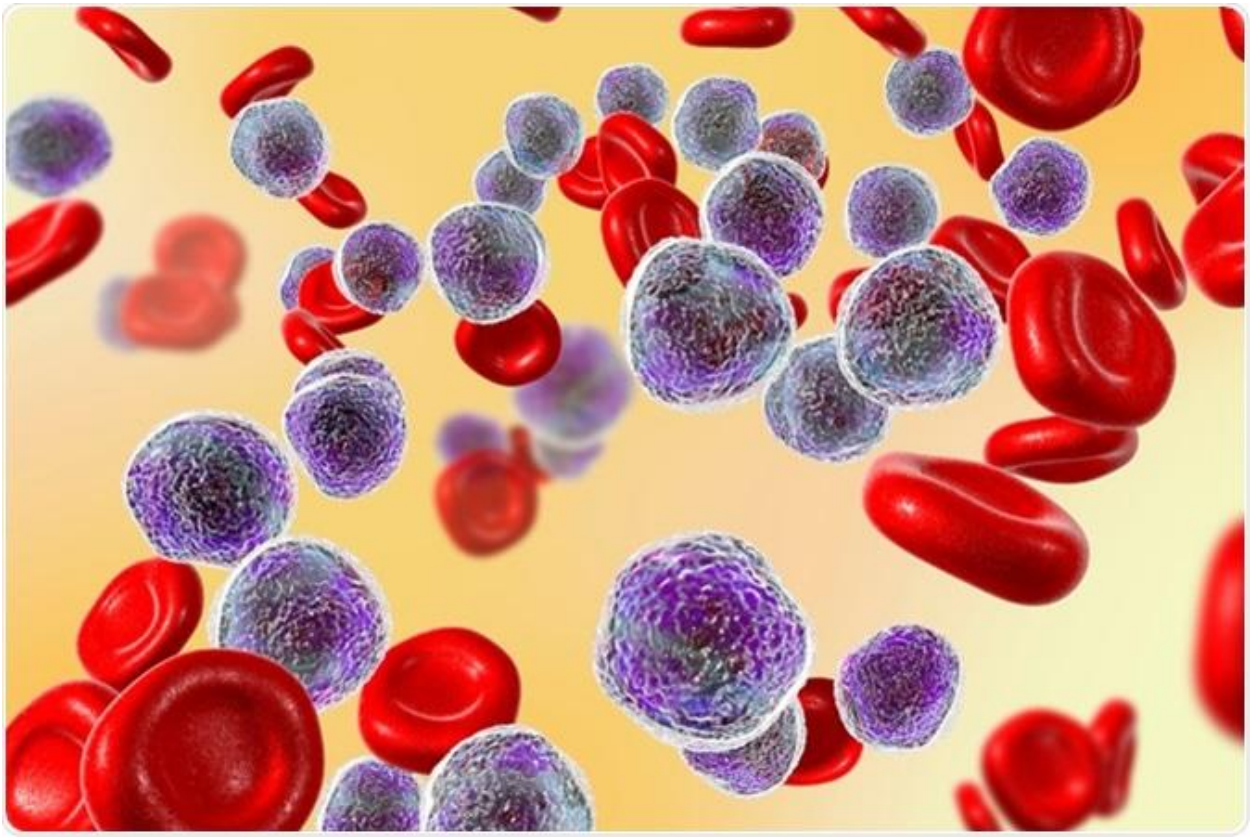
CAR T-cell therapy

Applications in hematologic malignancies



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Abstract

Hematopoiesis is the development of blood cells, when this fails hematologic malignancies occur. Hematologic malignancies include lymphomas, myeloid and lymphoid leukemias. Standard treatment for these diseases is chemotherapy, but the problem with this is high relapse rates and side effects. Alternative therapy options are needed to increase survival rates and reduce the amount of relapses. Due to the important role of the immune system in cancer, an alternative treatment that has gained interest in the last couple of years is immunotherapy. A promising form of immunotherapy is the use of CAR T-cells, which are chimeric antigen receptors linked to cytotoxic T-cells. The CAR can specifically target tumor-associated antigens, and thus eliminate tumor cells via activation of the attached T-cell. Many studies and clinical trials have examined the potential therapeutic effects of CAR T-cells in lymphomas and lymphoid leukemia. In myeloid leukemia, only a few studies and clinical trials have been conducted to examine the therapeutic effects of CAR T-cells in this disease. Overall, CAR T-cells provide a very promising alternative treatment option for lymphomas and lymphoid leukemias, while they are harder to apply in myeloid leukemias, due high heterogeneity in markers among patients. Although CAR T-cell therapy can be applied in lympomas and lymphoid leukemias, there remain serious disadvantages of current CAR T-cell therapies, such as resistance, side-effects and individual engineering. However, more research could increase efficacy and specificity, and decrease resistance and side effects. This will expand knowledge and will help to increase the therapeutic value of CAR T-cell therapy even more.

