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## Targeting MHC-I related proteins for cancer diagnosis and therapy

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# ***Chapter 1:***

## ***Introduction to immuno-oncology***



## **Tumor development**

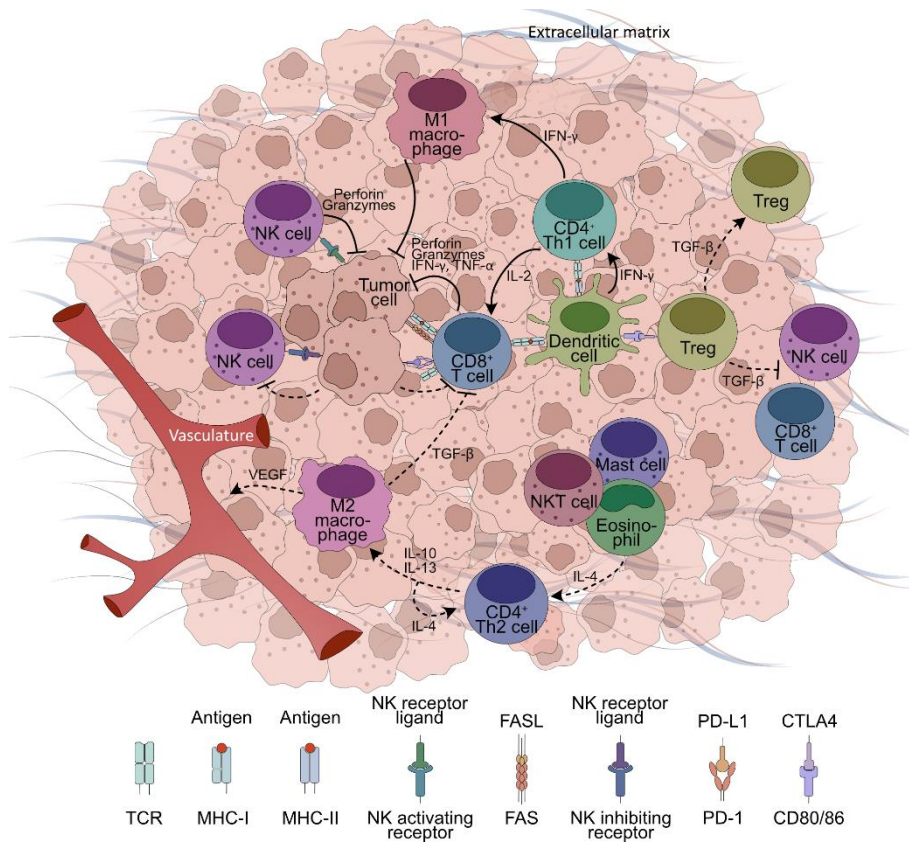
The World Health Organization reports approximately 20 million people in the world got diagnosed with cancer in 2022. The most common types of cancer are lung, colon, and prostate cancer for men, and breast, lung, and colon cancer for women. Although ongoing efforts to prevent and treat cancer have improved survival rates, an estimated 9.7 million patients worldwide died from cancer in 2022<sup>1</sup>. The most common cause of death is metastatic diseases, which is the spread of cancer from the localized origin to other sites in the body<sup>2</sup>. Metastases and cancer growth are facilitated by cellular changes, as well as by changes in the tumor microenvironment (TME)<sup>3-5</sup>.

Some well-studied changes in tumor cells that lead to cancer and metastases are the downregulation of certain tumor suppressor genes (TP53, BRCA1/BRCA2, and PTEN)<sup>6-13</sup>, or the increase in expression of oncogenes (RAS, MYC, and WNT)<sup>14-24</sup>. Other cellular changes include downregulation from the cell surface of certain MHC Class I (MHC-I) molecules such as HLA-A, -B, and -C, which renders the tumor cells invisible to CD8<sup>+</sup> T cells. Such downregulation applies to 40-90% of epithelial cancers and correlates with worse prognosis<sup>25-33</sup>. Alternatively, upregulation of other MHC-I molecules such as HLA-E and -G can lead to inactivation of immune cells<sup>34-37</sup>. Strategies of immune-evasion deployed by tumor cells will be described in greater detail below.

## **The tumor microenvironment and the immune system**

Besides the tumor itself, the TME consists of stromal cells, the extracellular matrix (ECM), the tumor vasculature, and immune cells (Figure 1). The ECM, comprised of connective tissue-specific molecules like collagen, hyaluronic acid, proteoglycans, and laminins, creates a dense environment that surrounds the tumor cells. This creates a diffusion barrier that inhibits access of drugs, nutrients, and oxygen to the tumor<sup>38</sup>. Furthermore, the ECM contributes to the epithelial-to-mesenchymal transition (EMT) and subsequent metastases. Epithelial cells are characterized by their apical-basal polarity and contact with adjacent cells through adherens junctions, tight junctions, and desmosomes. In the EMT, an epithelial cell transitions into a mesenchymal cell, characterized by a loss of the apical-basal polarity and separation of neighboring cells by interaction with the ECM. Mesenchymal cells can migrate out from the primary tumor and establish metastases at distant sites<sup>39-43</sup>. The ECM also determines the infiltration of immune cells

such as CD4<sup>+</sup> or CD8<sup>+</sup> T cells, natural killer (NK) cells, and antigen presenting cells (APC) like macrophages and dendritic cells (DC)<sup>44</sup> into the tumor. Such tumor-infiltrating lymphocytes can have either a tumor-promoting or tumor-inhibiting effect, which will be described in greater detail below.



**Figure 1.** The tumor microenvironment consists of stromal cells, the extracellular matrix, the tumor vasculature, and immune cells. Infiltration of lymphocytes in the tumor can have a tumor-promoting (dashed lines) or tumor-inhibiting (solid lines) effect.

## CD4<sup>+</sup> T cells

APCs present antigens in the form of peptides derived from extracellular proteins on class II MHC (MHC-II) molecules. CD4<sup>+</sup> T cells engage MHC-II via their T cell receptor (TCR) and the CD4 co-receptor. Naïve CD4<sup>+</sup> T cells differentiate into T helper cells (Th) under the influence of different chemokines and cytokines. Several types of Th cells exist: Th1, Th2, Th9, Th17,

Th22, follicular helper T cells (Tfh), and regulatory T cells (Treg). Each type of Th cell has a distinct function in pro- or anti-tumor immunity<sup>45</sup> (Figure 2).

