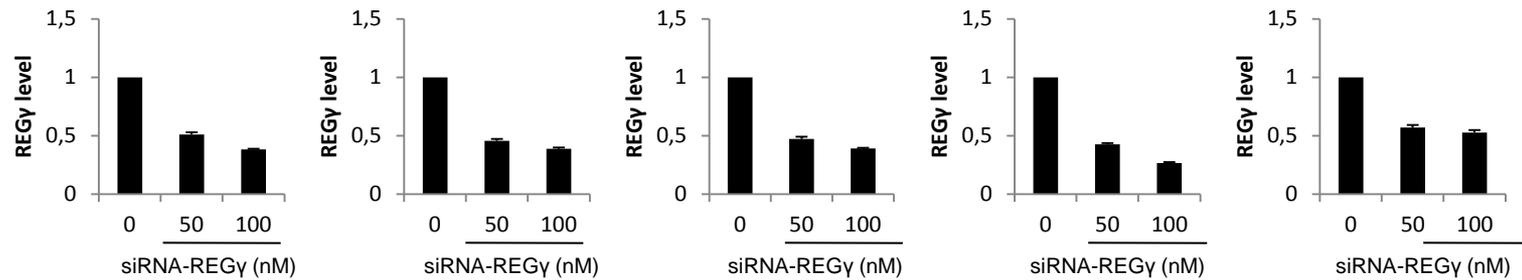
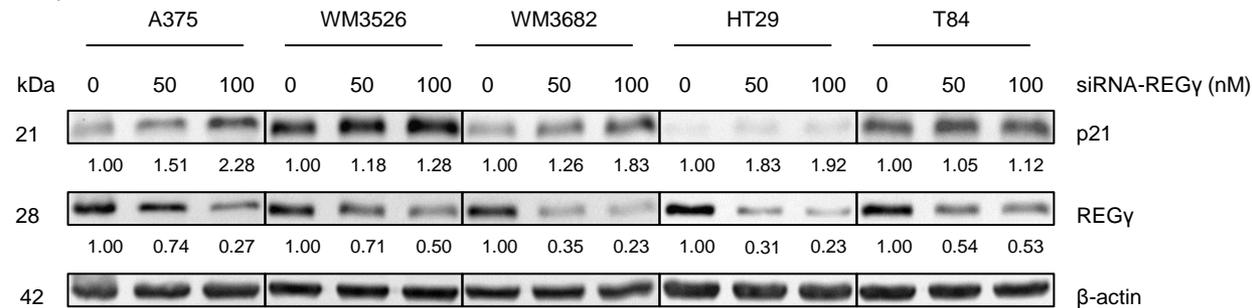
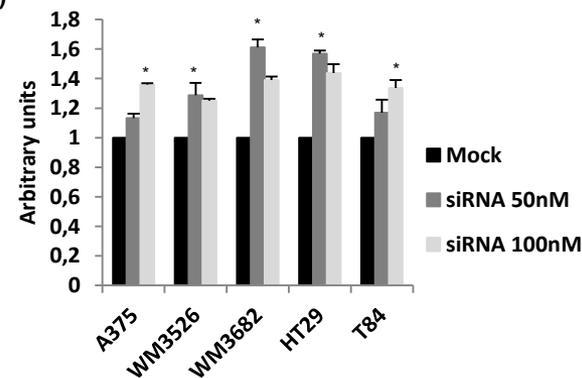
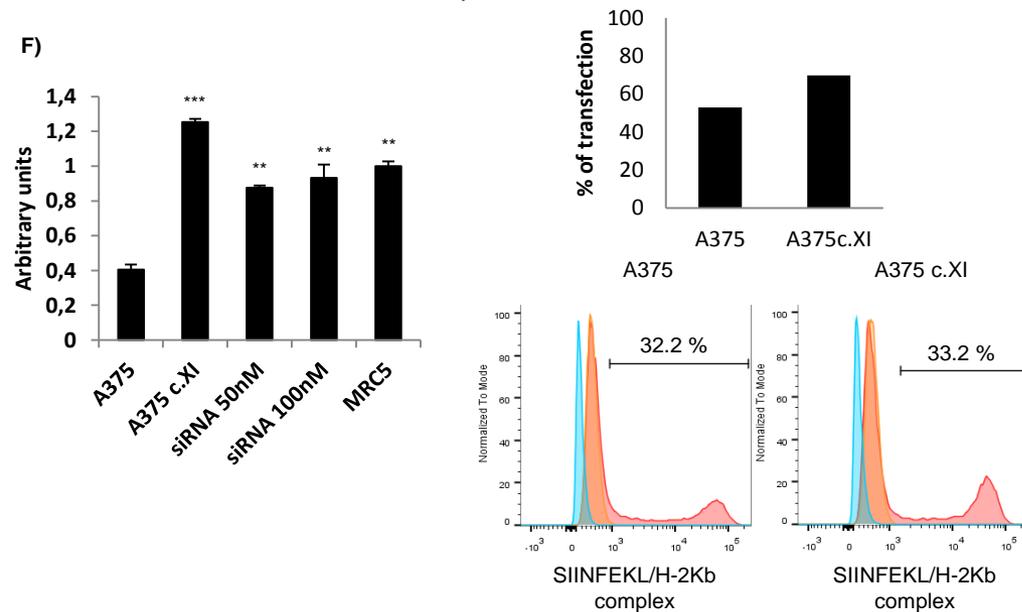