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Introduction

Hematopoiesis

Hematopoiesis, which is the process of blood cell development, starts during embryonic development and carries on throughout the whole lifespan of an organism in order to produce and maintain the blood volume and cells. In humans, two waves of hematopoiesis occur; the primitive and definitive wave. The primary wave occurs only during early embryonic development to quickly form red blood cells in order to promote rapid growth in this stage of life. The progenitor cells for this primitive wave are not renewable. The definitive wave involves hematopoietic stem cells (HSCs). These stem cells are found in the bone marrow and are multipotent, which means that they can renew themselves, as well as differentiate into different blood cell types (Jagannathan-Bogdan & Zon, 2013). Under the influence of different factors, the HSC differentiates into either a lymphoid or a myeloid cell lineage (Figure 1). The lymphoid lineage eventually gives rise to lymphocytes, which is a subtype of leukocytes (white blood cells). The myeloid lineage will differentiate into erythrocytes (red blood cells), platelets and the remaining types of leukocytes (Alberts et al., 2002).

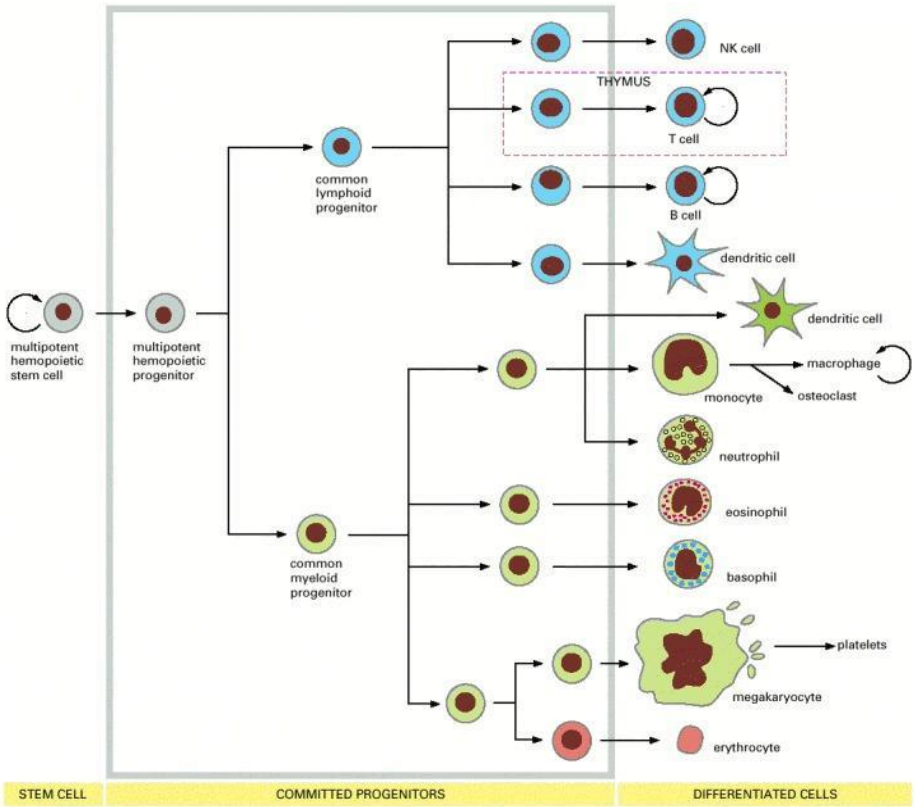

Figure 1: Schematic overview of hematopoiesis. The multipotent hematopoietic stem cell is self-renewable and can differentiate into all blood cell lineages (Alberts et al., 2002).

Malignant hematopoiesis

Hematopoiesis can fail in different parts of the process, which causes hematologic malignancies. The two types of hematologic malignancies are leukemias and lymphomas, affecting 437,033 and 589,580 people respectively worldwide in 2018. Besides, leukemia is the most common type of cancer in children (American Institute for Cancer Research, 2019).

Leukemia affects the leukocytes and starts either in the myeloid or in the lymphoid cell lineage. Therefore, these two types are referred to as myeloid and lymphoid leukemia. For both myeloid and lymphoid leukemia there is an acute and chronic form. The chronic type of leukemia progresses more slowly than the acute form (De Kouchkovsky & Abdul-Hay, 2016). The most common symptoms of acute leukemias are fever, pale appearance and bleeding disorders. Enlarged spleen, liver and lymph nodes also occur often, but are more common in acute lymphoid leukemia compared to acute myeloid leukemia. Patients with chronic leukemia often do not experience symptoms, therefore it is often detected during regular blood sampling (Shahab & Raziq, 2014).
In acute myeloid leukemia (AML), chromosomal rearrangements and somatic mutations occur that alter the myeloid progenitor cells and HSCs into poorly differentiated myeloid cells, resulting in replacement of normal leukocytes by leukemic leukocytes. A frequent cause of AML is a translocation of chromosome 8 and 21, resulting in the formation of the RUNX1-RUNX1T1 product (De Kouchkovsky & Abdul-Hay, 2016). Additionally, like normal hematopoiesis, AML includes different cells that originate from the leukemic stem cell (LSC) (Walter et al., 2012). LSCs have many characteristics in common with normal HSCs, like the ability to self-renew and repopulate, a mostly quiescent state, heterogeneity within stem cell compartment, and similar immunophenotypes. Simultaneously, it can be distinguished from HSCs because they express some markers differently. It is important that these LSCs are targeted, because they can cause a relapse when they are still present after therapy (Siveen et al., 2017).
In chronic myeloid leukemia (CML), chromosome 22 is often transformed into the Philadelphia chromosome in HSCs (Xie et al., 2019). This Philadelphia chromosome is present in 95% of all cases (Sawyers, 1999). The transformation is caused by the fusion of the ABL gene from chromosome 9 to the BCR gene on chromosome 22. This results in the BCR-ABL protein, which promotes cell survival and proliferation, and is thus responsible for the malignant hematopoiesis (Bartram et al., 1983; Xie et al., 2019).
Acute lymphoid leukemia (ALL) is induced by abnormal expression of proto-oncogenes, chromosomal translocations, and hyperdiploidy in more than 50 chromosomes. These mechanisms change regulatory processes in apoptotic pathways, proliferation, and self-renewable capacities of the B- or T-cells (Pui et al., 2004). It is unclear if there are also LSCs in ALL. There are controversial findings described in literature (Bernt et al., 2009).
In chronic lymphoblastic leukemia (CLL) there is a deletion at 13q14.3, which is a chromosome 13 deletion, in 50% of the cases. This contains two microRNA genes, which regulate gene expression. These microRNAs are either deleted or down-regulated in CLL (Chiorazzi et al., 2005).