Differentiation of naïve CD4<sup>+</sup> T cells into Th1 cells is facilitated by the cytokines interferon- $\gamma$  (IFN- $\gamma$ ), secreted by activated dendritic cells (DC), and interleukin (IL)-12 and IL-18, secreted by activated macrophages. Th1 cells produce IFN- $\gamma$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-2, which play a role in tissue-specific destruction during pathogenesis and autoimmune disease, as well as in elimination of cancer cells<sup>46</sup>. Th1 cells activate and regulate the persistence of CD8<sup>+</sup> cytotoxic T lymphocytes (CTL), maturation and activation of APCs, and induction of immunoglobulin class-switching, mostly to IgG2a, which increases tumor-specific antibody production<sup>47</sup>. Increased levels of Th1 cells in the TME are associated with a positive prognosis and an improved response to immunotherapy in cancer patients<sup>48-51</sup>.

Differentiation of naïve CD4<sup>+</sup> T cells into Th2 cells is induced by the extracellular pathogen pathway, primarily through the effects of IL-4 secreted by mast cells, eosinophils, and natural killer T cells (NKT cells), or by IL-25 and IL-33 produced by epithelial cells<sup>52</sup>. Th2 cells that have differentiated produce IL-4, which regulates immunoglobulin class switching to IgE in B cells and acts as a positive feedback loop for Th2 activation<sup>53</sup>. Although the precise role of Th2 cells in tumor proliferation is still unknown, some studies associate large numbers of Th2 cells in tumors with worse prognosis, for instance because Th2 cells drive the polarization of nM $\phi$  and M1-type macrophages towards M2-type macrophages through secretion of IL-4, IL-10, and IL-13<sup>54-56</sup>. The properties of macrophages are described in more detail later. Other studies ascribe a more ambivalent role to Th2 cells in the TME<sup>57</sup>, and even show that large numbers of Th2 cells are associated with a positive prognosis in patients with colon cancer<sup>58</sup>, pancreatic cancer<sup>58,59</sup>, melanoma<sup>60</sup>, breast cancer<sup>61</sup> and lymphoma<sup>62</sup>, possibly due to Th2-driven infiltration of anti-tumor immune cells like eosinophils, M1-type macrophages, and neutrophils<sup>57,58,62</sup>.

Th9 cells differentiate in response to transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-4. Th9 cells produce IL-3, IL-9, IL-10, and IL-21. Although the role for Th9 cells in tumorigenesis is not entirely clear, Th9-driven secretion of IL-9 and IL-21 primarily promotes anti-tumor immunity<sup>63-70</sup>. Th17 cells are induced by the synergistic action of IL-6, TGF- $\beta$ , and IL-21 or IL-23<sup>71,72</sup>. Th17 cells produce IL-6, IL-17, IL-17A, IL-17F, IL-21, and IL-22. The cytokines secreted by

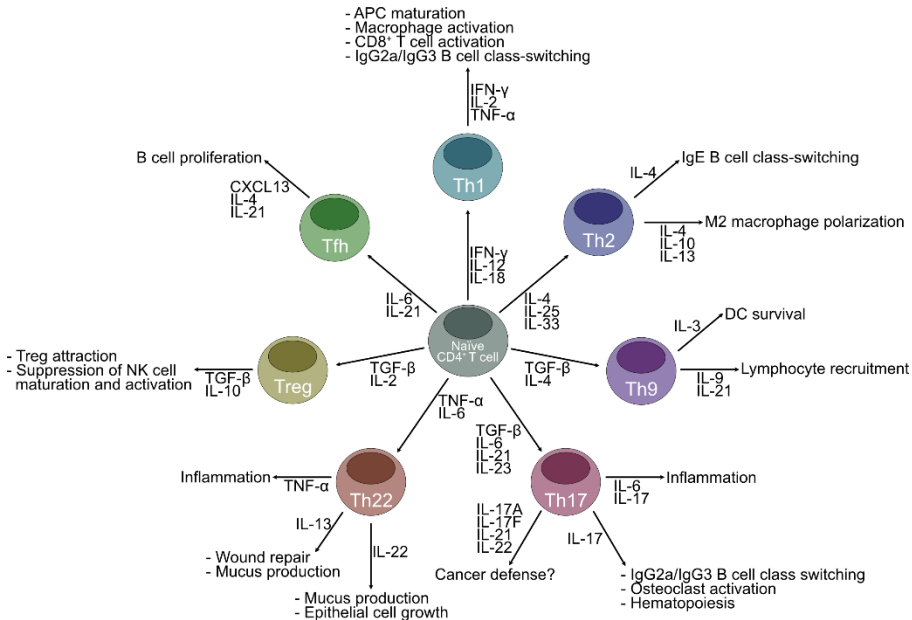
Th17 cells promote inflammatory reactions of endothelial cells, epithelial cells, and fibroblasts<sup>73</sup>, which primarily play a role in protection against bacterial and fungal infections but are also believed to take part in development of certain auto-immune diseases like rheumatoid arthritis<sup>74</sup>. Although the role of Th17 cells in cancer immunology is still poorly understood, higher levels of Th17 cells or Th17-associated cytokines in the serum of breast cancer patients are believed to have both a positive<sup>75,76</sup> and negative<sup>77,78</sup> effect on tumor prognosis and therapy.

Th22 cells arise through the combined action of IL-6 and TNF- $\alpha$ . Upon activation, Th22 cells secrete TNF- $\alpha$ , IL-13, and IL-22<sup>79-81</sup>. Th22 cells participate in induction of inflammation, mucus production, epithelial cell growth, and wound repair. High levels of IL-22 found in patients with hepatocellular carcinoma and lung cancer are associated with poor prognosis<sup>82,83</sup>. Furthermore, Th22 cells are suggested have a tumor-promoting effect in colorectal cancer<sup>84</sup>.

Tfh differentiate under the effects of IL-6 and IL-21. Tfh cells are found in the germinal centers and activates B cells to become plasma cells to induce antibody production<sup>85,86</sup>. Although the role of Tfh cells on cancer progression is still largely unknown, increased numbers of Tfh cells in patients with B cell-associated malignancies are associated with a poor prognosis<sup>87-90</sup>, whereas in patients with solid tumors the presence of Tfh cells is associated with a more favorable outcome<sup>91-95</sup>.

