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## **Immunochemical approaches to monitor and modulate the adaptive immune system**

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# 1

**General introduction and  
Scope of this dissertation**

## ADAPTIVE IMMUNITY

We are in constant battle against pathogens, and throughout evolution our bodies have developed sophisticated mechanisms to protect us. Our immune system consists of two branches to prevent, cure and suppress infection<sup>1,2</sup>. As immediate defense, the non-specific innate immune system recognizes and responds to pathogens in a generic fashion. Recognition of danger- or pathogen-associated molecular patterns (DAMPs or PAMPs) induces inflammation, membrane attack and phagocytosis of pathogens<sup>3</sup>. Common PAMPs that trigger innate immunity are motifs not found in vertebrates, such as dsRNA, glycans, lipopolysaccharides or endotoxins. DAMPs can be host-derived constituents, such as DNA or RNA, that are normally contained in the nucleus or cytosol. The second branch of the immune system, adaptive immunity, is acquired throughout life<sup>4</sup>. This highly specialized response is the main focus of this dissertation. Its key mediators are B (bursa-derived) and T (thymus-derived) lymphocytes, which are typically activated by specific antigens<sup>5</sup>. In contrast to the immediate and short-lived innate immune response, the adaptive response results in long-term protection by creating immunological memory following the initial infection<sup>6</sup>.

To evoke potent adaptive responses against pathogens without inducing auto-immunity T cells must be able to distinguish between 'self' and 'non-self' antigens. The first step in the generation of functional T cells is positive selection by matching cell surface receptors, such as CD4 and CD8<sup>7</sup>. Those that match undergo a second round of (negative) selection: self-reactive T cells are deleted, thus preventing autoimmunity and establishing central tolerance<sup>8</sup>. Stringent selection assures that only cells with functional T cell receptors (TCRs) that are not auto-reactive will leave the thymus. But before doing so, they downregulate one of the two coreceptors, maturing into either CD4<sup>+</sup> (T helper) or CD8<sup>+</sup> (cytotoxic) T cells<sup>9</sup>. Aberrations in T cell selection mechanisms can result in immune deficiency or autoimmunity, often causing severe disease.

### Cell-mediated immunity

In adaptive immunity two pathways are distinguished: cell-mediated and humoral. The first adaptive immune pathway allows the immune system to catch a glimpse inside most cells for signs of infection or mutation and to take action if needed. The pathway is mediated by major histocompatibility complex (MHC) class I molecules that present peptides derived from intracellular proteins on the surface of all nucleated cells (Fig. 1, left panel)<sup>10</sup>. CD8<sup>+</sup> T cells scan the repertoire of peptide-MHCI (pMHCI) complexes and, when they recognize peptides originating from viral or mutated (onco)proteins, set a cytotoxic response in motion<sup>11-13</sup>. The majority of peptides presented by MHC I are the product of proteasomal degradation of proteins that have fulfilled their function and are no longer

needed, but about 30% of newly synthesized proteins is degraded immediately after synthesis, as a result of defects in protein transcription, translation or folding<sup>14-16</sup>. Presentation of fragments derived from these proteins, collectively termed defective ribosomal products (DRiPs), allows processing and display of even long-lived proteins to CD8<sup>+</sup> T cells as soon as 1.5 hours post infection, thus accelerating the detection rate of infection<sup>17</sup>. A more recently discovered source of T cell epitopes is protein splicing: protease-mediated transpeptidation by the proteasome<sup>18,19</sup>. This ligation of peptide fragments broadens the repertoire of presented peptides by extending past the expressed peptidome<sup>20,21</sup>.

Peptide fragments generated by the proteasome are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where MHCI molecules await to be loaded<sup>22</sup>. Peptide receptive MHCI is assembled in the ER as a heterodimeric complex unstable in the absence of peptide and thus requires stabilization by chaperones<sup>23</sup>. Upon loading of a cognate peptide, typically comprised of 8-10 amino acids, MHCI acquires enough stability to be released from the ER and transported to the cell surface, where it can present its peptide to CD8<sup>+</sup> cells. When a naïve CD8<sup>+</sup> T cell encounters a non-self antigen, it becomes activated, resulting in proliferation of the antigen-specific T cell and lysis of the antigen-presenting cell. After an infection is cleared, most mature CD8<sup>+</sup> T cells undergo apoptosis, but a few remain in the form of memory T cells<sup>6</sup>. In general, the initial response is slow, while the one initiated from memory progresses much quicker. Hence, in case of challenge with a previously-encountered virus or pathogen, the infection is likely to be cleared even before symptoms occur.

### **Humoral immunity**

The second adaptive immune pathway protects the extracellular space (body fluid – humor) and is mediated by MHC class II complexes (MHCII). These present antigens derived from extracellular proteins on the surface of professional antigen-presenting cells (APCs), such as dendritic cells (DCs), B cells and macrophages (Fig. 1, right panel)<sup>16</sup>. APCs can internalize proteins in various ways, including receptor-mediated endocytosis (B cells), phagocytosis (DCs) or macropinocytosis (macrophages)<sup>24</sup>. In lysosomes the proteins are cleaved into peptides, generally 15-24 amino acids in size, which can bind to major histocompatibility complex class II (MHCII) molecules in late endosomes<sup>25</sup>. Endosomes carrying peptide-loaded MHCII complexes are then transported back to the cell surface for presentation to CD4<sup>+</sup> T cells. Upon binding of non-self antigens, CD4<sup>+</sup> T cells become activated, inducing the release of cytokines that stimulate clonal expansion of B cells, thus promoting antibody production. Antibodies are subsequently released into the plasma, where they can bind cognate antigens. By doing so, antibodies are able to confer protection through three main modes of action: inhibiting infectivity

