Bioinformatic avenues to improve the specificity of Chimeric Antigen Receptor (CAR) immune therapies

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Abstract

Chimeric Antigen Receptor (CAR) T-cell therapy has proven to be a successful alternative to more traditional treatment methods for patients with B-cell malignancies. This success has not yet been repeated in other cancer types (especially solid tumors) which requires further development of CAR technology. Several aspects of CAR development can be aided with bioinformatics methods, and potentially new *in silico* methods need to be developed alongside the improvements of CAR-based immune therapies. We examine various proposed adaptations of CAR-T cell therapy, and the role bioinformatics plays in this. The use of nanobody-based targeting domains, using Natural Killer (NK) cells instead of T-cells, improved downstream signaling and a combinatoric approach to targeting domains seem to be useful improvements to CAR T-cell therapy that will help to improve the next generation of immune therapy. For these to succeed, we need to adapt existing bioinformatics methods to include the combined molecular modeling for several antigen-antibody pairs and a good estimate of the downstream response of CAR cells. Overall, the improvements to CAR-based immune therapy give reason to be optimistic about future treatment options.

Plain Language Summary

Usually, cancer patients are treated with chemotherapy, radiation, or surgery or a combination of these. This often has a positive outcome, but sometimes the treatment takes a large physical toll on the patient or does not reach the desired outcome. To improve this, immune therapies are developed as an alternative to the currently used treatments with the goal of improving the cure rate and the treatment impact on patients' health. Immune therapy uses mechanisms that are based on the human immune system to rid the body of cancer cells. This can be achieved in many ways, one of which is by using so-called CAR immune cells. CAR therapy involves human immune cells (white blood cells) that are extracted from a patient (or healthy donor's) blood, and genetically engineered to contain a new molecule – the Chimeric Antigen Receptor (CAR). After this, the patient is infused with the new CAR cells. These cells are now better at recognizing cancer cells in a patient's body and help with the removal of cancer. Human cells contain many molecules on their surface, and CAR immune cells need to react to cancer cells only so we search for molecules that can be used as 'targets'. These targets are what a CAR cell latches on to. Specifically latching on to and destroying targets that only occur as part of cancer cells prevents side-effects like the ones experienced by patients undergoing chemotherapy – where damage from the therapy affects many cells in the body. After discovery of a target, a CAR needs to be designed in such a way that it reacts to the target. This is achieved by using antibodies that are developed with a combination of experimental work and bioinformatics methods.

Currently, the challenge lies in finding suitable targets and the design of the antibody part of the CAR cells. We examine the development so far of CAR therapy, its success in treating a type of B-cell lymphoma, and opportunities for further development of the technology. We consider several opportunities and pitfalls for the development of CAR cells. Firstly, CAR technology has mostly been developed with T cells while there are many other immune cell types such as Natural Killer (NK) cells which have unique properties that could be leveraged in immune therapy. This includes the NK cell's inherent response to tumor cells, a lower risk of side-effects, and a lower risk of disease by using incorrectly matched donor cells to treat a patient. Secondly, nanobodies are a new type of antibody derived from llama's immune systems that can be used instead of antibodies from a human or mouse. Nanobodies are easier to produce, and offer fewer side-effects especially compared to mouse antibodies. Thirdly, we consider increasing the specificity by designing CARs that are sensitive to multiple targets instead of just one. This can help in recognizing cancer types that have varied targets on the cells, especially when we can engineer CAR cells to only attack a given target when there is a specific other target nearby or missing. Several steps in the process of developing a CAR therapy require bioinformatics analysis or predictions. These vary from modeling the molecular structure and interaction of a CAR cell to the target to searching for the target itself in genetic/molecular data of cancer cells. With development of new CAR therapies, we might need to change the bioinformatics methods used or develop new methods to accommodate the design and usage of this new generation of immune therapy.

Introduction

The human immune system has the inherent ability to detect and remove (potentially) malignant cells; an important function because mutations that can lead to cancer accumulate in these cells over time. Malignant cells are removed frequently in a healthy human body, either by cytotoxic (CD8+) T-cells after MHC class I presentation of mutated self (neo-antigens) or (when malignant cells escape this through MHC down regulation) by Natural Killer (NK) cells recognizing a lack of MHC I on the cell surface. Malignant cells that survive the inherent defense mechanisms can become a larger malignancy that requires treatment. Treatments for cancer have long been one (or a combination) of three possibilities: chemotherapy, radiation therapy, and surgery. These approaches result in positive treatment response for many patients, especially when the disease is detected in an early stage. Over the decades prevention, early detection programs, and improvements to treatment for specific cancers have resulted in higher cure rates and progression free survival (Jemal et al., 2010). However, these methods can be only partly effective or completely ineffective for specific cancers. Research into immunotherapies has shown to pay off, with several new treatments being approved and others in development (Labanieh & Mackall, 2023).

Immunotherapy leverages the inherent ability of the immune system to eliminate malignant cells. It exists in several forms such as monoclonal antibodies, immune checkpoint inhibitors, or adoptive cell therapy. Monoclonal antibodies are derived from a single B-cell lineage and are used to target a single antigen such as cancer cells or immune inhibitors. Immune checkpoint inhibitors stimulate the immune system by binding to immune checkpoint proteins (e.g. PD-L1) which normally inhibit an immune response and are often overregulated in cancer cells. Adoptive cell therapy involves isolation, modification, and re-infusion of immune cells from a patient. This can be achieved without a modification step by using already specific tumor-infiltrating lymphocytes (TIL) from a resected tumor which are then clonally expanded ex vivo. Other patient derived lymphocytes need to be genetically modified to reach specificity for a given tumor. To this end, T-cells modified with specific T-cell receptors (TCR) or chimeric antigen receptors (CAR) are used (Rohaan et al., 2019). CAR modification can also be applied to NK cells, however this technology is not as far developed as T cells (Laskowski et al., 2022). Modification of lymphocytes allows for cancer specific receptors (Safarzadeh Kozani et al., 2022) and target elimination efficiency that would not normally occur in the body's own immune response (Sterner & Sterner, 2021).

Molecular structure and function of chimeric antigen receptors

CAR T-cells have been through several generations of development so far (Figure 1). Structurally these receptors consist of several domains. An extracellular targeting domain determines antigen recognition. These are often single chain fragment variable antibodies (scFv), which are a heavy- and light chain variable domains derived from human immunoglobulin G (IgG). The targeting domain is connected to a transmembrane domain via a linker, which is in turn connected to the activating domain. This first generation design was effective in binding to a target antigen but could not achieve cell persistence and continual activation, therefore costimulatory (CS) domains such as CD28 (Wu et al., 2023) were added in consecutive generations to increase activation and cell expansion (Safarzadeh Kozani et al., 2022). The current FDA approved therapies consist of CARs with CS domains CD28 and 4-1BB from the second and third generation. Other therapies stemming from the variety of newer-generation CARs involving novel domains and improved signaling are under development

(Honikel & Olejniczak, 2022; Labanieh & Mackall, 2023). Fourth and fifth generation receptors include cytokine expression and/or -sensitivity domains (CY domain, figure 1), allowing immune modulation and tumoricidal properties of cytokines on the location of the tumor. This improves the effectiveness of CAR based therapies against solid tumors (Safarzadeh Kozani et al., 2022).