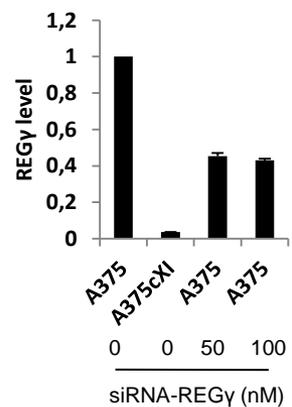
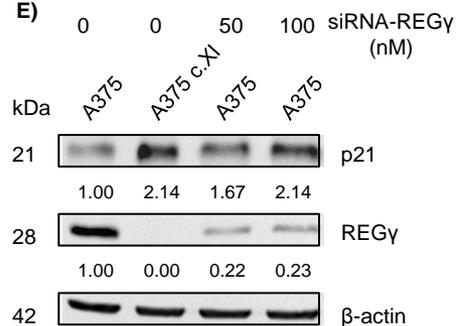
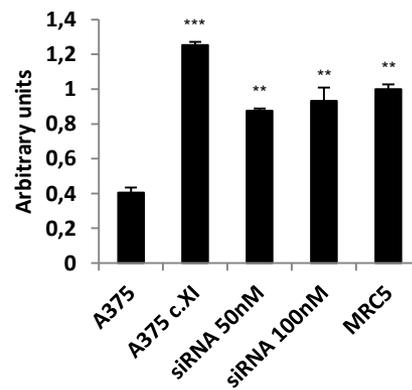