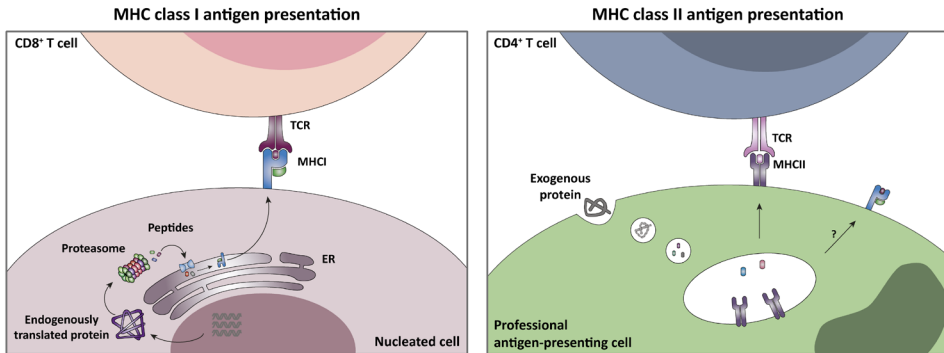


Figure 1. Schematic representation of major histocompatibility complex (MHC) class I and class II-mediated antigen presentation.

or toxicity by binding proteins on the pathogen's cell surface to neutralize them; marking a pathogen for phagocytosis; or activating the innate classical complement pathway.

The pathways described above are not strictly distinct: certain APCs can also process and present extracellular peptides on MHC I in a process referred to as cross-presentation. This is particularly useful for protection against pathogens that have developed strategies to evade immune detection<sup>26</sup>. For example, some herpes viruses produce specific proteins to interfere with host protein synthesis, mainly targeting those involved in MHC I antigen presentation and thus escaping detection by CD8<sup>+</sup> T cells<sup>27</sup>. Through cross-presentation of endocytosed antigen fragments on MHC I (that would normally be presented on MHC II) CD8<sup>+</sup> T cells can still get activated. It is apparent that primarily DCs are capable of cross-presenting, but the exact mechanisms by which cross-presentation occurs are still under debate<sup>28</sup>. Various mechanisms have proposed, yet many questions remain unanswered.

### Immune response in three signals

TCR activation by recognition of an antigenic peptide on MHCs is only the first of three signals required for mounting a full-blown immune response<sup>29</sup>. Whether or not a peptide is presented to its matching T cell depends on a number of factors. Firstly, peptides processed by the proteasome and downstream peptidases need to be of compatible size. Peptides presented by MHC I are typically nine amino acids long, while the proteasome generates peptides of various lengths. Peptides, which are too short (less than seven amino acids), will not interact with TAP and will therefore not be translocated to the ER lumen<sup>30</sup>. On the other hand, long peptides (more than sixteen amino acids) will require further trimming by peptidases to enable efficient loading onto MHC I<sup>31,32</sup>. Secondly, the peptide sequence needs to match one of the expressed MHC I allotypes<sup>23</sup>. Lastly, the affinity of the peptide for its cognate MHC allele should be sufficient to sustain

presentation at the cell surface and prevent exchange for exogenous peptides<sup>33</sup>. The frequency of a specific T cell clone in circulation is generally low prior to encounter with its cognate peptide, so it could take a while before a presented antigen comes in contact with the right CD8<sup>+</sup> T cell. Once a peptide manages to reach the cell surface—on average a chance of 1:200—the pMHC is ready to be scrutinized by passing T cells<sup>34</sup>. TCRs interact not only with the exposed residues of the peptide, but also with the residues on top of the two  $\alpha$  helices, which make up the MHC's binding groove. This interaction is highly selective: a TCR will only be potently activated by a specific combination of peptide and allele<sup>33,35</sup>. This feature is referred to as MHC restriction, which is essential for mounting an appropriate immune response, while maintaining self-tolerance<sup>36</sup>.

Researchers only learned in the late 1980s of a second immune signal, provided by costimulation predominantly through the B7-1/B7-2:CD28 pathway<sup>37</sup>. The CD28 receptor is expressed on 95% of CD4<sup>+</sup> T cells and 50% of CD8<sup>+</sup> T cells, and constitutively on naïve T cells in humans<sup>38</sup>. Engagement by costimulatory ligands B7-1 (CD80) or B7-2 (CD86) on APCs provides the signals needed for T cell activation and survival, including production of the master regulator of T cell activation, IL (interleukin)-2<sup>39-42</sup>. A second costimulatory factor in the B7:CD28 family is CD28 homologue ICOS (inducible costimulator) and its ligand ICOS-L (B7h), a homolog of B7-1 and B7-2<sup>43,44</sup>. ICOS is expressed on TCR-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells and upregulates expression of helper T cell (Th)1- and Th2-polarizing cytokines, but in contrast to CD28, it does not activate IL-2 production<sup>43,45</sup>. ICOS and CD28 seem to work synergistically to regulate CD4<sup>+</sup> T cells<sup>40</sup>. In absence of costimulation, T cells activated by pMHC I will become anergic, although in some cases, when the interaction between pMHC I and TCR is very strong, T cells may remain activated, resulting in peripheral tolerance<sup>46,47</sup>.

Although proliferation of naïve T cells can be initiated when TCR and CD28 signals are present, a productive response will only be established once specific cytokines are produced<sup>48</sup>. Cytokines that provide this third immune signal, required for proper development of CD8<sup>+</sup> T cell effector and memory functions, are IL-12 and type I interferons (IFNs)<sup>29,49</sup>. For CD4<sup>+</sup> T cells, IL-1 can be considered a general third signal, in addition to cytokines that prompt differentiation into one of the Th subsets, such as polarization towards Th1 or Th2 by IL-12 and IL-4, respectively<sup>50-52</sup>.