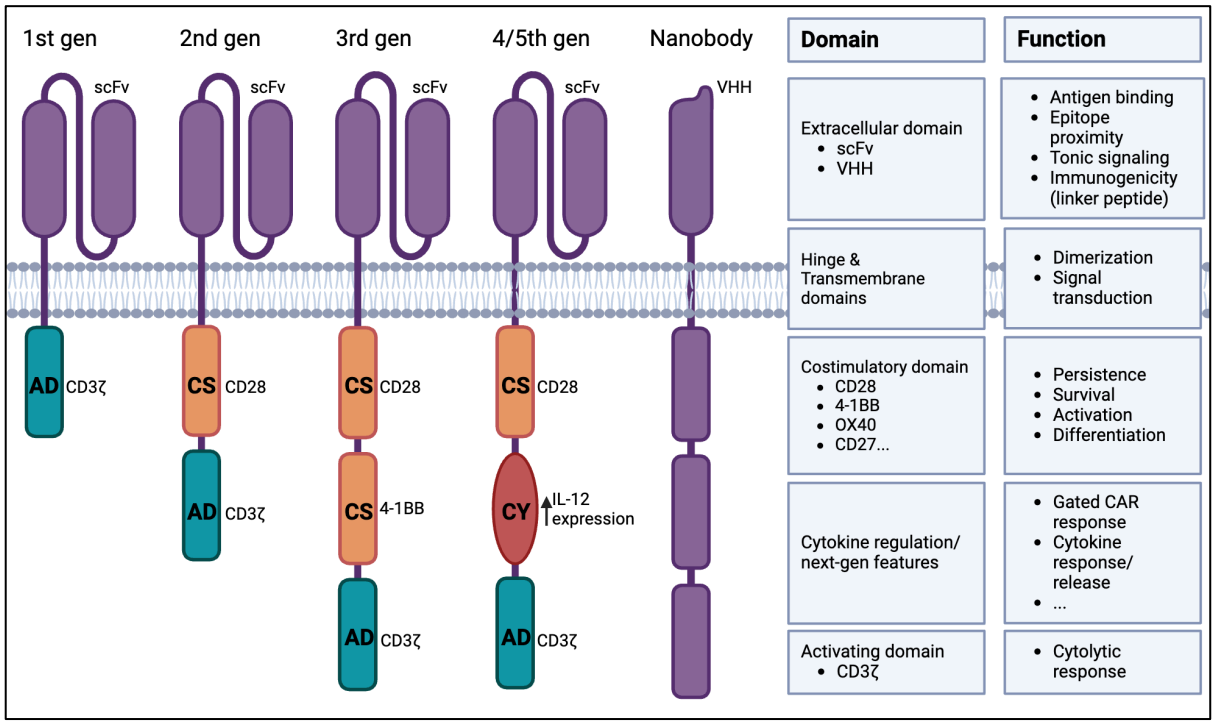

Figure 1 – Generations of chimeric antigen receptors, their domains (with example proteins) and function. AD = activation domain, CS = costimulatory domain, CY = cytokine response. Based on (Safarzadeh Kozani et al., 2022), (Honikel & Olejniczak, 2022), and (Labanieh & Mackall, 2023).

In addition to developments in the intracellular tail, the extracellular part of the protein can be designed differently, here research into nanobodies seems promising. Nanobodies consist of the variable domain of heavy chain only antibodies (VHH) derived from Llamas and are generally more stable than scFv's. They solve several problems inherent to scFv usage. For example, the variable heavy and light chains of two receptors can aggregate due to cross-linking, which results in tonic signaling followed by exhaustion of the CAR-T cell. The chance of aggregation is increased in CARs that are specific to multiple antigens (e.g. as used in TanCARs; two tandem domains designed for two epitopes on a target antigen). Another issue is the immunogenicity of murine-derived linkers used to link the VH and VL chains of a scFv; in case of nanobodies the immunogenicity of the linker is not an issue as a single VHH does not require a linker peptide. Introducing foreign (from mouse or camel) antibodies requires humanization to prevent anti-idiotypic immune reactions, which is also easier in nanobodies. A final advantage of nanobodies over scFvs is the long CDR3 region in VHH, enabling binding of some epitopes that could be out of reach for scFv (Safarzadeh Kozani et al., 2022).

Efficacy and challenges of CAR-T cell therapy

CAR T-cell therapies have been successful in treating certain B-cell lymphomas because they can invoke a strong immune reaction without MHC I involvement - a CAR could theoretically target any surface molecule. This has already been leveraged to develop 12 FDA approved CAR

T-cell treatments - 10 of which target CD19 and 2 target BCMA (Labanieh & Mackall, 2023). However, these treatments have the downside of severe side effects in the form of cytokine release syndrome and neurotoxicity, both of which occur frequently in patients (A. N. Khan et al., 2024). This and other practical barriers still prevent CAR T from being used to its full potential.

CAR T-Cell therapy still faces multiple problems of varying nature (Figure 2); most of which have to do with antigen targeting or lack thereof. Firstly, treatment resistance can develop in the form of antigen downregulation or loss (Figure 2A). This has been suggested to be the cause of relapse with CD19 and (to a lesser degree) BCMA treatments (Labanieh & Mackall, 2023). A solution to this would be targeting of multiple antigens on the tumor, which can be achieved with VHH (Safarzadeh Kozani et al., 2022) or scFv (Labanieh & Mackall, 2023) targeting domains. Targeting multiple antigens also has the advantage of increased specificity, however given that target search is challenging, for many cancers it might be difficult to find suitable multiple targets. Secondly, CAR-T cells could have on-target off-tumor effects due to targeting of antigens that are not exclusively present on the tumor (Figure 2B). This could in theory be solved with targeting of tumor neoantigens – a promising approach for highly specific cancer treatments. However, neoantigens are very diverse with tumors containing 50-1000 mutations that vary per patient (Bobisse et al., 2016), therefore this approach will require personalized treatment which is expensive and time consuming.