Lymphomas are tumors affecting lymphocytes. There are two types of lymphoid tissue: the central lymphoid tissue, which includes the bone marrow and the thymus, and the peripheral

lymphoid tissue, which consists of blood, spleen, lymph nodes and the mucosa. Lymphomas are categorized in two groups: Hodgkin and Non-Hodgkin lymphomas. Hodgkin Lymphoma (HL) affects peripheral lymph nodes. This type of lymphoma is characterized by unique malignant cells, called Hodgkin and Reed-Sternberg (HRS) cells. These cells are formed from B-cells, in which somatic mutated Ig heavy- and light-chain transformation occurred (Küppers et al., 2012). The other lymphomas are collectively referred to as Non-Hodgkin lymphomas. About 85-90% of all Non-Hodgkin lymphomas begin in the B-lymphocytes. The remaining arise in T-lymphocytes or NK-cells. It can start at different steps in the process of differentiation into these lymphocytes. In these lymphomas, chromosomes are translocated, which is the main driver for proto-oncogene activation and the formation of cancer cells (Evans & Hancock, 2003). Clinical presentation for both types of lymphomas is abnormal lymph nodes, enlarged spleen, fever, paleness, and weight loss (Evans & Hancock, 2003; Küppers et al., 2012).

Standard therapy

Chemotherapy is the common standard treatment for leukemia. However, chemotherapy is not curative in most cases. Data from the National Cancer Institute show that the 5-year relative survival rate is 28.7% for AML, 68.8% for ALL, 72.7% for non-Hodgkin lymphoma and 87.4% for Hodgkin lymphoma (National Cancer Institute, 2019). To illustrate, a large cohort study of Röllig et al., including 1557 AML patients, showed that patients often relapse after the first treatment (Figure 2A-2D). Additionally, the overall survival rate of patients younger than 60 years old is 10-70% after 5 years, and for patients older than 60 years this was 5-40% after 5 years (Figure 2E & 2F). Because patients are subcategorized based on genetic screenings in favorable, intermediate I, intermediate II or adverse groups, this study also demonstrates the importance of genetics in response to the treatment. The adverse group had poorer outcomes than the other groups after chemotherapy (Röllig et al., 2011). Similar data has been found for other types of leukemia and lymphoma (National Cancer Institute, 2019).