## MHC STRUCTURE AND FUNCTION

The functions of the two classes of MHC are similar: presentation of peptides to the immune system, but what peptides they present, in which tissues and to which cells differs between the classes. In short, MHC I presents peptide fragments

1 derived from intracellular proteins to CD8<sup>+</sup> T cells, resulting in a cytotoxic response against cells expressing an immunogenic antigen. MHCII, on the other hand, is only found on dedicated antigen-presenting cells and can activate CD4<sup>+</sup> T cells, leading to a B cell response. MHCI and MHCII both consist of two immunoglobulin (Ig)-like domains topped by two  $\alpha$  helices<sup>53-55</sup>. MHCI consists of one long heavy chain, the  $\alpha$ -chain, that forms an Ig-like transmembrane domain,  $\alpha$ 3, and the  $\alpha$ 1 and  $\alpha$ 2 domains that comprise the peptide-binding groove. The second Ig-like domain is provided by the light chain  $\beta$ 2m, which associates non-covalently with the heavy chain. In MHCII, heavy chains  $\alpha$  and  $\beta$  combine to form the  $\alpha$ -helices of the peptide-binding groove. Both chains harbor Ig-like domains ( $\alpha$ 2 and  $\beta$ 2) that anchor the complex in the membrane. The groove of MHCI is closed at both ends wherein only short peptides of 8-11 amino acids can fit, whereas MHCII has an open groove able to accommodate peptides in the range of 15-24 amino acids<sup>56-58</sup>. These structural features dictate binding of certain sized peptides in general, but exact sequences depends on other factors described in more detail below.

### **MHC polymorphisms**

The three major groups of human MHC genes, referred to as HLA (human leukocyte antigens) class I, II and III, are located on chromosome 6, together with many more immunity-associated genes<sup>59</sup>. Unlike the HLA class I and II regions, the gene-dense HLA class III region has been poorly defined<sup>60</sup>. The HLA class I gene region contains three loci coding for the classical HLAI proteins involved in antigen presentation, HLA-A, HLA-B and HLA-C<sup>61-64</sup>. Classical HLA class II proteins HLA-DR, HLA-DP and HLA-DQ are also expressed from three loci. The loci coding for the HLAI heavy chains are among the most polymorphic in the human genome, meaning that they contain many variations in their sequence, generated by mutation, recombination and gene conversion<sup>65,66</sup>. Different allotypes have evolved with selection pressure, yielding a distinct distribution of allele frequencies across the globe<sup>67-69</sup>. Individuals can carry three to six different MHCI allotypes and three to twelve different MHCII allotypes, depending on the inheritance of their parents<sup>33</sup>. The majority of single nucleotide polymorphisms and deletion/insertion polymorphisms are found in the regions that code for the peptide-binding groove, resulting in differences in nature and location of binding pockets, and consequently preferred peptide motifs per allele<sup>65,70-72</sup>. Since only peptides with a matching motif get presented, expressing multiple allotypes allows presentation of more fragments derived from the same protein and hence provides widespread protection against numerous pathogens. There is an obvious heterozygote advantage, explaining why expression of MHC genes is even suggested to play a role in mate selection<sup>73-75</sup>.

Over the years, vastness of information on MHC ligands and motifs has been



gathered in online databases, with SYFPEITHI and the IEDB (Immune Epitope Database) containing the largest collections<sup>76,77</sup>. The discovery of new alleles has greatly advanced since the development of Next-Generation Sequencing, resulting in the identification of over 15,000 subtypes registered in international databases<sup>64,78,79</sup>. These data are extremely valuable for immunogenicity research and the transplantation community<sup>80,81</sup>. For instance, using the data available, HLA types of donors and patients can be matched to increase survival rates of hematopoietic stem cell transplant recipients<sup>82,83</sup>. This is important because mismatch of only a single nucleotide polymorphism could affect the outcome of a transplant.

### **Peptide affinity and T cell activity**

Which peptides can stably associate with the MHC heavy chain depends on the interactions of the binding groove residues with the peptide backbone and occupation of defined binding pockets by the peptide side chains<sup>84-86</sup>. HLA-A, -B and -C heavy chains form six binding pockets, named A-F, that can accommodate a peptide's amino acid side chains<sup>87,88</sup>. Generally two amino acids, referred to as the anchor residues, position the peptide by docking in the pockets. Because theoretically any peptide with a matching motif will fit a certain allotype, predicting which peptides will strongly bind is extremely challenging.

The use of algorithms to predict peptide affinity *in silico*, such as those used by SYFPEITHI, IEDB and NetMHC, facilitates epitope prediction<sup>76,89,90</sup>. Although they provide an indication of binding strength, computational tools alone often fail to accurately predict immunogenicity. For a long time it was assumed that a high peptide affinity results in prolonged presentation to T cells, and hence in increased immunogenicity. Gradually, however, it became clear that binding affinity of a peptide to an MHC is not the only determinant for immunogenicity<sup>91-93</sup>. This is illustrated by a study by Speiser et al., who directly compared vaccination with wild-type melanoma antigen EAAGIGILTV or a higher-affinity altered peptide ligand (APL), ELAGIGILTV<sup>94</sup>. They found that, although more T cells were induced by vaccination with the APL, quality of the response in terms of tumor reactivity and T cell activation *in vivo* was higher after vaccination with the wild-type ligand. A similar observation was made by McMahan et al, who investigated T cell responses in mice with CT26 colon cancer<sup>95</sup>. When comparing vaccination with tumor-associated antigens or APLs, they observed proliferation of tumor antigen-specific T cells and elevated IFN- $\gamma$  in response to the high-affinity APLs, but this did not correlate with anti-tumor immunity. Although a certain affinity is required for efficient loading in the ER, other factors, such as stability, conformational flexibility and formation of the immunological synapse, are also important determinants for T cell activation<sup>92,93,96-99</sup>. More recent epitope prediction tools therefore also include pMHC stability as an extra parameter<sup>100,101</sup>.