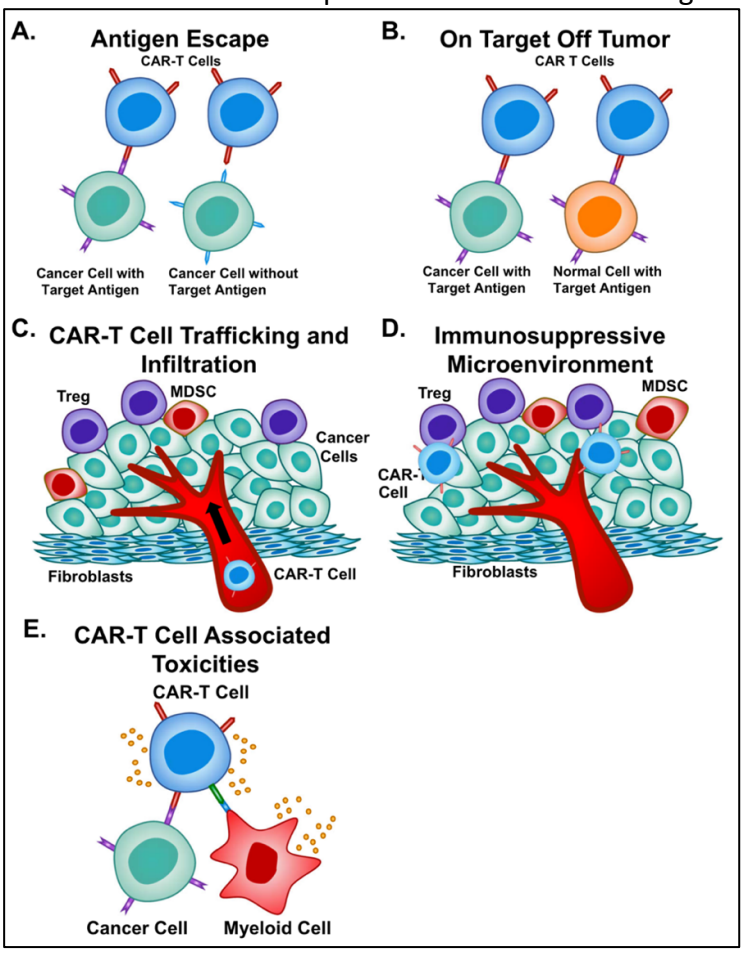

Figure 2 – Problems faced by CAR T-Cell therapy. A) immune escape via mutations or downregulation causing low antigen expression through selection. B) On target off tumor effects of CAR-T cells. C) CAR-T trafficking and infiltration. D) Immunosuppressive microenvironment. E) CAR-T cell associated toxicities. From (Sterner & Sterner, 2021).

Other issues faced have to do with CAR T-cell persistence and the tumor microenvironment (TME) (Figure 2C, D, E). One of these is CAR T-cell trafficking and tumor infiltration, especially important in solid tumor treatment. The TME is often immunosuppressive, limiting the CAR T-cell reaction. In addition, it is estimated that CAR T-cells requires more antigens (>1000 as compared to 100) than a normal T-Cell activation would be via TCR and the peptide-MHC complex (Labanieh & Mackall, 2023; Wu et al., 2021). There are many efforts to increase CAR T persistence, expansion, armoring, and fitness to improve the response of the engineered cells to cancer (Labanieh & Mackall, 2023). However, the major disadvantage of a strong reaction by CAR T-cells is that they also have toxic side effects. They are introduced quickly in a patient's bloodstream and can clonally expand in vivo, resulting in high numbers of T-cells without corresponding immune regulation. This leads to dangerous side-effects observed in patients, most of which are related to systemic cytokine release syndrome (CRS). This can be treated with IL-6 inhibition which is often not successful, resulting in patient lethality (Sterner & Sterner, 2021). An additional problem seen in practice is the decreased effectivity of CAR cells because of the host immune responses, e.g., anti-idiotypic response to the (murine) linker peptide that is used in scFv molecules. This leads to removal of the CAR-T cells, or quick induction of T-cell exhaustion. Just as is the case with the tonic signaling (see above) this might be improved with design iterations or by using nanobodies which are less sensitive to this due to lack of linker peptides. Note that the FDA approved therapies are all based on autologous T cells, with disadvantages in manufacturing time and low functionality of T cells due to prior treatment or disease. Research into allogenic T cell manufacturing and function promises to offer a higher level of activation and antitumor potential if graft vs host disease (GVHD) can be avoided. This would be in addition to 'off the shelf' benefits such as quick treatment time, low cost, and cheaper and more availability (Yang et al., 2023).

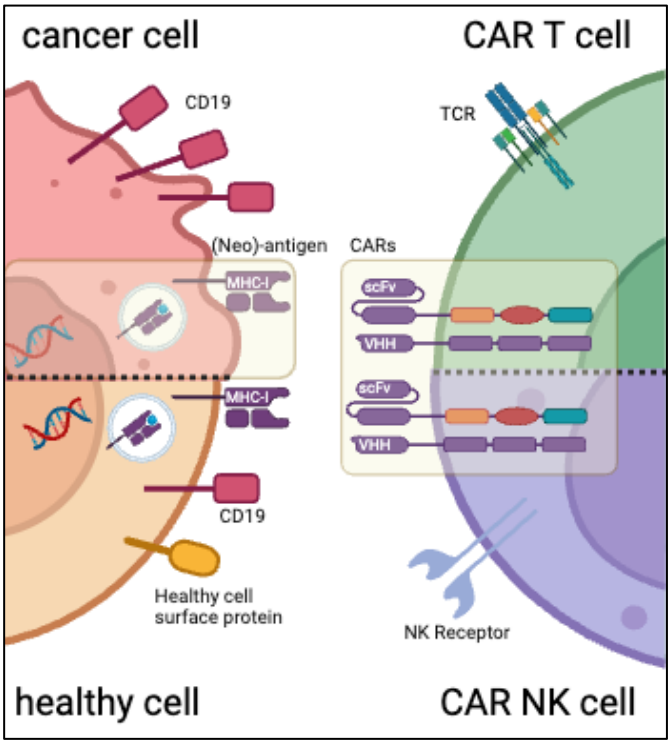

Figure 3 – **Regions of interest for bioinformatics approaches** in CAR T and CAR NK cells, highlighted in yellow. On cancer cells we investigate prediction of cancer specific epitopes that are presented by MHC class I, but also differentially expressed surface proteins like CD19 in B-cell cancers. On CAR cells we investigate specificity through modeling of molecular dynamics, and logic gating.