Treg cells can be produced by the thymus (natural Treg cells), or proliferated from peripheral naïve CD4<sup>+</sup> T cells under the influence of TGF- $\beta$  (adaptive Treg cells). Treg cells express CTLA-4 and CD28 on their cell surface, which bind to CD80 and PCD86 on APCs and inhibit T cell activation<sup>96,97</sup>. Tregs secrete cytokines TGF- $\beta$  and IL-10. TGF- $\beta$  not only attracts more Treg cells, it also suppresses the infiltration in tumors of CD8<sup>+</sup> cytotoxic T cells and natural killer cells (NK), and other inflammatory responses, thus promoting tumor development and progression<sup>98,99</sup>. In a tumor microenvironment, the Treg cells, tumor cells, and myeloid derived suppressor cells all produce TGF- $\beta$ , which suppresses the maturation and egress of NK cells from the bone marrow. In addition, TGF- $\beta$  downregulates the expression of Nkp30 and NKG2D receptors on NK cells, thereby impairing the recognition and activation of NK cells. NK cells and their receptors will be described in greater detail below.

High levels of TGF- $\beta$  are associated with poor prognosis in lung carcinoma, pancreatic cancer, colorectal cancer, gastric cancer, and hepatocellular carcinoma<sup>100</sup>.



**Figure 2. Proliferation and functioning of naïve CD4<sup>+</sup> T cells.** Naïve CD4<sup>+</sup> T cells differentiate into several different types of Th cells under the influence of different chemokines and cytokines. CD4<sup>+</sup> T cells can proliferate into Th1, Th2, Th9, Th17, Th22, Treg, and Tfh cells. Each type of Th cell has a distinct function in pro- or anti-tumor immunity.

## CD8<sup>+</sup> T cells

CD8<sup>+</sup> T cells engage with MHC-I via their T cell receptor (TCR) and CD8 co-receptor. All nucleated cells of vertebrates present antigens derived mostly from intracellular proteins in the form of peptides on MHC-I<sup>101</sup>. A healthy cell will present peptides derived from normal cellular protein turnover, to which a CD8<sup>+</sup> T cells will not respond due to the imposition of central and peripheral tolerance<sup>102</sup>. However, when a cell presents foreign peptides on MHC-I, for instance due to viral or bacterial infection, or due to malignant transformation, the CTLs will be activated. Most activated CD8<sup>+</sup> T cells differentiate into effector CTLs, which exert cytotoxicity through secretion of granzymes and perforins, cytokines like IFN- $\gamma$  and TNF- $\alpha$ , and induction of caspase-mediated apoptosis through Fas/FasL interactions<sup>103–108</sup>. High levels of CTLs in the TME are associated with better prognosis in cancer patients<sup>109–112</sup>.



Some cancer cells present tumor-specific antigens, also referred to as neoantigens, on MHC-I. Neoantigens could have oncoviral origins, such as the human papilloma virus-derived HPV E6 and E7 in cervical cancer<sup>113,114</sup>, mutated versions of proteins like Claudin 18.2 in several epithelial cancers<sup>115</sup>, Wilms-tumor gene 1 isoforms in leukemia<sup>116–118</sup>, and BRCA1/BRCA2 in ovarian and breast cancer<sup>119,120</sup>, or overexpression of tumor-associated antigens such as HER2 in breast cancer<sup>121,122</sup>, mesothelin in pancreatic cancer<sup>123</sup>, and CD19 in B cell lymphoma<sup>124</sup>. These neoantigens are recognized by the CD8<sup>+</sup> T cells, upon which these CTLs will be activated. Somatically acquired mutations in other genes can also specify neoantigens. Such mutations might be unique to a given cancer, and may or may not contribute to the transformation themselves. The various mutant versions of KRAS fall into the former category.

### Memory T cells

Although the majority of activated CTLs die once an infection is cleared, a small subset of activated CD8<sup>+</sup> T cells differentiate into memory CTLs and return to an inactive state. These memory CTLs contribute to the central memory immune response, of which memory B cells and memory helper T cells are also a part. When these memory T cells encounter the same antigen, they are quickly activated and differentiate into effector T cells<sup>106,125</sup>. Although the mechanism of differentiation into memory T cells is not completely understood, it is hypothesized that they arise from a population of activated T cells that, after pathogen clearance, turn off their effector functions<sup>126</sup>. Tumor-specific CTLs also require activation, presumably under inflammatory conditions, and are likely to behave similarly to their pathogen-specific counterparts. The genes encoding their effector functions are maintained in a state of low methylation, allowing rapid reactivation upon pathogen encounter<sup>127,128</sup>.

The memory T cell repertoire includes stem cell memory T cells (Tscm), central memory T cells (Tcm), effector memory T cells (Tem), and the more recently discovered tissue-resident memory T cells (Trm). Tcm and Tem cells are characterized by high CCR7 expression and mostly reside in the secondary lymphoid organs. Tem cells are also found in non-lymphoid tissues. CD4<sup>+</sup> and CD8<sup>+</sup> Trm cells are not circulating and are found in the peripheral tissues and mucosa. Trm cells are distinguished from other memory T cells by expression of CD69, CD49a, and CD103<sup>129–131</sup>.

CD103<sup>+</sup> Trm cells are found in various human cancers, and high levels are associated with better prognosis and improved relapse-free survival in patients with melanoma<sup>132–134</sup>, lung cancer<sup>109,135,136</sup>, breast cancer<sup>137,138</sup>, ovarian cancer<sup>139</sup>, and other solid tumors<sup>140</sup>.

## Macrophages

Macrophages are innate immune cells of the monocyte lineages. Their main function is the engulfment and digestion of micro-organisms, dead cells, and immune complexes. Macrophages stimulate other immune cells through secretion of chemokines and cytokines<sup>141</sup>. Macrophages are broadly divided into two distinct subtypes: M1 and M2 macrophages, which are polarized from undifferentiated macrophages (Mφ) through stimulation of different cytokines and other factors<sup>142–145</sup>.