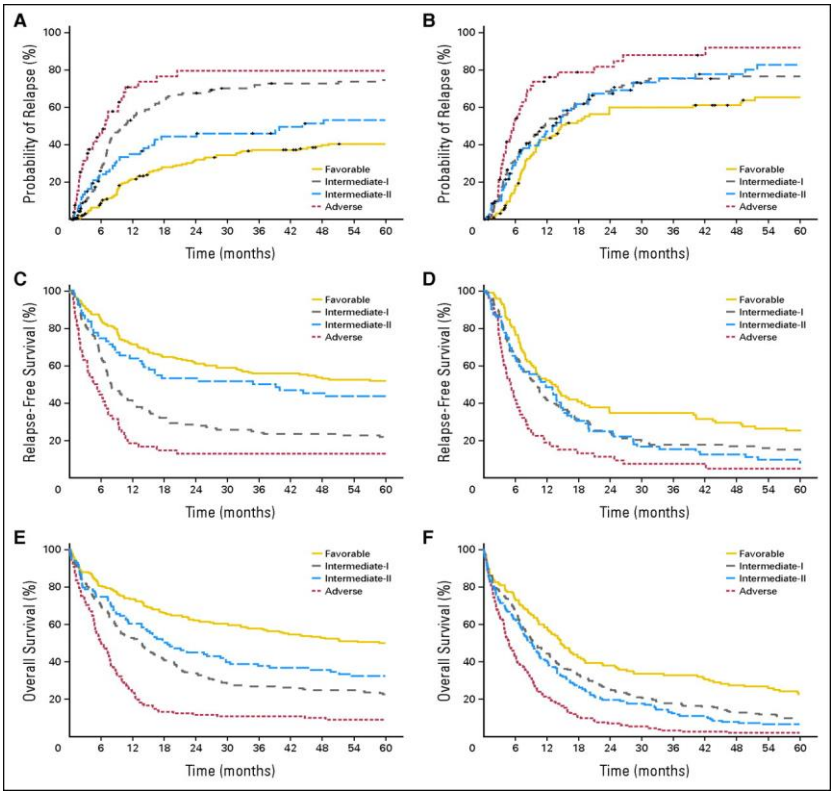

Figure 2: The outcome of young and old AML patients after first chemotherapy treatment, classified into four genetic groups. 2A: Probability of Relapse in young patients (under 60 years old). 2B: Probability of Relapse in old patients (above 60 years old). 2C: Relapse-Free Survival in young patients. 2D: Relapse-Free Survival in old patients. 2E: Overall Survival in young patients. 2F: Overall Survival in old patients (Röllig et al., 2011).

Besides the high relapse rates and low overall survival rates, chemotherapy is also damaging to healthy cells. Chemotherapy targets fast-growing cells, which includes cancer cells. However, there are several cell types in the body that grow quickly, which are not cancer cells. Examples are hair follicles and epithelial cells in the intestinal and oral mucosa. These cells are also likely to be damaged by the chemotherapy, resulting in serious side effects, such as hair loss, loss of appetite and nausea (Acheampong et al., 2018).

Aim

Regular cancer therapies are often not satisfactory in leukemias and lymphomas. Patients often relapse and these hematological malignancies still have high mortality rates, in particular in AML. Chemotherapy is also not specific, resulting in various side effects. Therefore, other therapies are being developed. An interesting and promising therapy is immunotherapy. The patient's own immune system could be used to target the tumor cells in the patient. For both leukemia and lymphoma, there seem to be possibilities for this. This thesis will focus on CAR T-cell therapy, which is a type of immunotherapy, and its possibilities in lymphomas, myeloid and lymphoid leukemias. In order to do so, the general principle of immunotherapy and CAR T-cells will be explained. Then, several studies on CAR T-cell therapy in different (pre-)clinical stages in hematologic malignancies will be discussed. Eventually, the main question will be answered: "In what way can CAR T-cell therapy be applied in hematologic malignancies as alternative for standard therapies?"

Immunotherapy

The role of the immune system in cancer

The immune system is a system that recognizes particles as self or foreign, and can thus eliminate the pathogens and malignant cells. The immune system plays two roles in cancer: on one hand, it suppresses tumor growth by eliminating dangerous cancer cells. On the other hand, it promotes the progression of tumor growth by immunoediting (Naijng & Hajjar, 2018).
The main function of the immune system in cancer is the prevention, in which the immune system plays three important roles. First of all, it protects humans from viral infection and thus prevents virus-induced cancer growth, like human papillomavirus-induced cervical cancer. Secondly, it prevents the formation of an inflammatory condition that can facilitate tumorigenesis. Finally, it eliminates tumor cells because cancer cells often express ligands for receptors on cells of the innate immune system (Schreiber et al., 2011). Many cells of the immune system play a role in the defense against cancer (Figure 3) (Borghaei et al., 2009).

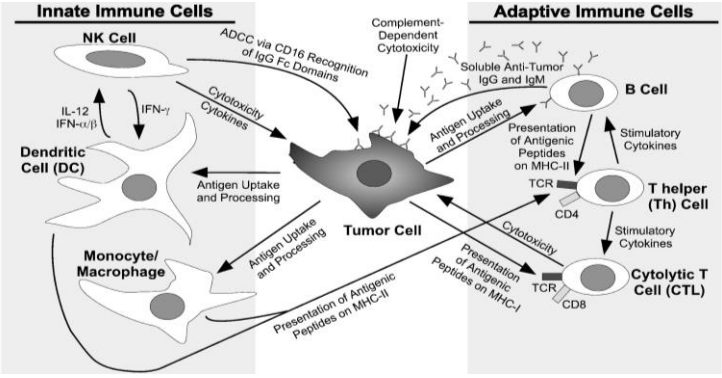

Figure 3: Cells of the immune system in response to cancer cells (Borghaei et al., 2009).

The immune system also contributes to the promotion of tumor growth by immunoediting. Immunoediting is a process that consists of three phases, of which the elimination phase or immunosurveillance is the first phase. In this phase, the immune system recognizes the differences between self and transformed self cells, which are malignant. The transformed self cells express antigens that are different from non-transformed cells, which are then recognized by the innate immune system. These antigens are called tumor-associated antigens (TAA) (Schreiber et al., 2011). The innate immune system presents these TAAs on so-called antigen-presenting cells (APCs) to the adaptive immune system by using a special presentation molecule called the major histocompatibility complex (MHC). This process is called antigen presentation. MHCs are highly polymorphic, meaning that every person has different MHC molecules. In humans, MHCs are also called human leukocyte antigens (HLAs). In the second phase of immunoediting, the equilibrium phase, the immune system is able to eliminate the malignant cells. At the same time, however, this phase gives rise to new tumor cell variants with reduced immunogenicity that can escape from the immune system. This results in a state of equilibrium. In the last phase, the escape phase, tumor cells find more ways to escape from immunosurveillance, such as downregulation of MHC, so the antigen presentation to the adaptive immune system is decreased (Naing & Hajjar, 2018). Both roles of the immune system in cancer are shown in Figure 4 (Schreiber et al., 2011).