Bioinformatics for CAR T-cell therapy improvement

From the bioinformatics point of view, an interesting problem is the prediction of CAR receptor specificity. Improved (novel) antigen targeting is not yet sufficient to generate high specificity but will certainly aid in the development of CAR based therapies by giving multiple targeting options (Fedorov et al., 2014). Experimental work to generate highly specific CAR receptors is labor-intensive and given the improvements in computational tools and artificial intelligence, there is a lot of potential in a bioinformatics approach to this problem. The aim of this section is to dive into the novel developments in the field of CAR immunotherapy and investigate bioinformatics methods that are (or could be) used in CAR immune therapy design. We focus on new technologies, expansion of CAR to different cell types, and tools that allow *in silico* identification of cell-surface targets and potentially the targeting of intracellular cancer markers that are presented by MHC class I (figure 3).

Specificity in CAR-T cell therapies

Given the abundance of potential antigens that could be a danger for the human body, the immune system faces a difficult challenge of finding targets that are specific to malignant cells or infections without attacking healthy cells or symbionts (Figure 3). The ability to recognize an antigen without reacting to closely related or very similar molecules is what we refer to as specificity. Specificity for an antigen is a balancing problem between giving an efficient immune response to pathogens and the negative outcome of autoimmunity. There is an ongoing search for antigens that are upregulated in or unique to specific types of cancer that can also effectively be targeted by immune therapies (Table 1). However, in addition to having very specific targets it is useful to have targets that are biochemically distinct from self-antigens to prevent autoimmunity as a side-effect of immunotherapy. Cancer neoantigens can be classified in two categories: public and private. Most mutations are unique per patient or tumor (i.e., private); however, some mutations related to cancer driver genes occur frequently in different tumors and patients (i.e., public). Common occurrence is what distinguishes public from private mutations, making them a useful group of targets in addition to upregulated cell surface proteins or tissue-specific proteins. These, together with upregulated antigens are currently being leveraged for CAR- or TCR-based therapies (Table 1).

Autoimmunity is prevented through negative selection of B cells and T cells during development (also known as central tolerance), and afterwards via T regulatory (Treg) cells that inhibit immune responses (peripheral tolerance). Immune cells modified with CAR receptors do not undergo central tolerance *in vivo*. Therefore, they require high specificity in the design and humanization to minimize the chance of autoimmunity. Tregs can be recruited to the tumor micro-environment (TME) by cancer cells that have evolved this mechanism of peripheral tolerance as a form of immune escape, thus decreasing the effect of immunotherapies (Figure 2D). In the case of CAR treatments, it has been shown that CAR Treg cells that are also produced during the viral transduction step can cause relapse due to their proliferation in the TME (Haradhvala et al., 2022). To resolve this, it has been suggested to deplete the CAR Treg fraction in a patient's infusion, which can be done via freezing or negative selection for $CD25^{+}$ cells (Haradhvala et al., 2022).

Table 1 – CAR-T and TCR-T cell therapy targets in varying phases of development. TCR-T therapy targets are presented by the HLA class I molecules, and therefore each target is patient specific as HLA type varies per patient. Note that this TCR-T list is not exhaustive as it is from 2019. Cancer types: B-ALL = B-cell Acute Lymphoblastic Leukemia, LCBL = Lymphocytic Choriomeningitis-associated Lymphoblastic Leukemia, MCL = Mantle Cell Lymphoma, FL = Follicular Lymphoma, HL = Hodgkin Lymphoma, MM = Multiple Myeloma, T-ALL = T-cell Acute Lymphoblastic Leukemia, TLBL = T-cell Lymphoblastic Lymphoma, AML = Acute Myeloid Leukemia, NHL = Non-Hodgkin Lymphoma, CLL = Chronic Lymphocytic Leukemia, NB = Neuroblastoma, DMG = Diffuse Midline Glioma, GBM = Glioblastoma Multiforme, BTC = Biliary Tract Cancer, MPD = Myeloproliferative Disorder, GC = Gastric Cancer, PC = Pancreatic Cancer, MCRPC = Metastatic Castration-Resistant Prostate Cancer, RR = Relapsed or Refractory. * = Approved therapies exist for the antigen, not necessarily for all cancer types mentioned. Adapted from (Labanieh & Mackall, 2023), (He et al., 2019) and (Saura-Esteller et al., 2022).

	Antigen	Cancer type(s)	Remarks	Source
FDA-approved therapies exist*	CD19	B-ALL, LCBL, MCL, FL	Liquid tumor target	(Labanieh & Mackall, 2023)
	BCMA	MM		
Therapies with clinical evidence of efficacy	CD20	LBCL		
	CD22	B-ALL, LBCL		
	CD30	HL		
	CD7	T-ALL, TLBL		
	CD38	AML	Solid tumor target	
	k-light chain	NHL, CLL, MM		
	GD2	DMG		
	HER2	Sarcomas		
	IL-13Ra2	GBM		
	EGFR	BTC		
	Mesothelin	MPD		
	Claudin-18.2	GC, PC		
	PSMA	MCRPC		
TCR-T therapy targets in clinical trials	MART-1	(metastatic) Melanoma	For specific TCR and MHC molecules, see source	(He et al., 2019)
	gp100	Melanoma		
	NY-ESO-1	Melanoma, synovial sarcoma, MM		
	CEA	Metastatic colorectal cancer		
	MAGE-A3	Metastatic/ulcerated melanoma, synovial sarcoma, esophageal cancer, myeloma		
	MAGE-A4	Esophageal cancer		
	WT1	AML, MDS		
CAR γδ T-cell therapy clinical trials	CD19	RR ALL, CLL, B-NHL		(Saura-Esteller et al., 2022)
	NKG2DL	RR solid tumors		
	CD20	B-NHL	Combined with IL-2	

Novel developments in molecular structure and function

Novel developments in CAR technology (Figure 4) mostly focus on 3rd generation CAR receptors to address therapeutic problems such as managing toxicity and the improvement of CAR T cell trafficking, persistence, and immune reaction instead of only improving antigenic specificity. For example, bivalent CARs with several targeting domains require at least one of the antigens to be present, which provides robustness to antigen loss (Labanieh & Mackall, 2023). There is also development in mitigation of toxicity by adding inhibitory mechanisms that can be triggered to reduce CAR T cell activity. For example, SNIP (signal neutralization via inhibitory protease) CAR cells contain a protease that without inhibition cleaves the intracellular domain of the CAR. Therefore, activity is only continued with administration of a corresponding protease inhibitor which allows researchers to 'switch off' the CAR T cells when needed (Labanieh et al., 2022). Efforts to address rejection of modified immune cells are also on-going; in Stealth CAR therapy gene knockouts prevent TCR and MHC expression and thus recognition by the host's immune cells. Cells with CAR receptors can now also be shielded from NK- or macrophage-based rejection through transcriptional reprogramming. Transcriptional reprogramming is also leveraged to prevent CAR T-cell exhaustion. Examples of this include TET2 and NR4A knockout or c-Jun overexpression (Figure 4), but also knockout of PD1, TGFBR2, or HPK1 (Labanieh & Mackall, 2023). Apart from transcriptional reprogramming, engineering tethered versions of cytokines such as IL-7 and IL-15 to the cell surface is also used to improve persistence of CAR T-cells. Other approaches include alternative intracellular signaling, which is known to be sustained at a higher level from a TCR compared to a CAR (Haradhvala et al., 2022). Fusion of an scFv to a TCR results in a CAR that is structurally very different from the first three generations. This is done differently with HIT or TRuC technologies (Figure 4) - the latter of which is referred to as the fourth generation of CAR (Chmielewski & Abken, 2015) – in order to overcome the problem of low sensitivity and low response to antigens (Labanieh & Mackall, 2023). With regard to increased specificity, combinatorial CAR systems such as SynNotch, LINK CAR (Figure 4), or aforementioned bivalent CARs with scFv or nanobody receptors (Safarzadeh Kozani et al., 2022) offer responses to multiple antigens, which allows for new approaches to the central problem of CAR specificity.