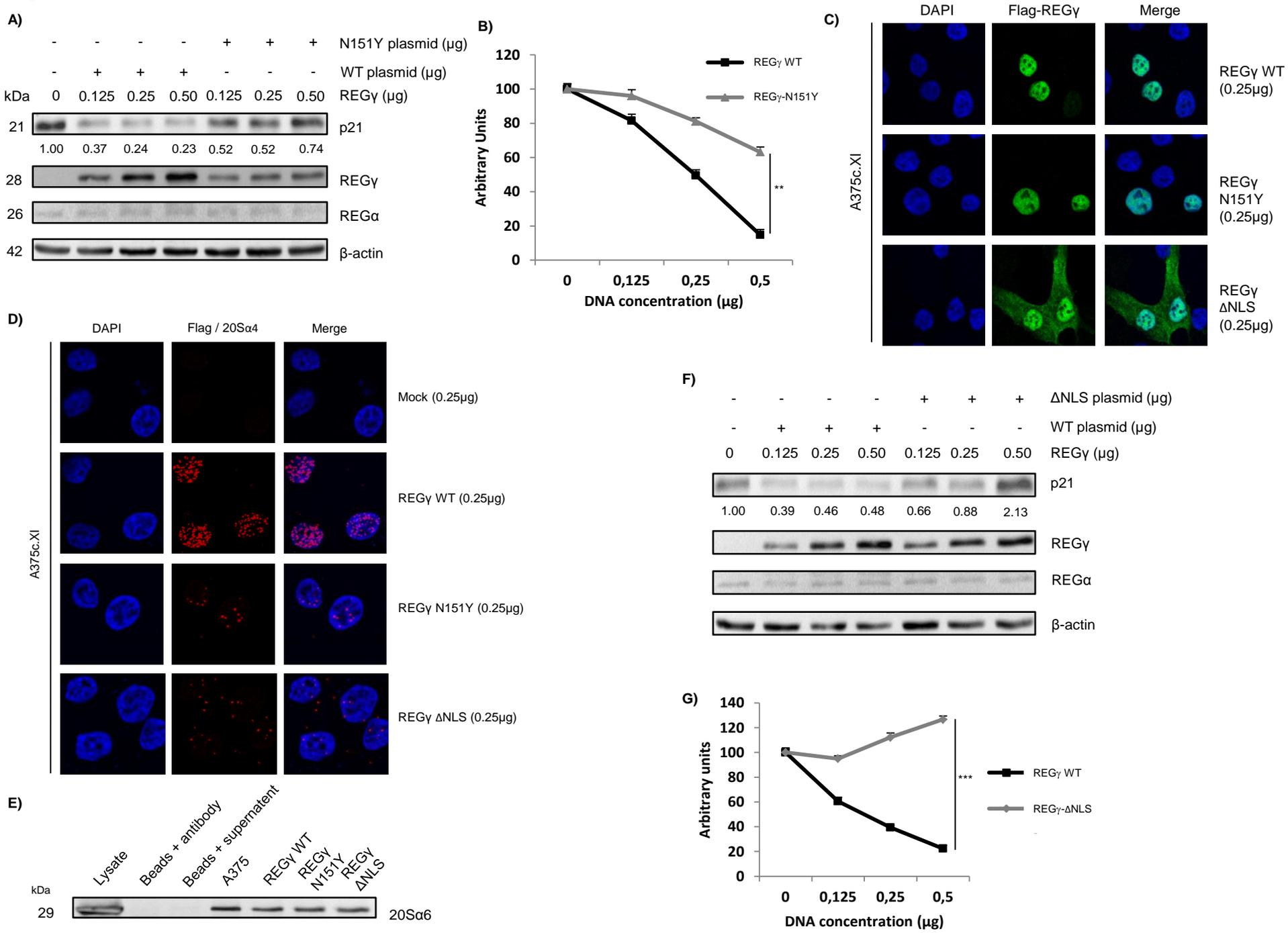
Figure 1



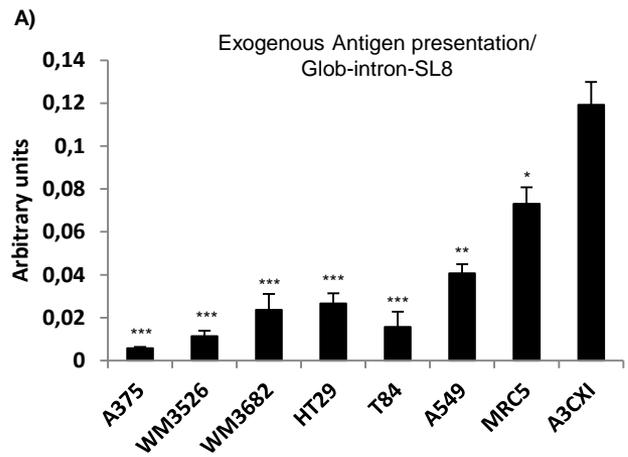
**Figure 2**



**Figure 3****A)****B)****C)****G)****D)****E)****F)**

**Figure 4**