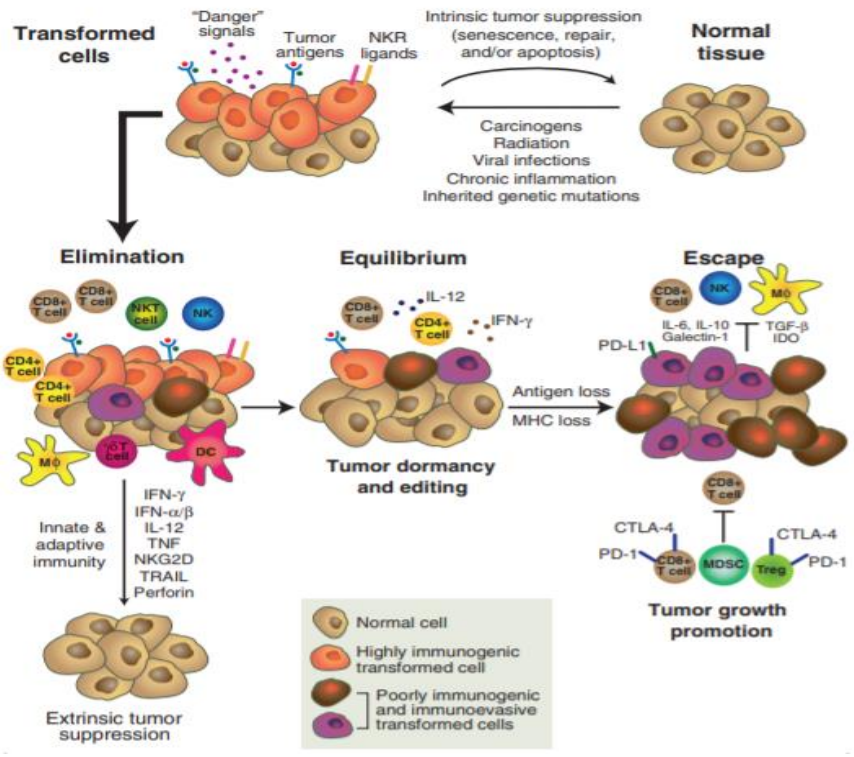

Figure 4: The dual role of the immune system in cancer. On the one hand it eliminates cancer cells, while on the other hand it contributes to tumor growth promotion (Schreiber et al., 2011).

The most important role of the immune system is cancer. Therefore, it could also be used to treat cancer. The use of the immune system to treat cancer is called immunotherapy. The goal of this type of therapy is to recognize cancer cells as dangerous and activate the immune system in order to attack and eliminate these cells (Facinelli, 2010). Currently, it is known that

immunotherapy can be mediated through both the innate and the adaptive immune system. From these two systems, the cytotoxic T-cells are seen as the most important aspects of the immune response against cancer (Zhang & Chen, 2018).

Types of immunotherapy

There are several ways to use the immune system in order to eliminate tumors. All the options are classified into either passive or active immunotherapy. In active immunotherapy, it is attempted to stimulate the immune system to attack tumor cells. In passive immunotherapy, drugs are used to improve the already existing anti-tumor response (Kakimi et al., 2016). A more specific classification is shown in Table 1 (Zhang & Chen, 2018).

Table 1: Classification of immunotherapy into passive and active immunotherapy (Zhang & Chen, 2018).

Passive immunotherapy		Active immunotherapy	
Immunomodulating antibodies	**Adoptive immunotherapy**	**Specific**	**Non-specific**
Immune checkpoint inhibitor	Tumor-infiltrating lymphocytes	Vaccines	Immune adjuvants
Immune co-stimulatory antibodies	TCR gene-modified lymphocytes		Cytokines
	Chimeric antigen receptors (CARs)		

Here, a few of the most promising strategies will be briefly discussed. First of all, monoclonal antibodies (mAbs), which are very specific antibodies. For the production of mAbs, an organism, often a mouse, is immunized with antigen X and produces anti-X antibodies. Mouse spleen cells are isolated from the mouse and are fused with a mutant myeloma cell line that is unable to grow in a specific selection medium. In this medium, unfused normal cells and tumor cells cannot grow. The fused cells, however, can grow in the selection medium, and these cells exclusively are selected. These cells are hybridomas and produce specific antibodies, called monoclonal antibodies (Abbas et al., 2015). mAbs stimulate an immune response through ADCC, which is antibody-dependent cell-mediated cytotoxicity. Immune cells, usually natural killer cells (NK cells), lyse the target cells during the process of ADCC. An Fc receptor on the surface of the NK cells bind to the IgG domain of the mAb that is bound to the target's cell surface, which causes lysis and killing of the target cell (Shepard et al., 2017). Therefore, mAbs can be aimed to target a specific element in a transduction pathway in cancer. Several mAbs are already used to treat cancer, also in hematological malignancies, such as alemtuzumab, which binds to CD52 on lymphocytes and is used to treat CLL (Zhang & Chen, 2018). Monoclonal antibodies can also be used to produce bispecific antibodies, which are antibodies that have two antigen recognizing parts. Bispecific antibodies have been developed in which one antigen recognizes CD3, which activates cytotoxic T-cells, and the other antigen recognizing domain recognizes a TAA. The activation of the cytotoxic T-cell promotes the elimination of the cancer cells (Sedykh et al., 2018). Another use of mAbs are antibody-drug conjugates (ADCs), which are monoclonal antibodies that are chemically linked to cytotoxic drugs. ADCs can also specifically target TAAs. When linked to the target cell, the cytotoxic drug enters and kills the cancer cells (Polakis, 2016).