Polarization of Mφ macrophages into M1 macrophages is facilitated by Th1 cells, and by secretion of signals such as bacterially derived lipopolysaccharides (LPS), IFN-γ and TNF-α. M1 macrophages are associated with an anti-tumor response. They secrete the pro-inflammatory cytokines IL-1β, IL-6, IL-12, IL-23, and TNF-α and chemokines CXCL9, CXCL10, CXCL11, CXCL16, and CCL5<sup>55,56,142,143,146</sup>. Polarization of Mφ macrophages into M2 macrophages is facilitated by the Th2 cell response through secretion of IL-4, IL-10, IL-13, IL-21, and TGF-β<sup>54–56,141–143,147–149</sup>. M2 macrophages can be further subdivided into M2a, M2b, M2c, and M2d macrophages, each polarized under the effect of different cytokines and chemokines<sup>141</sup>. M2 macrophages are often referred to as tumor-associated macrophages (TAMs). Typically, high levels of TAMs in the TME are associated with poor prognosis<sup>150–153</sup> in part because TAMs negatively influence the infiltration and function of Th1 and Th2 cells through secretion of IL-10 and TGF-β, the latter of which also suppresses CTL function<sup>154</sup>. TAMs secrete other tumor-promoting factors, such as vascular endothelial growth factor (VEGF), which contributes to neovascularization and lymphangiogenesis<sup>154–158</sup>.

## Natural killer cells

NK cells are CD3<sup>-</sup> and CD56<sup>+</sup>/CD16<sup>+</sup> cells that can be divided into two subsets: the naïve CD56<sup>bright</sup>/CD16<sup>dim</sup> and the mature CD56<sup>dim</sup>/CD16<sup>bright</sup> cells<sup>159</sup>. NK cells lack the antigen specificity of B or T cells and instead recognize infected and malignant cells through germline-encoded NK receptors (NKR). According to the 'missing self-hypothesis', coined in 1981, a major function of NK cells is to recognize and eliminate cells that do not express 'self MHC-I'<sup>160</sup>.

Because NK cells don't require prior antigen sensitization or presentation by MHC-I for activation, NK cells contribute to a rapid anti-viral and anti-tumor immune response<sup>161</sup>.

The activities of NK cells are regulated by NK-cell inhibitory or activating receptors on the surface of the NK cells, and NK-cell receptor ligands on the surface of target cells. Activating receptors, which include NKP46, NKP30, NKG2C, NKG2D, and CD16, are upregulated upon stimulation with IL-2, IL-15 or IL-1 $\beta$ , often released by activated dendritic cells and macrophages<sup>161-163</sup>. NK inhibiting receptors, like natural killer group 2 member A (NKG2A) and its splice variant NKG2B, and human killer cell immunoglobulin-like receptor (KIR) are constitutively expressed on NK cells<sup>164</sup>. They interact with ligands that are primarily expressed on healthy cells and thus contribute to regulation of autoimmunity<sup>165</sup>. For the context of this thesis, the NK cell receptors NKG2D and NKG2A and their ligands will be explained in greater detail in the section "Tumor targets".

## **Immunotherapy to treat cancer**

Treatment of cancer has long been based on surgical removal of the primary tumor and surrounding lymph nodes, localized radiation of the tumor, or administration of chemotherapeutic drugs. Immunotherapy is a concept in tumor treatment that, based on its success, has gained popularity and employs a patient's own immune system to fight or prevent cancer. Examples of immunotherapy are based on modulating the immune system with monoclonal antibodies acting as checkpoint inhibitors or targeting tumor-associated antigens, or adoptive cell transfer (ACT).

### **Monoclonal antibodies**

Monoclonal antibodies (mAbs), typically of the IgG class, are increasingly commonly used for the treatment of cancer. The FDA has approved the use of dozens of mAbs for cancer treatment, among which are mAbs that target tumor-associated antigens such as Herceptin in breast cancer (Trastuzumab<sup>166</sup>), CD20 in lymphoma (Rituximab<sup>167</sup>), epidermal growth factor receptor (EGFR) in head-and-neck and colorectal cancer (Cetuximab<sup>168</sup>), CD56 in several solid tumors (Lorvotuzumab<sup>169</sup>) and VEGF-A in several solid tumors (Bevacizumab<sup>170</sup>).

### **Antibody-drug conjugates**

mAbs can be employed as the targeting moiety of an antibody-drug conjugate (ADC). The cytotoxic payload of ADCs are often (micro)tubulin inhibitors

like Maytansine, DNA damaging agents like Exatecans, and immune modulators like STING agonists<sup>171,172</sup>. The mAb targets and binds its antigen and gets internalized through receptor-mediated endocytosis. Inside the cell, the cytotoxic payload is released and exerts its cytotoxic actions<sup>173</sup>.

For the research in this thesis, we employed the cytotoxic activities of Maytansine, fused to a nanobody as targeting moiety. Maytansine and its analogs (Maytansinoids, also referred to as DM1 and DM4), bind to the vinca site of microtubules, causing depolarization of the microtubules and subsequent mitotic arrest<sup>174–177</sup>. Due to this powerful cytotoxicity, the therapeutic window of Maytansine is small, with adverse effects often experienced on the gastrointestinal system. Conjugated to a monoclonal antibody, however, tissue-specific delivery of Maytansine is possible. This not only significantly improves anti-cancer therapy, it also decreases adverse effects. This has been shown in several clinical trials, for instance where DM1 was fused to Trastuzumab to treat breast cancer<sup>178</sup>, and Lorvotuzumab to treat several solid and hematopoietic tumors<sup>179,180</sup>.

A common effect of ADCs is called “bystander killing”, which occurs when the payload of the ADC is released from the target cell, either after internalization and degradation or by release of the drug in the extracellular space, leading to the uptake and killing of surrounding “bystander cells”, even if they don’t express the target antigen. Because DM1 has a positive charge, it is unable to permeate a cell membrane on its own. This drug is thus suitable for use without risk of the bystander killing effect<sup>181</sup>.