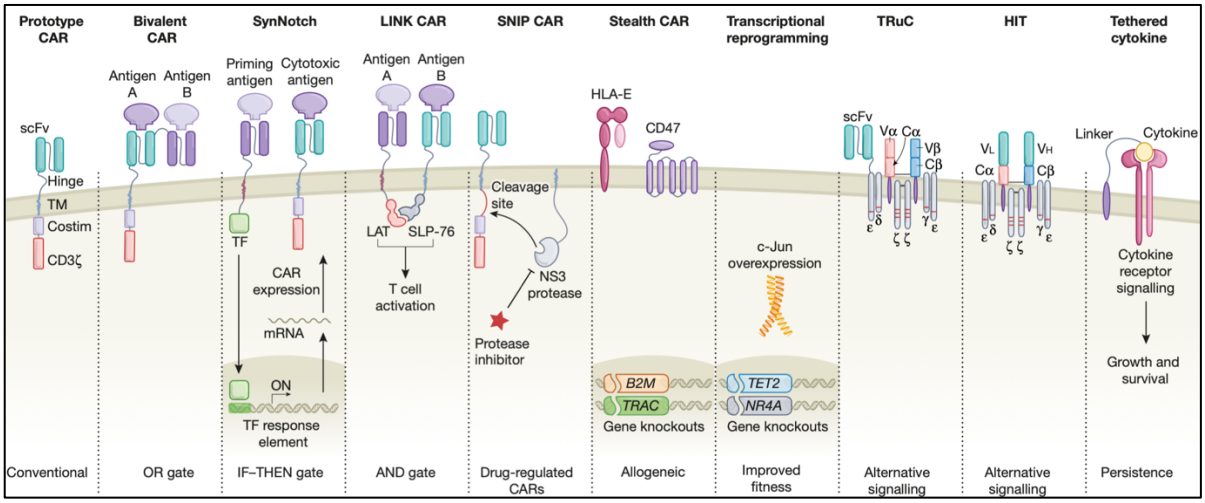

Figure 4 – Overview of novel CAR technologies. Technology names are shown at the top of the figure, respective novel features at the bottom. TF = Transcription Factor, TM = Transmembrane Domain. Adapted from (Labanieh & Mackall, 2023).

Combinatorial technologies and specificity

Given the novel technologies in CAR receptors and their intracellular signaling, we can now start to think about combinations of antigens present on the cell instead of searching for a single highly specific target, potentially boosting CAR specificity through a logic system. Combinatorial approaches to antigen recognition have been of interest for a few years (Lee & Wong, 2020). From the simple example of bi- or multivalent CARs that allow CAR T cells to recognize multiple different target cells or mitigate antigen escape, to more complex engineered behavior where multiple antigens need to be present (or absent) to evoke a response. This is why these receptors are referred to as "OR","AND" and "IF-THEN" gates (Figure 4). Combinatorial or Boolean logic can (by definition) only be achieved with multivalent CAR cells. For example, bi- or multivalent molecules that can be thought of as "OR-gates" since they will induce a response when antigen A, B, or both are bound (Figure 4). There are also receptors that would execute "IF-THEN" logic by release of a transcription factor upon antigen recognition. The ability to recognize multiple targets and to respond with transcription factors, albeit useful against antigen escape and orchestrate CAR cell behavior respectively, does not result in higher specificity. An approach that is more promising for higher specificity is "AND" logic, where activation only occurs upon binding two different antigens. For example, in LINK CARs the intracellular CD3ζ domain has been replaced with LAT and SLP-76, which propagate a signal after phosphorylation (Figure 4). This successfully repurposes the internal signaling of T cells, yielding lower off-target toxicity as a result (Tousley et al., 2023). Approaches like this offer a new search space of potential treatment targets consisting of antigen combinations which increases exponentially based on the number of different targeting domains involved in the signaling cascade.

Combinatorial behavior can also be achieved with nanobodies using bispecific VHH-based CARs, enabling faster development. Nanobodies have the advantage of being more flexible due to their small size and low immunogenicity (Safarzadeh Kozani et al., 2022). They are also smaller, more easily produced molecules with a physically extended CDR3 region that allow increased reach for potential antigens. Bioinformatics methods have allowed for improved design of anti- and nanobody structures with molecular modeling. For example, there are computational methods for modeling the docking of CAR with BCMA (Moazzeni et al., 2023) or to other novel targets (Mohanty et al., 2019). Molecular modeling can be done for any potential receptor-ligand combination, therefore *in silico* development of targeting domains is also possible such as was done with CD20 as a target for non-Hodgkin lymphomas (Poustforoosh et al., 2023). For combinatorial antibodies (such as bispecific nanobodies) where affinity optimization needs to be done for several receptor-ligand combinations simultaneously, *in silico* predictions might differ from those corresponding to these receptors separately – cross-linking of a bispecific nanobody, or one blocking the other's epitope once bound. If these methods are further developed an increased number of antigens to consider and an increasing number of receptors (and intracellular mechanisms) could require an integration of bioinformatics methods that model the affinity of CAR cells to targets. For example by combining molecular modeling/docking simulations (e.g. HDock) that are done now for single antibody-antigen pairs (Moazzeni et al., 2023), but with larger multi-specific molecules such as bispecific nanobody-based CARs to combinations of known cancer (neo-) antigen combinations like BCMA and CD19 (table 1) or CD20 and CD30 (Mohanty et al., 2022). However, combinations of 2-3 antigens strike an effective balance between precision and recall (Dannenfelser et al., 2020), in which case the modification of existing *in silico* methods

might be easy to accomplish. After designing the external domain with these simulations, analysis of the signaling cascade of the CAR molecule is required and can be done, for example by using the STRING database of protein interaction networks (Mohanty et al., 2022; Szklarczyk et al., 2015).