Another form of immunotherapy is cancer vaccines. This is a form of active and specific immunotherapy that works by stimulating the immune system to fight cancer. There are two general types of cancer vaccines: preventive and therapeutic vaccines. Preventive vaccines prevent cancer from developing, for instance, the vaccine against the human papillomavirus (HPV) that causes cervical cancer. Therapeutic vaccines target the immune system directly and enhance the immune system's attack on cancer cells (Zhang & Chen, 2018).
Immune checkpoint inhibitors are a form of passive immunotherapy because they are drugs that increase the immune response against cancer. Immune checkpoints prevent the immune system from attacking these cells. In tumors, immune checkpoints are upregulated, resulting in low immune responses. If these checkpoints are inhibited, the immune response can be used to attack cancer cells. Nivolumab is an example of a checkpoint inhibitor, it inhibits PD-L1 and is approved for Hodgkin Lymphoma (Zhang & Chen, 2018). The last promising immunotherapy possibility is CAR T-cell therapy, which will be more in depth discussed in the next section.

CAR T-cells

Structure of CAR T-cells and how they work

CAR T-cells are T-cells transduced with a CAR, which are chimeric antigen receptors. The extracellular domain of the CAR can recognize TAAs and bind to them. Consequently, signals will be sent via a signaling pathway to activate the T-cell. The T-cell will eventually kill the cancer cell with toxins (Jackson et al., 2016). CARs consist of three different domains; the ecto-, endo- and transmembrane (TM) domain (Figure 5). The ectodomain is the part of the CAR that is extracellular and contains a single chain variable fragment (scFv). The scFv consists of the light and heavy variable (V_L $_{\&}V_H$) fragments of monoclonal antibodies. These monoclonal antibodies are very specific for certain fragments on tumor cells, and can therefore specifically target tumor cells. Another option as binding part of the ectodomain is the NKG2D receptor, which recognizes stress proteins that are upregulated in tumor cells (Cartellieri et al., 2010; Zhang et al., 2017). The transmembrane domain is the end of the endodomain and contains a hydrophilic alpha helix that crosses the whole membrane. This part is responsible for the stability of the receptor. The endodomain is the intracellular part of the receptor, of which CD3ζ is the main component. This component compromises three immunoreceptor tyrosine-based activation motifs (ITAMs). After recognition of the TAAs in the ectodomain, the signal is transmitted via the transmembrane and endodomain to the T-cell (Zhang et al., 2017).

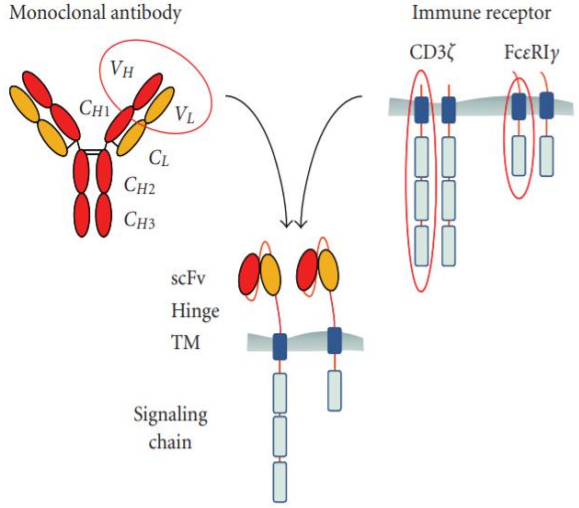

Figure 5: Structure of CAR T-cells. The ectodomain consists of the heavy and light chain (V_H & V_L) variables of mAbs. This is connected to the transmembrane domain and the endodomain, which consists of immune receptors like CD3ζ (Cartellieri et al., 2010).

CAR T-cells enable T-cells to specifically target cancer cells independently of HLA restriction, because they are derived from monoclonal antibodies. Other T-cell therapies are HLA-restricted. HLA is very polymorphic, making the other therapies more complex than CAR T-cell therapy (Davila et al., 2012).

Evolution of CAR T-cells

The initial development of CAR T-cells was in 1989, and since then CAR T-cells can be divided into three generations (Figure 6). This grouping is based on the structure of the endodomain, the inner part of the CAR T-cells. Recently, the fourth generation of CAR T-cells was developed; TRUCKs.