1 Passing T cells may sample various antigens, but will only respond to ligands with a certain affinity<sup>102,103</sup>. The TCR-pMHC contact should last long enough to induce signaling, but short enough to allow serial engagement and activation of multiple T cells<sup>104</sup>. Because infected or mutated cells do not always express many copies of an antigen, for example due to downregulation of MHCI or other proteins involved in antigen presentation, sometimes only a few peptides survive the journey to the cell surface. Activation of multiple T cells by one pMHC-complex amplifies the immune stimulus, thus ensuring high sensitivity needed to respond to low-frequency peptides. Of course this can only be accomplished if the association and dissociation kinetics of the TCR-pMHC interaction occur at a reasonable rate. This is in line with the observation that TCR affinity for pMHC is generally low, in the micromolar range<sup>105</sup>. Serial engagement becomes less important when density of a certain pMHC is high, thus an optimal half-life seems to only be required for low-density pMHCs<sup>106</sup>.

### MHC and disease

Predisposition to certain infectious, inflammatory or autoimmune diseases is known to have a genetic origin, in many cases located in the MHC genes<sup>107-112</sup>. An estimated 5% of the population suffers from autoimmune diseases, which include the well-known type I diabetes, multiple sclerosis and rheumatoid arthritis, all diseases that have been extensively studied in relation to MHC<sup>113-118</sup>. In the past few decades increasing numbers of HLA subtypes have been reported in concurrence with other autoimmune diseases not initially linked to MHC, such as celiac disease, systemic lupus erythematosus, ulcerative colitis, Crohn's disease and ankylosing spondylitis<sup>119-123</sup>. The latter example is associated with expression of HLA-B\*27:05<sup>124,125</sup>. It was discovered as early as 1973 that this subtype is expressed in 85-90% of ankylosing spondylitis patients, but how it relates to development of the rheumatoid disorder is still unknown<sup>124-126</sup>. Strikingly, individuals expressing the closely related HLA-B\*27:09 do not develop the disease, although the two subtypes only differ in residue 116 found at the bottom of the F pocket (Asp in HLA-B\*27:05 and His in HLA-B\*27:09)<sup>127</sup>. Crystal structures of the two alleles complexed with the same peptide are virtually indistinguishable, however, molecular dynamics simulations show that the flexibility of peptide-bound HLA-B\*27:09 is much higher than that of HLA-B\*27:05<sup>128</sup>. This implies that peptide dynamics may play an important role in the activation of T cells, and molecular dynamics studies should therefore be included in the experimental data used to build prediction algorithms<sup>129,130</sup>.

In some cases, combinations of MHCI and MHCII alleles convey a predisposition, such as the additional effect of HLA-A\*3 on the HLA-DR15-associated susceptibility to multiple sclerosis<sup>120</sup>. Often, even though a genetic association is established, the mechanism by which a given HLA allotype confers protection or causes disease

is unknown. Understanding the basis of these associations can help advance personalized disease prevention and treatment<sup>131</sup>.

A few relevant mechanisms can be envisioned, of which undesirable presentation of self-peptides or altered self-peptides is perhaps the most obvious. Alternatively, the association may have nothing to do with peptide presentation, but instead may affect the T cell repertoire, including regulatory T cells. Or there could be no effect at all, but the allotype could just be in linkage disequilibrium with another disease-causing gene and therefore act as a marker.

Altered self-peptides may arise from mutated proteins, but can also be derived from post-translationally modified proteins<sup>132,133</sup>. The latter appears to be the case with type 1 diabetes, where a number of modifications present on peptides have been found to trigger autoimmune responses<sup>134-136</sup>. In addition, citrullination, a post-translational deimination of arginine to form citrulline, is the hypothesized culprit in development and progression of rheumatoid arthritis<sup>137,138</sup>. Specifically, evidence points to dysregulation of protein citrullination in the rheumatoid joint, resulting in hypercitrullination and concurrent loss of tolerance<sup>139</sup>. Anti-citrullinated protein antibodies are detectable in early stages of the disease and hence provide valuable diagnostic and prognostic markers for rheumatoid arthritis<sup>140,141</sup>.

Certain MHC alleles are not directly associated with disease, but with susceptibility to adverse drug effects, ranging from mild skin reactions, fever and nausea to even fatal reactions upon re-exposure<sup>142,143</sup>. Patients are often genotyped for risk alleles prior to starting treatment with a drug known to have an association<sup>144</sup>. One of the best known HLA-related adverse drug effects is T cell-mediated hypersensitivity to treatment with abacavir, a nucleoside analog reverse-transcriptase inhibitor used to treat HIV<sup>145</sup>. Treatment with abacavir induces high frequencies of reactive CD8<sup>+</sup> T cells in individuals expressing HLA-B\*57:01, but not in those expressing any of the closely related allotypes HLA-B\*57:02/03 or HLA-B\*58:01, which only differ from HLA-B\*57:01 in three or four amino acids, respectively<sup>146</sup>. A crystal structure of HLA-B\*57:01 complexed with abacavir and an immunogenic peptide shows that abacavir binds specifically in the F pocket of HLA-B\*57:01 and may alter the specificity of the MHC to allow binding and presentation of self-peptides<sup>147-149</sup>.