Alternative adoptive cell therapies

CAR technology is versatile and is therefore applied using several different types of immune cells. The established technology of the first three generations has mostly been developed with αβ T-cells, with other T cells (namely T regulatory cells (Haradhvala et al., 2022)) being part of the treatment as a by-product of CAR T-cell production. However, major steps have been made in using NK-cells (Dagher & Posey, 2023; Laskowski et al., 2022) and γδ T-cells in CAR therapies (Jhita & Raikar, 2022; Saura-Esteller et al., 2022) in the recent years. Furthermore, the older technology of TCR transduced T-cells (TCR T, another allogenic immunotherapy (He et al., 2019)) is also still evolving and could be the method of choice for some (neo)antigens.

TCR T-cells and the immunopeptidome

Developments in the field of immunopeptidomics (which uses mass spectrometry to identify HLA-presented peptide epitopes) have allowed scientists to discover many cancer-related antigens. These are categorized in two groups: canonical tumor neoantigens which originate from protein-coding mutations, and noncanonical tumor neoantigens from sequences outside protein-coding regions. The first group has been known and explored quite thoroughly. However, they have not been widely used clinically as the neoantigens found in protein-coding regions seem to be patient specific and thus require development of personalized therapy. Luckily, recent developments and bioinformatics approaches now enable many novel noncanonical epitopes to be identified and added to immunopeptidomics databases (Chong et al., 2022). Together with the surfaceome (proteins expressed on the cell surface), the immunopeptidome makes up all proteins on the outside of a cell, which is where one hopes to find CAR targets. The MHC-peptide complex is, thus, a potential target for CAR (Wu et al., 2021), however previous research recommends that targets presented by MHC class I should be used in designing TCR-T-cell therapies (He et al., 2019). TCR T-cell therapy takes a similar approach to CAR T-cell therapy: both methods currently use allogenic T-cells from a patient transduced with genes expressing a new receptor (a TCR in the case of TCR T-cell therapy). However, TCR T cell therapy has some advantages compared to CAR T: *in vivo* TCR signaling is more intense and retains at a higher level than CAR signaling (Wu et al., 2021). TCR T-cells also require fewer peptides to be present on an antigen presenting cell for activation (~100) compared to CAR (>1000 antigens per cell) (Labanieh & Mackall, 2023). TCR-T is an older technology but can still be used when targeting tumor neoantigens (peptides) that are presented by the MHC complexes. The bioinformatics methods for prediction of peptide presentation by MHC were developed using neural networks and large datasets, and predictions are now offered with tools like NetMHCpan (Reynisson et al., 2020). A predicted MHC-peptide combination can then be used to design TCR-T cell treatments. Due to difficulties in predicting the interaction between TCR and peptide-MHC this occurs *in vitro* with patient-derived T-cells. Epitope-specific T-cells are then clonally expanded and used in therapy. Sequencing developments and murine models have helped this process (Baulu et al., 2023). Hypothetically, bioinformatics methods that can simulate VDJ recombination and consecutive TCR recognition to peptide-MHC would be of great help in this process.

It might also be possible to design a CAR to target a specific peptide-MHC complex after it has been predicted to occur frequently. The bioinformatics methods here are similar to nanobody target search: molecular modeling techniques can determine binding affinity between the CAR and antigen. Currently there is a separation of the CAR- and TCR- T-cell technologies where (depending on the target) TCR T can be more beneficial than CAR T. The significant challenge in neoantigen targeting persists, as the mutations remain highly individualized to each patient. However, finding methods to easily produce patient specific receptors could unlock the immunopeptidome as a target for CAR and result in increased specificity for (combinatorial) immune therapies. Effects of MHC targeting by CAR can be seen from CAR T-cells targeting pancreatic MHC class II-peptide complexes to modulate autoimmune diabetes *in vitro* and for several weeks post-treatment *in vivo* for a murine diabetes model (Zhang et al., 2019). This was done with CD4+ (cytotoxic) CAR T-cells with a murine antibody in the extracellular domain.

CAR NK-cells

Natural Killer (NK) cells are well suited for tumor cell targeting and elimination because this is part of their original function in the immune system – in healthy people they frequently prevent the occurrence of cancer. This makes them interesting candidates for modification with CAR for immunotherapy purposes. NK cells are recognizable by CD16 and CD56 surface proteins and were historically viewed as part of the innate immune system, but their complex integration of inhibitory and activating signals and their role in cytokine production (which mediates adaptive immune response) indicate a role in adaptive immunity as well (Islam et al., 2021; Vivier et al., 2011). They can be further classified based on CD56 density; high density NK cells show more cytokine and chemokine production (e.g. IFN-γ and TNF-α) and dim cells show more cytotoxic properties and express Ig-like receptors. NK cell response is regulated via inhibitory and activating signals. Upon activation NK cells play a role in immune checkpoint inhibition (M. Khan et al., 2020) which can be very beneficial in a immune suppressed TME. Activation of NK cells can occur by recognition of antibody-covered cells using CD16 or from target cells lacking MHC class I (inhibition occurs when HLA expression is normal).

The KIR family of surface molecules (Killer cell Immunoglobulin-like Receptors, or: CD158) which regulates the NK cell response to MHC type I molecules. Because of how this response works, CAR NK-cells can be less likely to induce graft vs host disease (GVHD) than T-cells - a step towards development of off-the-shelf immune therapy. KIRs contain several immunoglobulin domains on the outside of the cell membrane, and a short or long intracellular tail corresponding to activating or inhibiting function respectively. KIRs are encoded in the Leukocyte Receptor Complex (LRC), which is polymorphic like the MHC complex and are used to match donors to recipients in the immuno-polymorphism database (Robinson et al., 2013). Due to stochastic expression (Mehta & Rezvani, 2016), KIR activity varies among NK cell lineages which allows for a varied response to foreign cells with some NK cells being more prone to initiation of GVHD than others (Islam et al., 2021; Thielens et al., 2012). This can be used to generate an NK cell lineage that does not induce GVHD. Cases where the donor NK cells are HLA matched and KIR mismatched can even result in a higher anti-tumor effect than a patient's own NK cells due to a stronger response to "missing self" (Mehta & Rezvani, 2016) while preventing GVHD.