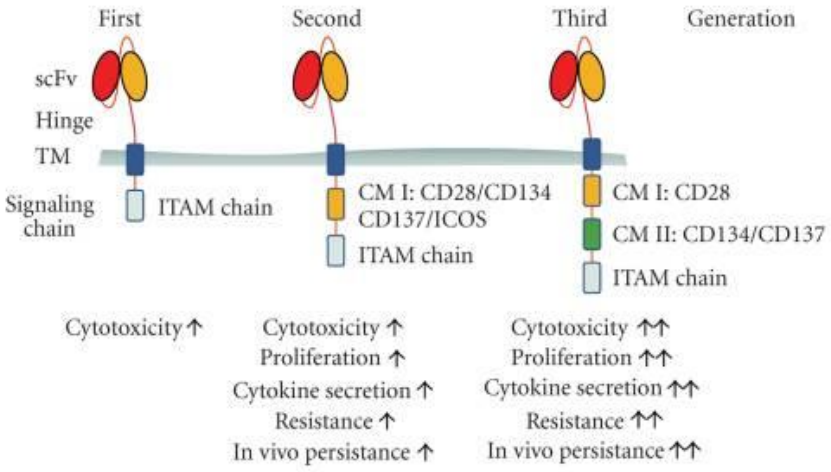

Figure 6: The evolution of the three generations CAR T-cells in structure and characteristics. First generation consists of the ITAM chain only. In the second generation, costimulatory domains were added. In the third generation, a second signaling domain was added (Cartellieri et al., 2010).

The first generation CAR T-cells often consist of the CD3ζ ITAM chain as endodomain. The problem with this generation is that they do not produce enough interleukin 2 (IL-2). To actually use the first generation of CAR T-cells, it was needed to administer IL-2 to the patient as well. On top of that, the first generation have a short life span *in vivo* and proliferate inadequately (Zhang et al., 2017). In the second generation, intracellular domains from costimulatory receptors were added for more signaling to the T-cells, such as CD28, CD134 or CD137. They were added to the endodomain to improve the proliferation, cytotoxicity and cytotoxic response in CAR T-cells. The life span *in vivo* of this generation also increased. An example of second generation CAR T-cells is scFvCD19-CD28-CD3ζ-CAR T-cells. These CARs target CD19 on B-cell malignancies, and have better outcomes than the first generation (Zhang et al., 2017; Cartellieri et al., 2010). In the third generation, multiple signaling domains were combined to increase the efficacy of the cells. The intracellular domains from costimulatory receptors, like CD28, CD134 or CD137, were combined (Zhang et al., 2017). The CD28-CD3ζ chain construct was significantly better in antitumor response than other combinations (Brentjens et al., 2007). By adding interleukin 12 to the base of the second generation CAR T-cells, the fourth generation was created. These are also called T-cell redirects for universal cytokine-mediated killing (TRUCKs). The addition of IL-12 to CAR T-cells improved the activation of the T-cells,

adjusted the immunological tumor environment, and recruited other immune cells, which can eliminate antigen-negative cancer cells in the tumor site (Chmielewski & Abken, 2015).

Production of CAR T-cells

To produce CAR T-cells, there are two main steps: isolation of T-cells and activation of T-cells to transduce the CAR. For the isolation of T-cells, a patient's leukocytes are first separated from other blood cells in a blood sample by a technique called leukapheresis. Afterwards, the T-cells are separated from the other leukocytes by enriching and washing them. Enrichment occurs through counterflow centrifugal elutriation. In this technique, cells are separated by size and density, while the cells remain viable. T-cells can then be separated into CD4+ and CD8+ T-cells by using antibody bead conjugates or other markers. The remaining cells are washed out of the buffer, and only T-cells are left. Then the T-cells need to be activated, which is normally done by the APCs. However, it is labor intensive to collect APCs from the patient to activate T-cells. Therefore, another standardized method is used. This is done by beads coated with anti-CD3/anti-CD28 monoclonal antibodies, anti-CD3 antibodies alone, or in combination with feeder cells and growth factors like IL-2. Finally, the CAR is transduced to the T-cells. The T-cells are incubated with the viral vector that contains the CAR genetic material. The viral vector attaches to the cells of the patient, enters the cell, and introduces the genetic material as RNA into the T-cells. With reverse transcriptase, the RNA is transcribed into DNA and integrates into the genome. When the cells divide, the CAR will be expressed on the surfaces of the cell (Levine et al., 2017; Zhang et al., 2017).

Applications of CAR T-cell therapy in hematologic malignancies

Applications in lymphoid leukemia

A possible target for CAR T-cell therapy in lymphoid leukemia is CD19. This antigen is expressed on normal and most malignant B-cells, such as B-cell ALL and CLL. However, it is not expressed on the HSCs. Targeting this antigen could thus be a possibility to destroy malignant and healthy B-cells while keeping the HSCs viable. Targeting the malignant and healthy B-cells results in side effects like B-cell aplasia. Nevertheless, the remaining HSCs can differentiate into B-cells again to overcome the B-cell aplasia (Brentjens & Curran, 2012).