## CANCER IMMUNOTHERAPY

According to its definition, i.e. 'treatment designed to produce immunity to a disease or enhance the resistance of the immune system to an active disease process', immunotherapy has been around for centuries, with first evidence of inoculation with smallpox dating from tenth century China<sup>150</sup>. Examples of

1 modern day immune-activating therapies are vaccination or immunization, whereas immune suppression is used to treat autoimmune diseases or prevent transplant rejection. A role for the immune system in the clearance of cancer has been studied for decades, and this has led to the perception that primary tumor development, especially of cancers that are virus-induced, is suppressed by the immune system<sup>151-155</sup>. As long as the 'cancer immunity cycle' proposed by Chen and Mellman functions properly, even distant tumor cells are eradicated<sup>156</sup>. Those sporadic tumors which escape, likely develop mechanisms to induce tolerance, for example by promoting expansion of anergic CD8<sup>+</sup> T cells or induction of CD4<sup>+</sup> T cells<sup>157-161</sup>. Immunotherapy may then restore the cycle and concurrently reestablish anti-cancer immunity.

The advance of DNA and RNA sequencing techniques has made it possible to identify tumor-associated mutations or aberrations, and to target these to cure disease. Several therapeutic strategies targeting T cell immunity are described in the next sections and in more detail in **Chapters 2, 3 and 4**.

### **Peptide vaccines**

The majority of cancer immunotherapy efforts involve vaccination, which is not surprising in light of historical achievements of vaccination to prevent or cure disease<sup>162</sup>. Despite this potential, only minor successes have been accomplished using preventive or therapeutic vaccines as anti-cancer strategy<sup>163,164</sup>. Preventive vaccines are designed to induce humoral immunity through engendering a pool of memory B cells and antipathogenic antibodies. On the other hand, therapeutic vaccines are designed to treat an established disease by activating cellular immunity through T cells. Preventive vaccines are primarily employed against cancers caused by viruses, such as in the case of the human papilloma virus vaccine used to prevent cervical cancer<sup>165</sup>. The first generation of therapeutic vaccines, consisting of adjuvants or microbial or tumor preparations, was not particularly specific and chiefly aimed at establishing an inflammatory environment<sup>166</sup>. Current anti-cancer vaccines are more specific, comprising antigens released by tumors often complemented with adjuvants<sup>167</sup>. Vaccination with epitope-based peptides to specifically induce relevant T cells targeted to infected or mutated cells potentially provides effective prevention or treatment of infection or cancer. Peptide vaccines are usually aimed at activation of CD8<sup>+</sup> T cells, because of their cytolytic activity directly targeting cells that present the antigen, also at distant sites. Antigenic peptides can be self or non-self when cancer is caused by viruses<sup>168-170</sup>. Self-antigens can originate from highly upregulated proteins necessary for tumor growth and formation, peptide splicing by the proteasome or tumor-associated antigens such as melanoma-associated antigen (MAGE) or cancer testis antigen 1 (CTAG1, also known as NY-ESO-1)<sup>169-174</sup>. In many cases the TCR affinity for these self-antigens is low, which is why they could escape negative

selection<sup>175,176</sup>. Vaccination with such self-antigens supports T cell activation, augmenting anti-tumor responses.

Neoantigens are mutated self-antigens that arise from tumor-specific somatic DNA mutations<sup>177</sup>. Because neoantigens are not expressed in healthy tissue, vaccination induces only tumor-specific responses<sup>178</sup>. It is not difficult to imagine that for this reason neoantigens are hot targets in the development of cancer immunotherapeutics<sup>179,180</sup>. Spontaneous immune recognition of neoantigens is inefficient, for tumors are poor antigen-presenting cells, but anti-tumor immunity can be greatly enhanced by neoantigen-based vaccination<sup>181</sup>. Many pharmaceutical companies endeavor to discover neoantigens that can be exploited for treatment options<sup>182,183</sup>. Multiple modes of neoantigen-focused treatment have been successfully demonstrated, including vaccination with peptides or neoantigen mRNA, or adoptive transfer of neoantigen-specific T cells<sup>184,185</sup>. Since neoantigens are patient-specific, their identification needs to be performed on an individual basis. Somatic mutations can be discovered through sequencing and comparison of expression profiles between healthy and tumor tissues. Using binding algorithms, transcribed neoantigens may be matched to MHC I or MHC II to predict presented neoantigens<sup>186-188</sup>. Only a few of these predicted neoantigens will actually be expressed and presented on MHCs and even fewer will be immunogenic<sup>189,190</sup>. Therefore, screening of T cells using neoantigen-loaded MHC multimers, as described in **Chapter 7** of this thesis, or validation by peptide elution, is necessary to reveal true neoantigens<sup>191,192</sup>.

Despite 20 years of peptide vaccine studies and numerous clinical trials, none have made it to the clinic yet<sup>193,194</sup>. Peptides alone are poorly immunogenic and consequently improving the immunogenicity of known MHC I antigens by altering amino acid sequences has been the central focus in the field<sup>195-198</sup>. Substitutions are primarily introduced in the anchor positions, to increase the number and quality of the interactions in the binding pockets, while the central amino acids are kept unaltered. Mutating the central amino acids can result in hyperstimulation of T cells with the risk of inducing a pool of T cells that is reactive against the altered peptide, but not the wild-type epitope<sup>199</sup>. This off-target activation can even be caused by modifying only the anchor residues, since they can induce conformational changes in both MHC and peptide, thus altering T cell reactivity<sup>130</sup>. Design of APLs that contain not only the 20 proteogenic amino acids, but also amino acids with chemically-modified side chains is elaborated upon in **Chapters 2 and 3**.