Moreover, several disadvantages related to T-cell usage with CAR can be negated by using NK cells. Firstly, CAR NK cells retain their innate cytotoxic properties when modified, and thus affect cancerous cells that have evolved antigen escape (Figure 5). This property is most useful in solid tumors where cancer cells located closely together have antigenic heterogeneity (Dagher & Posey, 2023). Secondly, a major downside of CAR T treatments is the aforementioned risk of Cytokine Release Syndrome (CRS), which can be fatal. So far, this risk seems decreased with CAR NK cells compared to CAR T in clinical trials due to their more diverse cytokine signaling mechanism. In addition to this, NK cells are relatively short-lived (~ 2 weeks), and any toxic side-effects should be mitigated in this timespan due to natural decay of the cells. This does mean that NK-cell treatment requires more frequent administration (Mehta & Rezvani, 2016; Pan et al., 2022). Finally, when keeping in mind the potential for large-scale production of allogenic therapies, NK cells are relatively easily clonally expanded from e.g. cord blood, pluripotent stem cells (iPSCs) or other donor sources (Dagher & Posey, 2023; Islam et al., 2021). Given the relatively new development of CAR NK cell technology compared to CAR T and especially TCR T in immunotherapy, there are still many enhancements to be made that are expected to increase the utility of these cells. An example of which is PDZ NK cells (Figure 5D) that have an enhanced immunological synapse resulting in prolonged survival and more effective elimination of tumor cells, also in solid tumors (Chockley et al., 2023; Dagher & Posey, 2023).

CAR γδT-cells

Another cell type that functions in between adaptive and innate immunity that also has potential in CAR based immunotherapy are γδ T-cells. These represent 1-10% of the CD3+ T cell in humans and are distinguished by expression of Vγ and Vδ chains as opposed to alpha and beta chains in other T cells (Saura-Esteller et al., 2022). Functionally, these cells can eliminate target cells independently of MHC-peptide resulting in similar advantages as NK cells have. Different from NK cells, the γδT cells target intracellular phosphorylated metabolites that can accumulate in the tumor environment due to dysregulation (Saura-Esteller et al., 2022). γδT cells are categorizable in Vδ1 and Vδ2 subsets; the former occurs mostly in tissue and the latter mostly in circulation. Vδ2 also have more pro-inflammatory features. Initial studies showed high safety due to mild immune-related adverse events, however their clinical effect was also limited. This has not held back the field; therapy based on γδT-cell transfers (without CAR) as well as CAR γδT-cell immune therapy are undergoing clinical trials, with several different targets for the CAR involved (Table 1). Overcoming issues of effectiveness is still a work in progress, but specifically Vδ1 and Vγ9Vδ2 T-cells are promising avenues for CAR therapy (Saura-Esteller et al., 2022).

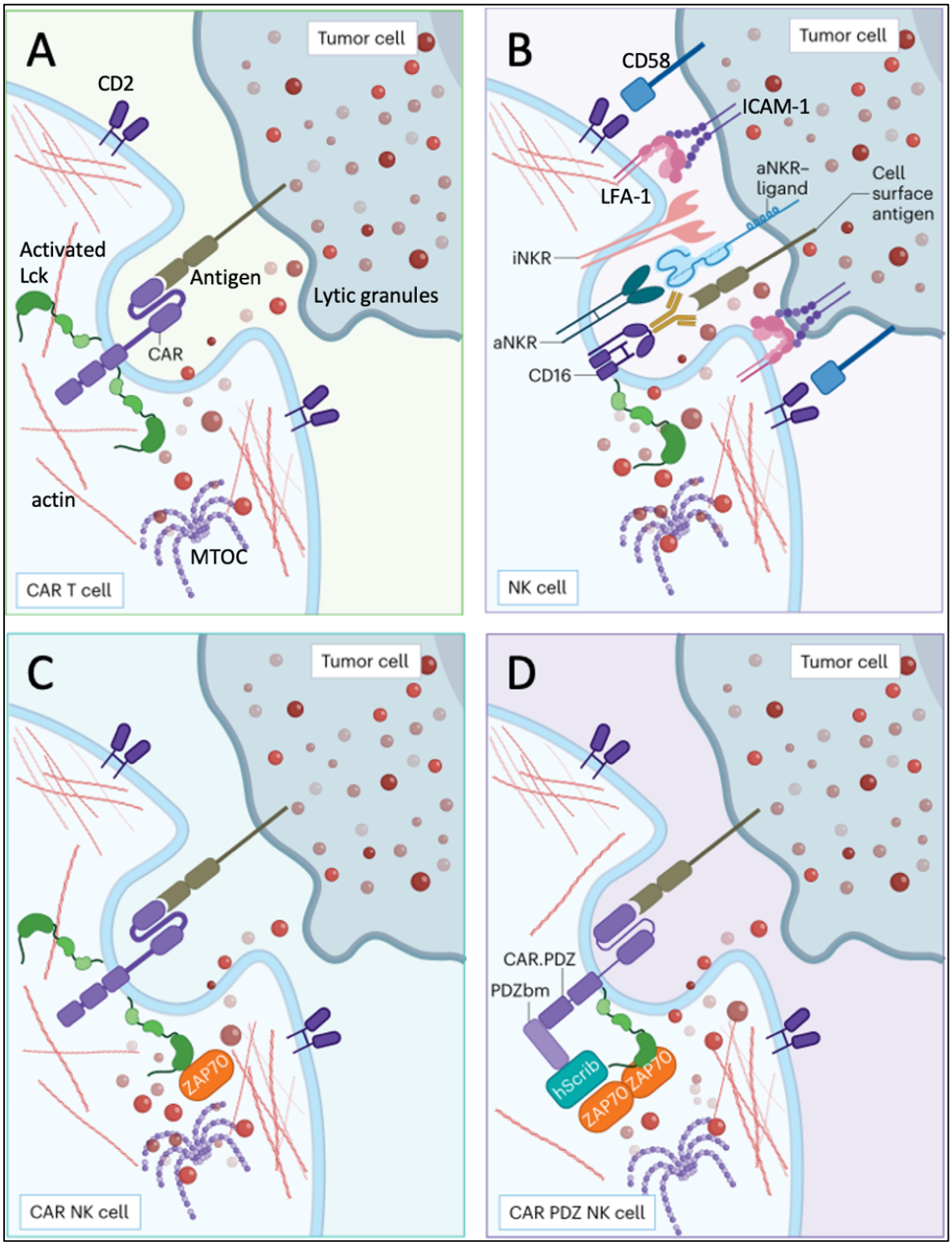

Figure 5 – CAR T- and CAR NK-Cell modes of action. A) T-cell recognizing a tumor antigen and giving a regular T cell response. MTOC = Microtubule organizing center, Lck = lymphocyte-specific tyrosine kinase. B) CD16-driven NK cell response to a tumor cell. aNKR = activating NK cell Receptor, iNKR = inhibiting NK cell Receptor. C) CAR NK cell tumor recognition of tumor cell without antigen escape. D) CAR PDZ NK cell with enhanced immunological synapse due to hScrib scaffolding protein incorporation. Adapted from (Dagher & Posey, 2023).