### **Immune checkpoint inhibitors**

Cancer cells develop defense mechanisms by downregulation of MHC-I, secretion of perforin-degrading enzymes, and overexpression of programmed cell death ligand 1 (PD-L1). PD-L1 is found on healthy cells and interacts with programmed cell death protein 1 (PD-1) found on T cells. The interaction between PD-L1 and PD-1 inactivates the T cells and prevents cytotoxicity, a mechanism employed in healthy tissue to prevent T cell exhaustion and auto-immunity<sup>182,183</sup>. PD-L1 is frequently overexpressed on cancer cells, rendering them resistant to T cell cytotoxicity<sup>182,183</sup>. Checkpoint inhibitors are antibodies that target the PD-1 or PDL-1, thereby inhibiting the interaction between them<sup>184–186</sup>.

CTLA-4 is found on CD8<sup>+</sup> T cells and T<sub>reg</sub> cells. It interacts with B7-1 and B7-2 (also known as CD80/86) on the surface of APCs. This interaction inhibits

T cell activation<sup>96,97</sup>. CTLA-4 targeting antibodies are used to inhibit the T cell inactivation. CTLA-4 inhibitors are sometimes administered together with PD-1 or PDL-1 inhibitors<sup>187</sup>.

### **Adoptive cell transfer and CAR therapy**

Adoptive cell transfer is a type of immunotherapy in which a patient receives T cells to fight cancer. As explained in a previous section, the tumor microenvironment can contain tumor infiltrating lymphocytes that recognize and eliminate cancer cells. These tumor infiltrating lymphocytes can be sourced from the tumor after surgical resection, expanded *ex vivo* with the help of IL-2 and CD3, and reintroduced in large numbers into the patient. Treatment with tumor infiltrating lymphocytes has been successful for metastatic melanoma in a phase 3 clinical trial<sup>188</sup>. Other clinical trials are on the way for treatment of gastrointestinal cancer (NCT01174121), HPV-associated cancers (NCT01585428), breast cancer (NCT05250336), and other solid tumors (NCT05087745, NCT06047977).

### **CAR T cell therapy**

In another form of adoptive cell transfer, a patient's circulating T cells are engineered with a chimeric antigen receptor (CAR) that targets the tumor cells and exerts cytotoxic activity upon binding. CAR constructs encode a protein that comprises an antigen-binding extracellular domain and intracellular signaling domains, connected to each other via a hinge and a transmembrane domain (Figure 3).

The extracellular antigen-binding targeting portion often consists of a single-chain variable fragment (scFv), composed of the heavy and light chain variable regions of an immunoglobulin, connected by a linker segment<sup>189</sup>. scFvs are around one-fifth the size of a conventional immunoglobulin, at 30 kD compared to 150 kD. Their small size imparts excellent solubility while maintaining antigen-recognition. However, the linker that connects the heavy and light chains, as well as the (often) mouse origin of the source immunoglobulin, could be immunogenic and both have been shown to elicit an antibody-response in patients, limiting the anti-tumor response of the infused CAR T cells<sup>190-194</sup>. Instead, the more recently discovered heavy-chain only variable fragments (VHH, also referred to as nanobodies) from camelid-derived heavy-chain only antibodies are suggested to be superior as the antigen-binding portion of CARs. Chapter 2 will go into more detail on the properties of nanobodies. Briefly, nanobodies are characterized by their small

size (15kD), solubility, ease of production, and excellent antigen-binding properties compared to full-length immunoglobulins<sup>195</sup>. Moreover, nanobodies are poorly immunogenic in humans due to the high homology between camelid and human heavy chain variable region sequences<sup>196,197</sup>.

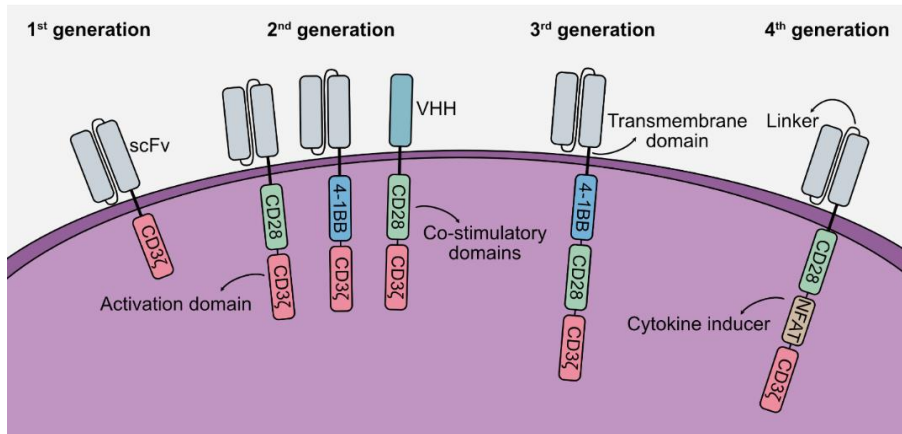
The intracellular signaling domains of a CAR harbor an activation domain and one or two co-stimulatory domains. First-generation CARs were engineered with only the cytoplasmic activation domain of CD3 $\zeta$ . These CAR T cells were unable to direct lasting T cell responses or sustained cytokine release and were thus considered clinically non-effective<sup>198</sup>. Second-generation CARs combine the CD3 $\zeta$  domain with additional co-stimulatory domains such as those derived from the cytoplasmic tail of CD28 or 4-1BB, which enhances survival and expansion of T cells *in vivo*<sup>199,200</sup>. CD28/CD3 $\zeta$ -based CAR T cells are believed to elicit superior cytotoxic capacity *in vivo*, whereas 4-1BB/CD3 $\zeta$ -based CAR T cells show higher *in vivo* expansion and persistence<sup>201</sup>. The FDA approved six second-generation CAR T cell therapies for hematopoietic cancers such as relapsed or refractory B-cell lymphoma or acute lymphatic leukemia based on CD19 targeting with an scFv (Axicabtagene ciloleucel, brexucabtagene autoleucel, lisocabtagene maraleucel, and tisagenlecleucel), and relapsed or refractory multiple myeloma, based on B-cell maturation antigen (BCMA) targeting with an scFv (idecabtagene vicleucel) or a VHH (ciltacabtagene autoleucel)<sup>202</sup>.

Third-generation CAR T cells combine the potential of the two costimulatory domains to enhance both the T cell response and the *in vivo* survival and expansion of the CAR T cells. Fourth-generation CAR T cells are enhanced by inclusion of other transgenes, for instance those promoting autologous cytokine secretion or other costimulatory ligands, into the T cell<sup>203</sup>.