One of the reasons for low efficiency of peptide vaccines is the absence of the second signal required for immune activation; namely, costimulation. Peptides presented by MHC I are usually derived from cytosolic or nuclear proteins and undergo trimming and loading in the ER. Circulating peptides are internalized in endosomes: the archetypal MHC class II compartments. How exactly peptide

1 vaccines administered in the blood eventually end up in class I MHCs on the cell surface is not completely understood, but this presumably takes place through cross-presentation, exchange on the cell surface or simply by cytosolic uptake of cell-permeable peptides<sup>200-203</sup>. These mechanisms bypass processing by professional APCs, such as DCs, and as a result, costimulatory signals necessary for activation of T cells are insufficiently provided, which may ultimately lead to tolerance<sup>47,204,205</sup>. Additional immune stimulation can be provided by CD4<sup>+</sup> T cells. Accordingly, the most successful peptide vaccines to date encompass long peptides that contain both MHCI and MHCII epitopes, thus triggering both cytotoxic (CD8<sup>+</sup>) and helper (CD4<sup>+</sup>) T cell responses<sup>204</sup>. Long peptides are processed by professional APCs and have been found to induce more competent anti-viral responses in multiple studies, with anti-human papilloma virus vaccines to prevent recurrent vulvar intraepithelial dysplasia as the greatest success story<sup>206-209</sup>. These long peptides ideally contain multiple potential epitopes able to bind various MHC allotypes, providing intrinsically broader application. Herein lies also the risk of off-target effects, since allotypes differ per individual and therefore the epitopes within a vaccine can unfavorably activate T cells in different individuals.

### Cell-based therapies

By directly administering autologous T cells, the peptide vaccination step can be skipped. Tumor-specific CD8<sup>+</sup> T cells can be isolated from peripheral blood mononuclear cells or tumor tissue, stimulated and expanded *ex vivo*, and then readministered to specifically attack the tumor<sup>210,211</sup>. Especially in the treatment of melanoma, adoptive transfer of tumor-infiltrating lymphocytes has shown remarkable responses<sup>212-215</sup>. To further enhance efficacy and tumor specificity, T cells can be genetically engineered through lentiviral or retroviral transduction or transfection with DNA or RNA to express novel tumor-specific TCRs or chimeric antigen-receptors<sup>216-218</sup>.

Another cell type that has been successfully used in cancer treatment are DCs<sup>219-221</sup>. They are at the center of antigen processing and presentation and activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, providing both activation and costimulation<sup>222,223</sup>. DCs can be used in various ways, but the most successful strategies include vaccination with antigens coupled to DC-antibodies (e.g. DEC-205) or DCs loaded with antigens *ex vivo*<sup>220,224-228</sup>. The first therapeutic anti-cancer vaccine to get approval from the US Food and Drug Administration (FDA, in 2010) is such a DC-based vaccine: Provenge®, also called Sipuleucel-T (Dendreon, Inc.), for treatment of prostate cancer<sup>229,230</sup>. This vaccine contains DCs that are activated *ex vivo* with a prostate cancer-specific antigen, prostatic acid phosphatase, to stimulate tumor-specific CD8<sup>+</sup> T cells<sup>231-233</sup>. Especially in these cases it is of the utmost importance that antigens are only expressed on tumors to avoid off-tumor effects<sup>217,234</sup>.

### Checkpoint inhibition

Widespread success and fame of cancer immunotherapy came with the discovery of checkpoint inhibition. Inhibitory receptors on T cells, such as cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and programmed death (PD)-1, are negative regulators of the costimulatory signal necessary for T cell activation<sup>235-238</sup>. The balance between inhibitory and stimulatory immune checkpoints ensures optimal immune protection, while maintaining self-tolerance and preventing autoimmunity<sup>239</sup>. By blocking inhibitory pathways, the brakes on immune responses are released, resulting in a boost of preexisting anti-tumor responses<sup>240-242</sup>. Checkpoint inhibition offers great opportunities, especially when treating cancers harboring high mutational burden and thus likely to express higher frequencies of neoantigens<sup>243-246</sup>. For their roles in this discovery James P. Allison and Tasuku Honjo were awarded the 2018 Nobel Prize for Physiology or Medicine<sup>247</sup>. The group of Allison was the first to demonstrate increased antitumor activity in vivo using antibodies blocking CTLA-4 in murine colorectal carcinoma and one year later in murine prostate cancer<sup>240,248</sup>. CTLA-4 is a homologue of CD28 and binds both B7-1 and B7-2, with a higher affinity than CD28<sup>249</sup>. This negative regulation results in inhibition of IL-2 production and blocking of cell cycle progression, thus functioning as an immune checkpoint to control lymphocyte homeostasis<sup>250-252</sup>. A quick search for clinical trials shows roughly 50 active and 150 recruiting/enrolling trials targeting CTLA-4 as a single or combination therapy<sup>253</sup>. Ipilimumab, a blocking antibody against CTLA-4, has demonstrated durable clinical responses and was approved for treatment of metastatic melanoma by the FDA and the European Medicines Agency (EMA) in 2011<sup>254,255</sup>.

A wide range of tumors express PD-L1, the ligand for immune checkpoint receptor PD-1, thus creating an immunosuppressive environment and escaping immune surveillance<sup>256-258</sup>. Blocking the PD-L1/PD-1 interaction is therefore even more effective against cancer than anti-CTLA-4. Indeed, as a monotherapy, antibodies against PD-1 cause a remarkable reduction of tumor metastasis spread in mice, owing to enhanced recruitment of effector T cells<sup>259,260</sup>. Their efficacy is reflected in the high number of clinical trials targeting PD-1 (almost 200 active and over 800 recruiting/enrolling trials) or PD-L1 (almost 200 active and over 500 recruiting/enrolling)<sup>253</sup>. Two PD-1 antibodies, nivolumab and pembrolizumab, have shown durable clinical responses in various cancer types and have been approved by the FDA and EMA<sup>261,262</sup>. Currently, one PD-L1 antibody, atezolizumab, has been approved by the FDA and EMA for treatment of urothelial cancer and non-small cell lung carcinoma<sup>263</sup>. Since PD-1 and CTLA-4 function in different stages of immune activation, combination of therapies targeting both pathways leads to additive anti-tumor effects<sup>264,265</sup>.