Discussion

CAR T-cell therapy has been through many improvements over the recent years and has earned a place among approved therapeutics for specific malignancies as an addition to classical chemotherapy and radiation therapy. However, CAR-based therapies remain an area of active research with many varieties in different stages of development and ongoing fundamental research to the biology behind them. This ranges from improvements in clinical efficacy and safety to a search for technologies that increase CAR specificity or allow for 'off-the-shelf' immune therapy to replace the more costly autologous approach. Here we discuss different novel applications of CAR such as nanobody-based extracellular domains (Safarzadeh Kozani et al., 2022), usage of alternative immune cells (Pan et al., 2022; Saura-Esteller et al., 2022; Wang et al., 2024) and additional variants of the signaling cascade (LINK CAR, SNIP CAR, etc.) (Labanieh & Mackall, 2023). Ongoing research on CAR therapy requires the usage of several types of omics data and bioinformatic modeling (Yang et al., 2023), which could require adaptation or integration as CAR therapies mature.

With regards to alternative cell types, we discussed CAR NK- and CAR γδT-cells showing promise for future development of therapeutics. CAR γδT-cells have innate anti-tumor capabilities through targeting of lipid metabolites, but low clinical efficacy due to their inherent less-aggressive immune response to cancer cells. CAR γδT-cells lag behind in development compared to CAR T and TCR T which are already used in far-developed clinical trials and approved therapies (table 1). CAR NK cells also leverage their innate tumor killing capabilities, which can be beneficial in cases where the tumor evolves antigen-escape as there remains a 'missing self' response to tumor cells lacking MHC I. Once activated, NK cells play a role in immune checkpoint inhibition which is beneficial in the TME but further research is required to improve the understanding of TME dynamics (M. Khan et al., 2020; Wang et al., 2024). To understand these dynamics we need further research that combines omics data from multiple sources (Yang et al., 2023), and potentially include dynamic models of the interactions between the cells. Donated NK cells can respond more effective than a patient's own NK cells if there is a KIR mismatch (Mehta & Rezvani, 2016) while keeping a reduced risk of GVHD if the HLA between donor and patient is matched. However, NK cells (as opposed to T cells) require signaling pathways to be added during transduction (Dagher & Posey, 2023). Despite the requirements for further development and testing in clinical trials there remains a lot of promise in the use of CAR NK-cells for the next generation of immune therapy, especially regarding off-the-shelf application due to their universal anti-tumor properties and the treatment of solid tumors due to their role in breaking up the TME (Wang et al., 2024).

High specificity for malignant cells is a crucial feature of immune therapy. A major issue of chemo- and radiation therapy is that they also affect healthy cells, resulting in varying side-effects. Immune therapy also has (sometimes major) side effects, but these are potentially more manageable if the toxic effects of modified immune cells are limited directly to malignant cells (Ying et al., 2019) and/or the TME through fine-tuned recognition (Mohanty et al., 2019). This can be achieved by searching for specific cancer neo-antigens, which are often unique to a patient, or by searching for differentially expressed antigens. Finding these kind of targets for immune therapy is an ongoing effort, the search space of which could be widened with the help of different omics methods (Yang et al., 2023). Bioinformatics plays a major role in validating and searching for targets, from interpreting different types of omics data to modeling the specific interaction between a CAR and an antigen. The greatest

challenge with using tumor neoepitopes is that they are often unique for a patient, and therefore not suitable as therapeutic targets. A major step towards more specific targeting might not be the result from single antigen search but from engineering novel features based on 3rd generation CAR systems. Bivalent receptors using nanobodies (Safarzadeh Kozani et al., 2022) and intracellular signaling pathways that are introduced to transduced cells alongside CAR could lead to logic gated CARs. These allow for a new approach to antigen search: from one antigen that is highly specific to several less-specific antigens that need to occur in a combination that can also reach high specificity (Lee & Wong, 2020). Finding relevant combinations of cell surface antigens (optionally including the immunopeptidome) then becomes a new challenge where new bioinformatics approaches are required that integrate information about exposed proteins on all patients' cells with that on cancer cells. This will require integration of already used approaches for antigen search: cancer epitopes (or lack thereof) and predicted MHC-peptide epitope combinations should be searched for common occurrence in certain cancer types. Candidate combinations of 2-3 (Dannenfelser et al., 2020) epitopes can then be tested with molecular modeling against nanobodies or scFv with the required signaling cascades. The search space of antigen combinations and the surfaceome is large, and if we let go of the limitation of 2-3 antigens there will be an exponential increase that might require heuristics such as machine learning to search for these combinations more effectively.

Ideally, novel CAR-based immune therapies are off-the-shelf and therefore derived from donor sources such as cord blood. The reason for this is that there is a very high cost to designing new therapies for a small part of the population. There are many steps required to get to the point of widely-useable donor cells with immunogenicity of the CAR and of the allogenic immune cells as the main caveat. One way to reduce immunogenicity in autologous CAR cells is by using nanobodies, which have a lower immunogenicity, higher specificity, and are easier to design and obtain than the often used scFv domains (Safarzadeh Kozani et al., 2022). These properties are already leveraged in the development of novel CAR-based therapeutics that are still based on patient derived immune cells. Viral transduction allows for the combination of different receptors and signaling cascades to be engineered in different immune cells to enhance the immune system (Labanieh & Mackall, 2023). For future developments, we propose to consider the full width of currently developing variants of CAR technology and different adoptive cell therapy approaches when designing new CAR immune therapies to solve specificity, immunogenicity, and the requirement of patient derived cells at the same time. Especially promising avenues are the usage of Nanobody extracellular domains and CAR NK-cells that are HLA matched to the patient. Given the amount of development in this field there is reason to be very optimistic about the next generations of immunotherapy that leverage these technologies (Pan et al., 2022; Wang et al., 2024).

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Footnote: Statement on the use of generative A.I. and corresponding reference

For this project ChatGPT version 3.5 was used for broad initial research. Example prompts include but are not limited to ‘explain CAR-T cell therapy’ or ‘is the Leukocyte Receptor Complex as complex as the MHC?‘. The full prompts and history are available in the history of the author’s ChatGPT account. ChatGPT was not used to write or improve paragraph text of this writing assignment. ChatGPT was used to generate the list of acronyms found in the description of table 1, due to the speed in typing and checking the names. The full prompt can be found here: https://chat.openai.com/share/5850c8ed-248d-46b5-8b3f-d9f3be9fe81d

Reference: OpenAI. (2023). ChatGPT (Mar 14 version) [Large language model]. https://chat.openai.com/chat