### **CAR NK cell therapy**

Just like therapies based on T cells, NK cell-based therapies have proven promising in clinical trials treating both hematological and solid cancers<sup>204</sup>. CAR NK cells can be produced from NK cells derived from the patient's or a donor's peripheral blood, from a placenta or umbilical cord blood, existing immortalized NK cell lines (NK-92 or NK-92MI) or manufactured from induced pluripotent stem cells (iPSC)<sup>205-210</sup>. There are a few advantages of treatment with CAR NK cells versus CAR T cells. First, unlike T cells, NK cells do not form the risk of Graft-versus-Host disease (GVHD) in an allogeneic setting. In fact, NK cells are believed to protect against GVHD in T cell-based

cancer treatments<sup>211–215</sup>. Furthermore, NK cells allow for the inclusion of a wider range of co-stimulatory domains, using not only traditional intracellular domains derived from CAR T therapies based on CD28, 4-1BB, and CD3 $\zeta$ , but also NK-specific domains such as CD244 and NK-ARs<sup>209,216,217</sup>. Moreover, if a tumor were to downregulate the CAR's target in an attempt at immune escape, the NK cells would still be effective against the tumor cells due to the intrinsic cytotoxic capabilities of NK cells. Lastly, a major reported side-effect of CAR T therapy is cytokine release syndrome, which is systemic inflammation caused by a large amount of cytokines released by the CAR T cells. The cytokines released by NK cells (IFN- $\gamma$ , IL-3, and TNF- $\alpha$ ) do not induce such inflammation, and thus do not cause cytokine release syndrome<sup>217</sup>. For these reasons, CAR NK cell therapy is potentially a more effective and a safer alternative to CAR T cell therapy.



**Figure 3. Composition of common CAR constructs.** The extracellular antigen-binding targeting portion often consists of an scFv, composed of the heavy and light chain variable regions of an immunoglobulin connected by a linker segment, or a VHH, the variable region of camelid heavy-chain only antibodies. The intracellular signaling domains harbor the cytoplasmic CD3 $\zeta$  activation domain (first generation) or a the cytoplasmic CD3 $\zeta$  activation domain in combination with a CD28 or 4-1BB co-stimulatory domain (second generation). Third-generation CAR T cells combine the potential of the two costimulatory domains. Fourth-generation CAR T cells are enhanced by inclusion of other transgenes, for instance those promoting autologous cytokine secretion.

## **Tumor targets**

Although often effective in treating tumors and metastases, a huge disadvantage of current (immuno)therapies is the large number of side-effects that patients experience. Most cancer drugs target proteins that are expressed on a wide variety of rapidly dividing cells, which include healthy cells, such as cells of the skin, stomach, gut, and hair. This explains the most frequently reported side-effects of rashes, nausea, diarrhea or constipation, and hair loss. An ongoing quest in clinical research has been the identification of tumor-specific targets to treat and/or prevent cancer. Tumor-associated and tumor-specific antigens, like those mentioned earlier, are important for therapies that involve CARs and antibodies. We propose the targeting of two MHC-I associated proteins: MICA and HLA-E.

### **MICA**

The MHC-I chain-related proteins A and B (MICA and MICB) are encoded within the family of human HLA class I (MHC-I) genes on chromosome 6. MICA/B consists of 3 extracellular, immunoglobulin-like domains ( $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ). The protein has a molecular mass of 36 kDa, but contains 8 potential N-linked glycosylation sites, some of which are used, resulting in an apparent molecular mass of approximately 56 kDa when the protein is examined by SDS-PAGE. Unlike conventional MHC-I proteins, MICA and MICB do not associate with beta-2-microglobulin and do not present antigen, but rather act as ligands for the NKG2D receptor on NK cells, CD8<sup>+</sup> T cells, and  $\gamma\delta$  T cells<sup>218</sup>. Upon binding, these cells can eradicate MICA/B-positive targets through cytotoxicity and secretion of cytokines<sup>219–221</sup>.

### **NKG2D signaling**

MICA/B, as well as other proteins such as the UL-16 binding proteins (ULBP) in humans, and members of the H6o, RAE and MULT1 protein families in mice, act as ligands for the NK cell-activating receptor NKG2D. Upon binding to ligands expressed on tumor cells or virus-infected cells, NKG2D pairs with DNAX-activating protein-10 (DAP-10). The complex transmits intracellular signals via the Phosphoinositide 3-kinase (PI3K) and Growth Factor Receptor Bound Protein 2 (GRB2) signaling pathways through tyrosine phosphorylation. This triggers activation of the AKT/MAPK or NF $\kappa$ B/NFAT pathway, causing NK-mediated cytotoxicity and production of cytokines, chemokines, and granzymes<sup>220,221</sup> (Figure 4).



The protease Granzyme B (GrzB), together with the glycoprotein perforin, participates in the induction of apoptosis of NK and T cell targets. GrzB has hundreds of substrates, most of them involved in induction of apoptosis, inflammation, and remodeling of the extracellular matrix. In the anti-tumor response, GrzB enters the target cell with the help of perforin, or by endocytosis facilitated by binding to the negatively charged heparan sulphate-containing receptors on the surface of the target cells. Inside the target cell, GrzB cleaves and activates initiator caspases 8 and 10 and executioner caspases 3 and 7, which triggers the apoptosis pathway<sup>222,223</sup>.

### **MICA/B as target**

The MICA/B proteins are expressed only weakly on healthy cells but are overexpressed on the surface of cells under stress, for instance due to infection or malignant transformation<sup>224</sup>. High levels of expression of MICA/B have been seen in both hematopoietic malignancies and in a wide variety of epithelial solid tumors such as colorectal cancer<sup>225</sup>, ovarian cancer<sup>226</sup>, cervical cancer<sup>227</sup>, breast cancer<sup>228</sup>, pancreatic cancer<sup>229</sup>, melanoma<sup>230</sup>, and cholangio-carcinoma<sup>231</sup>. Surface expression of NKG2D ligands can be regulated transcriptionally, translationally, and post-translationally by the tumor microenvironment. Post-translationally, the surface expression of MICA and MICB on tumor cells can be downregulated through shedding. Shedding is mediated by proteolytic cleavage at the  $\alpha 3$  domain involving the disulphide isomerase ERp5 and ADAM-type proteases such as ADAM10 and ADAM17<sup>232-236</sup>. Increased levels of soluble MICA/B in the serum of patients are associated with poor prognosis and worse disease progression<sup>29,225,237</sup>. Loss of surface-bound MICA renders tumor cells less sensitive to NKG2D-positive NK cells. Furthermore, soluble MICA might occupy the NKG2D receptors on NK and CD8<sup>+</sup> T cells and thus inhibit the cytotoxic activity on cells that express MICA/B at the surface<sup>238,239</sup>.