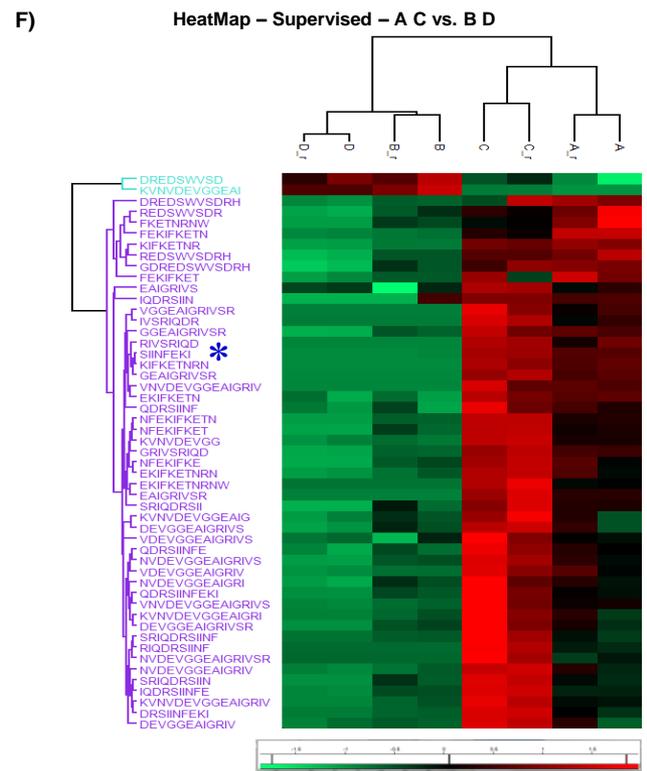
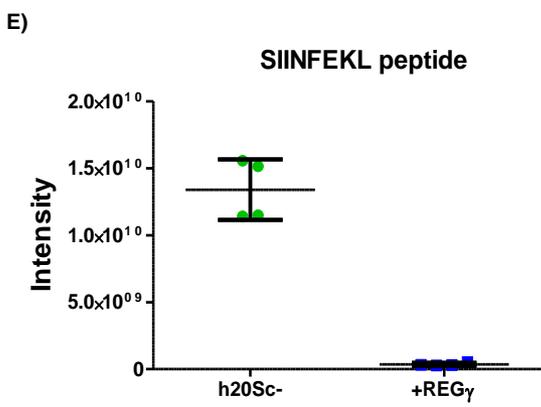
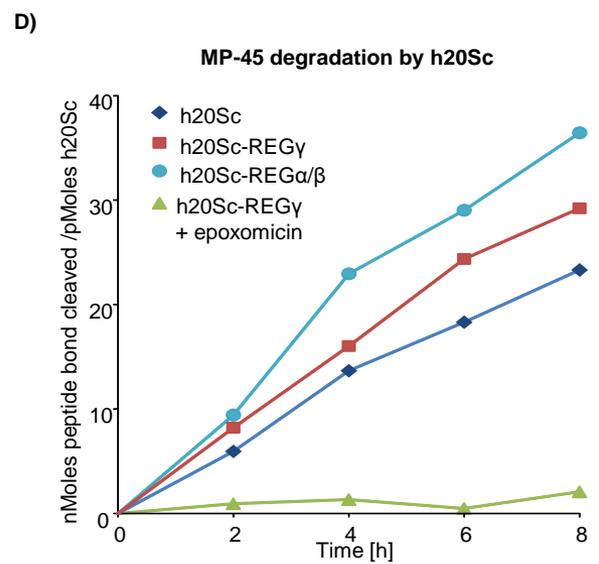
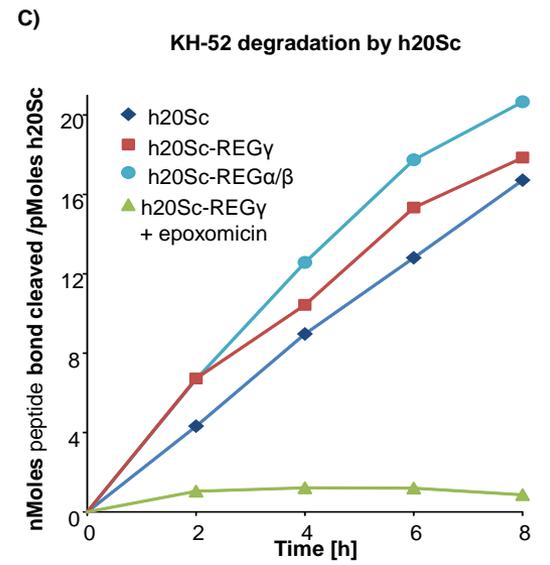
**Figure 5**