CTLA-4 and PD-1 were the first of many targets for immunotherapy and the



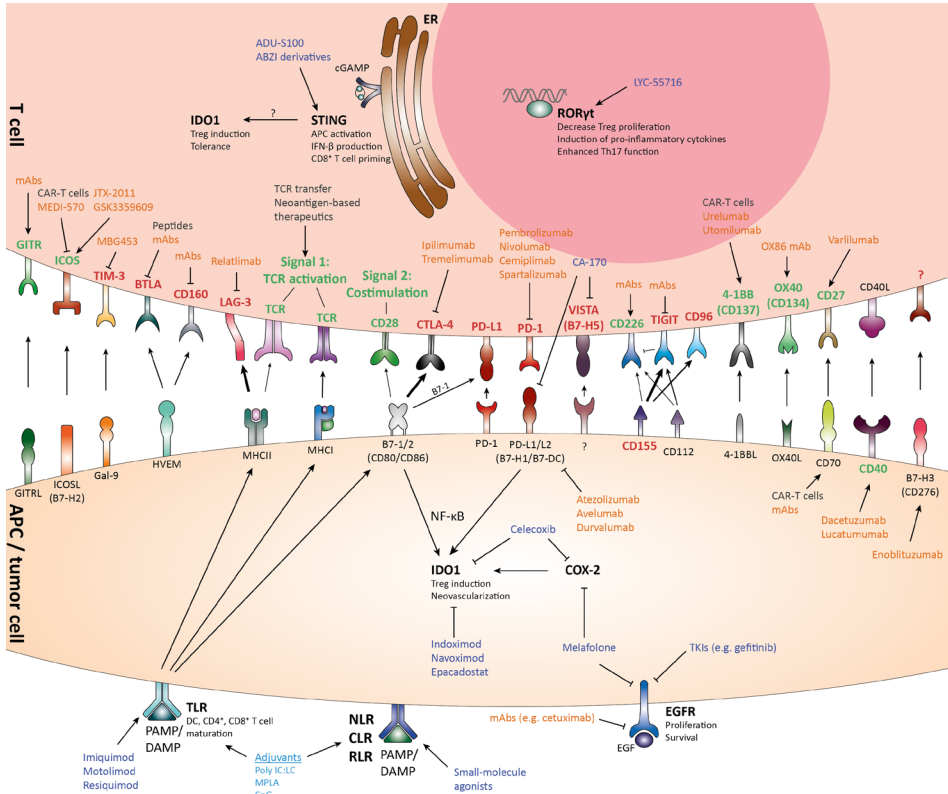


Figure 2. **Overview of checkpoint molecules and a selection of current preclinical and clinical therapeutics.** Green indicates stimulatory- and red inhibitory checkpoint molecules. Antibody therapies are depicted in orange; small molecules in dark blue; T cell therapies in grey. Arrows represent stimulation and T-bars represent inhibition. Arrow thickness corresponds to relative affinity compared to other ligands.

list is expanding immensely, as depicted in Figure 2. Both negative and positive regulators of the immune system are promising targets for cancer immunotherapy and autoimmune treatment and are actively pursued by pharmaceutical companies<sup>266</sup>. Combination with other treatment modalities has also led to synergistic effects, and consequently many ongoing clinical trials focus on combination therapies. Our perspective on the future of cancer immunotherapy is elaborately described in **Chapter 4**.

## T CELL DETECTION THROUGH pMHC I MULTIMERS

The study of T cell interactions and specificities has immensely benefited from the development of pMHC tetramers<sup>267,268</sup>. These tetramers conventionally consist of



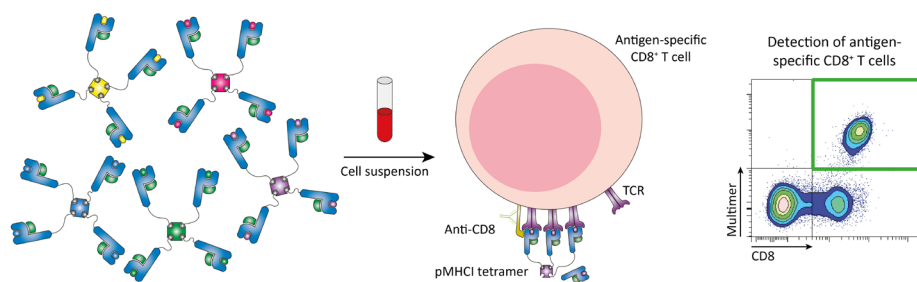


Figure 3. **Visualization and isolation of antigen-specific T cells using pMHC tetramers.** Peptide-major histocompatibility complex class I (pMHC) monomers are loaded with antigenic peptides and multimerized on fluorescently-labeled streptavidin. These reagents are widely used for the detection, isolation and characterization of antigen-specific CD8<sup>+</sup> T cells using flow cytometry.