In clinical settings, patients with melanoma who received a GM-CSF secreting cell-based cancer vaccine (GVAX) and anti-CTLA-4 antibodies generated high titer antibodies against MICA<sup>240</sup>. These antibodies inhibited the immune suppression caused by soluble MICA, and increased innate and adaptive anti-tumor immunity by CD8<sup>+</sup> T cell and NK cell responses. The increase in anti-MICA antibodies resulted in a decrease in soluble MICA in the patient's circulation<sup>241</sup>. The increase of humoral anti-MICA antibodies and its benefit in cancer therapy suggests a useful role for MICA/B-based vaccination. Indeed, by vaccinating mice with the conserved  $\alpha 3$  domain of MICA/B,

proteolytic shedding of MICA/B from the surface of murine-derived B16F10 melanoma cells transfected to express human MICA/B, was prevented both *in vitro* and in a mouse model. Furthermore, mice immunized with the MICA/B  $\alpha 3$  domains showed significantly reduced tumor growth of MICA/B<sup>+</sup> B16F10 melanoma and EL-4 T cell lymphoma tumors.

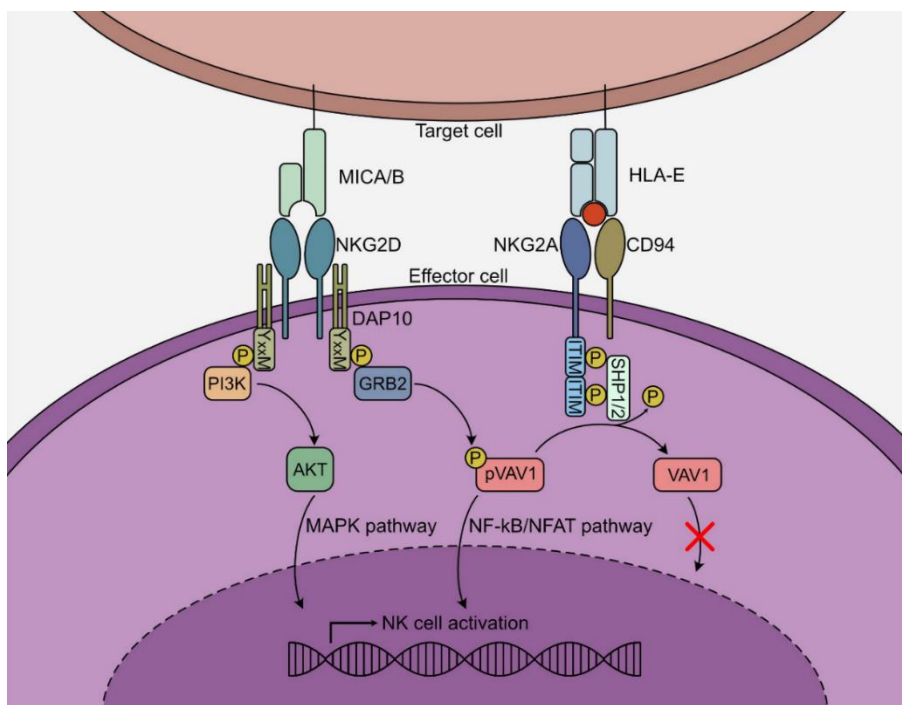
The vaccine safety and immunogenicity was examined in rhesus macaques which, unlike mice, endogenously express MICA/B proteins homologous to human MICA/B. High serum titers of anti-MICA/B antibodies were found following immunization with macaque MICA/B  $\alpha 3$  domains, while no clinical side effects were observed<sup>242</sup>. The monoclonal antibody “7C6” specifically targets the  $\alpha 3$  subunit of the MICA/B protein, thereby inhibiting shedding by the TME through obstructing access of ERp5. Mice treated with monoclonal antibody “7C6” showed significant reduction in tumor growth and metastases formation of MICA<sup>+</sup> B16F10 tumors<sup>243</sup>.

The absence of MICA/B on the surface of healthy cells, and the ability to overcome proteolytic shedding from the tumor cell membrane, makes MICA/B an appealing target for tumor therapy.

## HLA-E

MHC-I molecules are found on the surface of all nucleated cells in vertebrates. Assembly of MHC-I with  $\beta 2M$  is facilitated in the endoplasmic reticulum (ER), where the complex is loaded with peptides with the help of Tapasin and TAP<sup>244</sup>. Peptide-bound MHC-I rapidly exits the ER, traverses the secretory pathway, and is expressed at the cell surface<sup>101,245–250</sup>. MHC-I presents fragments of intracellular proteins in the form of peptides to cytotoxic T cells. As discussed earlier, healthy cells will display peptides from normal cellular proteins on their MHC-I, to which the CTLs will not react due to imposition of central and peripheral tolerance<sup>102,251</sup>. When cells express foreign proteins on MHC-I, like those found intracellularly after a viral infection or malignant transformation, the cytotoxic T cells will recognize and kill the affected cell<sup>103,106</sup>. The MHC-I molecule HLA-E presents a unique case, as it is specialized in the presentation of so-called “VL9” peptides (VMAPRT(L/V)(L/V/F)L). These peptides are derived from the signal sequences of other MHC-I products, or of viral type I membrane glycoproteins<sup>252–260</sup>.

Virus-infected and malignantly transformed cells can escape immune cell recognition by down-regulation of MHC-I, which can be achieved transcriptionally and post-transcriptionally<sup>261-265</sup>. Human cytomegalovirus (CMV) expresses the VL9 peptide in the leader sequence of its UL40 protein. This peptide can be loaded onto HLA-E in a TAP-independent manner in the ER<sup>266,267</sup>. This is sufficient to upregulate the expression of HLA-E on the cell surface, preventing NK cell-mediated cytotoxicity of the infected cells through interaction with NKG2A. Thus, if a virus succeeds in down-regulation of the classical Class I HLA-A, -B and -C products, VL9 peptides would continue to be produced and could serve as peptide cargo for HLA-E, rendering the infected cell resistant to NK and T cell lysis<sup>266-268</sup>.