**B)**

**MP-45: Peptide exon-SIIN-His**  
 MVHLTDAEKSAVT **SIINF**EKLALWAKVN  
 PDEVGGEALGRLLVVYP

**KH-52: Peptide intron-SIIN-His**  
 KVNVDDEVGGEALGRLVSRRLQDR **SIINF**E  
**KL**FKETNRNRNWACGDREDSWVSDRH



**Titre :** Rôle des Pioneer Translation Products (PTPs) dans la réponse immunitaire anti-tumorale.

**Mots clés :** PTPs, CMH de classe I, antigènes tumoraux, inhibiteurs de l'épissage, LT cytotoxiques

Les vaccins thérapeutiques anti-tumoraux reposent sur l'activation du système immunitaire adaptatif et sont basés sur la reconnaissance des antigènes tumoraux (AT) par les lymphocytes T auxiliaires et cytotoxiques spécifiques. Leur efficacité nécessite une sélection méticuleuse des antigènes cibles ainsi qu'une présentation antigénique par le CMH de classe I (CMH-I) dans les tumeurs non modifiées. Alors que l'attention s'est d'abord portée uniquement sur les AT dérivés de séquences exoniques, ceux dérivés d'événements de traduction alternatifs ont été montrés comme ayant un fort potentiel en tant que cibles. Ces derniers peuvent dériver d'une traduction de séquences dites « non traduites », initiée par des codons alternatifs ou dans un cadre de lecture non canonique. Une telle traduction alternative des ARN épissés dans le noyau et donnant naissance aux Pioneer Translation Products (PTPs) a été décrite. Ces derniers ont été montrés comme source de peptides pour la voie de présentation des antigènes par le CMH-I.

Récemment, nous avons montré que les antigènes dérivés des PTPs et présentés par les tumeurs sont capables d'entraîner une réponse lymphocytaire T cytotoxique *in vivo* et de contrôler la croissance tumorale. De plus, nous avons identifié la molécule inhibitrice de l'épissage isoginkgetin comme modulateur positif de cette présentation dans les cellules cancéreuses. Nous avons observé qu'un de ses dérivées, l'IP2, qui est soluble dans l'eau et moins toxique que l'isoginkgetin, est de même capable d'augmenter la présentation des antigènes dérivés des PTPs dans les tumeurs *in vitro*, ainsi que de réduire la croissance tumorale *in vivo* de manière dépendante de la réponse immunitaire. Ainsi, le composé IP2 se révèle être un immunomodulateur de la réponse anti-tumorale efficace et prometteur pour le développement de nouvelles stratégies thérapeutiques.

**Title:** Role of Pioneer Translation Peptides (PTPs) in the anti-tumor immune response.

**Keywords :** PTPs, MHC class I, splicing inhibitors, tumor antigens, cytotoxic T lymphocytes

Anti-tumoral therapeutic vaccines rely on the activation of the adaptative immune system and are based on the recognition of tumor antigens (TA) by specific helper and cytotoxic T lymphocytes (CTL). Their efficacy requires a careful selection of the targeted antigens as well as an unaltered MHC class I (MHC-I) antigenic presentation in tumors. While the focus was first put on exome-derived TA, evidences highlighted the ones derived from alternative translations as having a high potential as T-cell targets. These can be derived from translation of allegedly non coding sequences, initiated at alternative codons or performed in non-canonical open reading frames. Such an alternative translation occurring from pre-spliced mRNAs in the nucleus has been described as giving rise to the Pioneer Translation Products (PTPs), which constitute a source of polypeptides for the MHC-I pathway.

Recently, we showed that PTPs-derived antigens presented by tumors are able to elicit a CTL response *in vivo* that controls tumor growth. Here, we identified one positive modulator of PTPs-derived antigen presentation in cancer cells: the splicing inhibitor isoginkgetin. Then, we provided one of its derivatives, the IP2, which is water soluble and less toxic than the isoginkgetin, and showed that IP2 treatment increases PTPs-derived antigenic presentation of cancer cells *in vitro* and reduces tumor growth *in vivo* in an immune-dependent manner. Hence we describe here the IP2 as a new efficient immunomodulator of the antitumor response, promising for the development of innovative therapeutic strategies.