MHCI monomers that are folded with a specific antigenic peptide, enzymatically biotinylated and subsequently multimerized on streptavidin. It is imperative that pMHC complexes are multimerized so that they can bind multiple TCRs on their corresponding CD8<sup>+</sup> T cell, to reduce typically high monomeric dissociation rates and remain attached to the T cell during further experimental analysis<sup>269</sup>. When labelled with a fluorophore these tetramers can be used to directly visualize (by flow cytometry) T cells specific for the bound antigen in a cell suspension, as depicted in Figure 3. Using this technology T cell responses can be quantified, characterized and monitored, providing invaluable information on an individual's immune status and responses to treatment<sup>270,271</sup>. One of the main advantages over traditional assays, such as ELISpot (enzyme-linked immune absorbent spot), cytokine staining or single-cell PCR, is that cells can even be sorted using FACS for further studies<sup>272</sup>. Besides diagnostics, pMHC tetramers are widely used to study basic principles of ligand specificity, kinetics and dynamics of immune responses and employed in epitope mapping<sup>273-276</sup>. Although MHCII multimer technology is improving, considerable efforts are required to reach the same standards as those of MHC I multimers<sup>277</sup>. Progress is hampered by the difficulty to generate stable soluble forms of biotinylated MHCII, low frequency of CD4<sup>+</sup> T cells in circulation and generally low affinities of MHCII peptides<sup>272</sup>.

Since first reports in 1996 MHC I tetramer technology has markedly improved<sup>267</sup>. A major step towards high-throughput analysis was made by the advance of technologies to exchange peptides on MHC I. Because MHC I molecules require a peptide (or chaperones) for stability, every specific pMHC I had to be folded with the desired peptide, and production of tetramers was therefore laborious and costly<sup>278</sup>. Where at first production of one or a few tetramers would take more than a week of work, the development of exchange technologies now allows generation of numerous MHC I tetramer variants in parallel. This approach involves folding of a large batch of MHC I monomers with a peptide that upon

1 applying a trigger exchanges for a peptide of interest. Various techniques have been investigated, utilizing dipeptides or chemicals, such as dithionite or periodate, as stoichiometric reagents<sup>279-282</sup>. One of the most successful exchange technologies was developed in a collaboration between the Ovaas and the Schumacher labs, and employs UV radiation as a trigger<sup>283,284</sup>. The peptide used for folding contains a central photocleavable amino acid, which becomes cleaved upon UV irradiation, resulting in dissociation of peptide remnants and liberation of the binding groove for association with a peptide of choice. This approach was easily extended to other alleles by incorporating the UV-cleavable amino acid in allele-specific epitopes<sup>285</sup>. More recently, I have developed an exchange technology based on temperature, which is described in detail in **Chapters 5 and 6**<sup>286,287</sup>. Unlike UV, which damages proteins and bleaches fluorophores, this exchange can be performed on already multimerized pMHC. Exchange is induced by simply warming up the multimers, without the need for chemicals or specific lab equipment, and is therefore the easiest and most flexible exchange technology available to date.

In flow cytometry, the maximum number of detectable T cell specificities is limited by the number of available fluorochromes. Since sample volumes are often small, it is preferred to stain for as many specificities as possible in one sample. With development of combinatorial coding the number of simultaneously detectable reactivities increased from eight single stains to 28 dual combinations<sup>288</sup>. Adopting more complex coding strategies using six colors raises the number of detected specificities to 63, but also adds to the complexity of spectral overlap<sup>289</sup>. The most recently published strategy to scale up detection uses DNA labels instead of fluorophores<sup>290</sup>, where pMHC multimers are conjugated to a dextran backbone carrying a unique 25-oligonucleotide barcode sequence and a common fluorochrome. After assembly, different multimers are mixed and used to stain T cells in a similar fashion to conventional tetramer staining. Consequently, fluorophore-labelled T cells are isolated by FACS and their associated DNA is amplified and sequenced. This approach allows detection of over 1000 specificities in one sample in a high-throughput fashion and can be used for screening of epitopes in small sample volumes. First steps towards creating thermally-exchangeable DNA-labeled pMHC multimers are described in **Chapter 7**.

## SCOPE OF THIS DISSERTATION

The work described in this dissertation highlights how the adaptive immune system can be used to our advantage, either from a therapeutic or diagnostic perspective. In a therapeutic setting tumor- or pathogen-specific T cells can be activated to eliminate mutated or infected cells. **Chapter 2** describes the design and use of chemically enhanced altered peptide ligands as therapeutic vaccines. By modifying their anchoring residues, the affinity of wild-type epitopes to their corresponding MHC, HLA-A\*02:01, could be markedly increased with the goal of improving pMHCI stability and prolonging recognition by antigen-specific T cells. The study described in **Chapter 3** then sets out to chemically enhance three HLA-A\*02:01- and three HLA-A\*03:01-presented influenza A epitopes, of varying affinity and immunodominance, to serve as a preventive vaccine. In vitro and in vivo assays demonstrate that affinity and immunogenicity of HLA-A\*02:01 epitopes could be improved by modifying the anchoring residues, but that immunogenicity did not directly correlate with affinity. Peptide vaccines alone may not induce full anti-tumor responses, but they efficiently initiate T cell activation, thus supporting other immunotherapies. Our opinion on the potential contribution of small-molecule drugs is detailed in **Chapter 4**.

The classic reagents for studying antigen-specific T cell responses are pMHCI multimers. **Chapter 5** summarizes the development of a novel technique to exchange peptides on MHCI multimers. This method, described in a step-by-step protocol in **Chapter 6**, allows the generation of large panels of pMHCI multimers in parallel. Implementation of DNA barcoding increases the scale of detectable specificities. Such high-throughput approaches may prove particularly useful in neoantigen identification, as described in **Chapter 7**.

The findings of the research described in this dissertation are recapitulated in the final chapter, where we also provide suggestions for future directions.

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