**Figure 4. Activating and inhibiting receptors on NK cells.** The NK cell-activating receptor NKG2D is activated by MICA/B in humans. Upon binding to its ligand, NKG2D forms a complex with DAP-10, resulting in tyrosine phosphorylation of DAP-10. The complex transmits intracellular signals via the PI<sub>3</sub>K and GRB2 signaling pathways, triggering activation of the AKT/MAPK or NFκB/NFAT pathways, causing NK-mediated cytotoxicity and production of cytokines, chemokines, and granzymes. The NK-cell inhibiting receptor NKG2A is activated by the ligand HLA and forms a heterodimer with CD94. The interaction of NKG2A with HLA-E causes phosphorylation of the NKG2A ITIM motifs, which recruits SHP1/2. SHP-1/2 in turn dephosphorylates signaling molecules such as VAV1, blocking downstream NK cell activation signals.

### **NKG2A signaling**

HLA-E serves as a ligand for CD94/NKG2A and NKG2C on NK cells and T cells, and causes inhibition of the cytotoxic activity of such cells<sup>258,260,269-279</sup>. The interaction of NKG2C The interaction of NKG2A/CD94 with peptide-presenting HLA-E causes phosphorylation of the intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) of NKG2A. This recruits the activating Src homology 2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2. SHP-1/2 dephosphorylates signaling molecules such as VAV1, blocking downstream NK cell activation signals<sup>278,279</sup> (Figure 4).

### **The cytoplasmic tail of HLA-E**

The ectodomains of the MHC-I products, including that of HLA-E, are highly homologous. There are few locus-specific features present in the ectodomains that would allow an unambiguous assignment of a sequence to the HLA-A, -B or -C locus. In contrast, the cytoplasmic tails of the classical MHC-I products show such locus-specific features, shared among virtually all alleles at that locus (Chapter 7, Figure 1).

The cytoplasmic tail of MHC-I is involved in trafficking peptide-bound MHC-I from the ER to the cell membrane, and in endocytosis<sup>280</sup>. In the case of HLA-E, surface-disposed HLA-E is unstable and rapidly internalized, causing HLA-E to be enriched in endosomal structures. HLA-E is also retained in an immature state in the ER, as defined by the sensitivity of HLA-E to Endoglycosidase H, and intracellular accumulation seen by immuno-fluorescence<sup>281</sup>.

The cytoplasmic tail plays a role in ER retention. This has been confirmed by swapping the cytoplasmic tail domains of HLA-E and HLA-A3, creating HLA-E(A3) and HLA-A3(E). HeLa cells transfected with these transgenes showed a 1.7-fold increase in expression of HLA-E(A3) compared to HLA-E, and a reduction (0.7-fold) in expression of HLA-A3(E) compared to HLA-A3. Furthermore, the surface half-life of HLA-E(A3) molecules was twice that of HLA-E, confirming that the cytoplasmic tail of HLA-E also plays a role in its endocytosis and relocation of HLA-E to late and recycling endosomes<sup>281,282</sup>. The rapid turnover of surface-disposed HLA-E is also attributed to the binding affinity of VL9 to HLA-E, which is much lower than the average binding affinity of other MHC-I binding peptides<sup>253,283-285</sup>.

### **HLA-E targeting by CMV-based vaccines**

Peptide-presentation of HLA-E is further exploited in the more recently developed cytomegalovirus (CMV)-based vaccines, studied in rhesus macaques which express the HLA-E homologue Mamu-E. rhCMV-vectored vaccines against genes of the simian immunodeficiency virus (SIV) elicited a strong HLA-E-restricted CD8<sup>+</sup> Tem cell response to SIV peptides causing efficient eradication of a subsequent SIV infection, compared to a relatively slower Tcm response from comparable adenovirus-vectored vaccines.

It is hypothesized that the rhCMV-derived VL9 peptide stabilizes the hydrophobic binding groove of Mamu-E, allowing a broader range of SIV-derived peptides to bind, improving presentation of SIV-derived antigens to non-classical CD8<sup>+</sup> T cells. These findings, combined with the lack of polymorphism of HLA-E in the human population<sup>286</sup>, show a promising role of CMV-based vaccination against HIV and other viruses in humans.

### **HLA-E as target**

HLA-E is overexpressed on various types of hematopoietic and solid tumors, and is associated with worse prognosis and disease outcome in lung cancer<sup>287</sup>, glioma<sup>288,289</sup>, renal cell carcinoma<sup>290</sup>, colon cancer<sup>37,291–293</sup>, breast cancer<sup>36,228</sup>, and ovarian cancer<sup>275,294,295</sup>. Tumor infiltrating lymphocytes in certain cancers show higher expression of NKG2A, which is also correlated with poor prognosis<sup>296–298</sup>. Because overexpression of HLA-E on cancer cells is a mechanism of immune-evasion, blockade of the interaction between NKG2A and HLA-E may enhance the anti-tumor immune response and cancer therapies. In fact, the monoclonal NKG2A-targeting antibody Monalizumab has successfully been used in combination with PD-L1-targeting or EGFR-targeting therapies to treat colorectal cancer and squamous cell head-and-neck cancer respectively. Blocking of NKG2A alone had no effect on cancer growth<sup>296,299</sup>.

The role of the cytoplasmic tail on HLA-E trafficking and peptide presentation deserves further study. As mentioned, the cytoplasmic tail is also the feature distinguishing HLA-E from other MHC-I molecules. Thus, antibodies against the HLA-E cytoplasmic tail could provide a useful tool for studying the cytoplasmic tail interactions, as well as for other purposes where targeting of HLA-E specifically is necessary, such as staining of tumor tissues for diagnostics.

Targeting of proteins can be done with monoclonal antibodies, an approach we used for the cytoplasmic tail of HLA-E, or camelid-derived heavy-chain only fragments, called nanobodies or VHHs, which we use for targeting MICA. The difference between conventional antibodies and VHHs is described in Chapter 2.