Because of the expression of the CD19 marker on most lymphoid leukemia cells, this is an interesting marker to target with CAR T-cells. Several anti-CD19 CAR T-cells have already been generated in order to treat lymphoid leukemia. To illustrate, Davila et al. generated anti-CD19 second generation CAR T-cells. A B-cell ALL cell line was isolated, using Eμ-myc C57BL/6 transgenic mouse with progressive B-ALL disease. This cell line was injected into wild-type C57BL/6 (B6) mice to induce B-ALL in these mice, resulting in B-ALL in the B6 mice at genetic, cellular and pathologic levels. After treatment with chemotherapy, the anti-CD19 second generation CAR T-cells were given to these B-ALL mice. As a result of the CAR T-cell therapy,

the B-cells were killed. The survival rates of the mice treated with CAR-T cells was significantly increased compared to a chemotherapy treatment only, indicating that the mice were cured (Figure 7) (Davila et al., 2013).

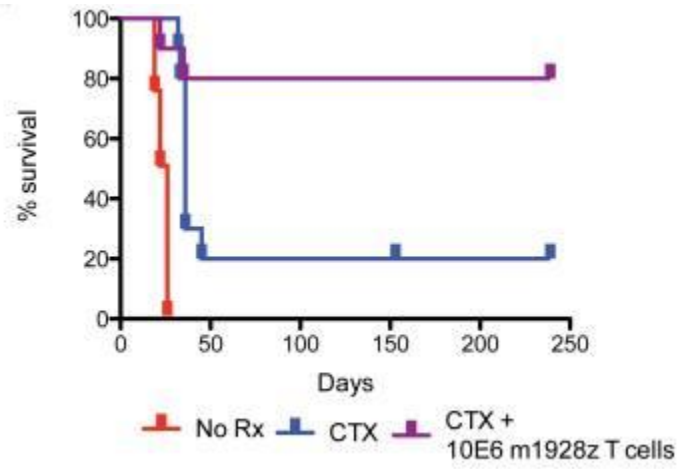

Figure 7: Survival rates of B-ALL mice model not treated (Rx), treated with chemotherapy only (CTX) and CAR T-cells in combination with chemotherapy (CTX = 10E6 m1928z T cells) (Davila et al., 2013).

Similarly, Brentjens et al. used CD19-specific CD28/CD3ζ second generation CAR T-cells to treat B-cell ALL in five adult patients, who were all relapsed. Before treatment, all patients included in the study were minimal residual disease positive (MRD+), which means that malignant cells were still present. After treatment, the tumors decreased quickly and bone marrow samples were MRD- (Figure 8A). On top of that, the expression of leukemic markers CD19 and CD10 were decreased after treatment (Figure 8B) (Brentjens et al., 2013).

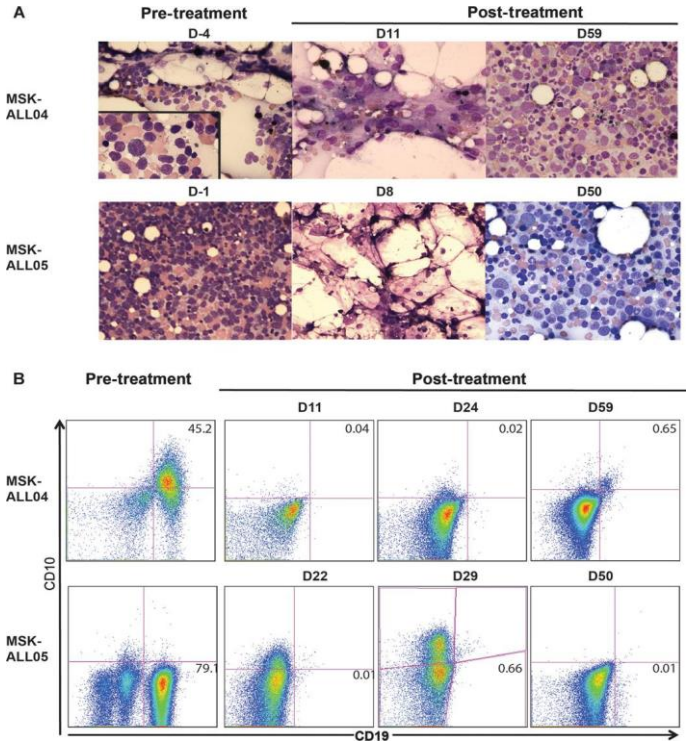

Figure 8: Effects of anti-CD19 CAR T-cell therapy. 8A: comparison of bone marrow cells before and after CAR T-cell treatment. In the pre-treatment sample, there are relatively many leukemic cells, which can be recognized by a large nucleus, and little cytoplasm. After treatment, on D59, there are various types of cells with different and smaller nuclei and relatively more cytoplasm. 8B: Comparison of flow cytometry data before and after CAR T-cell treatment. After the treatment, expression of leukemic markers CD10 and CD19 was lower in the samples (Brentjens et al., 2013).

Although this study showed promising results, the sample size was very small, which reduced the power of the results. Another study included 30 children and adult patients in a clinical trial to receive CTL019 against relapsed or refractory ALL. This is a CD19 specific CAR with CD3ζ domain and costimulatory signals by CD137. In 90% of the patients, there was complete eradication of the tumor cells. After the follow-up period of 2-24 months, 19 patients still had sustained remissions (Maude et al., 2014).

Next to CD19, CD22 is also present on B-cells. CD22 has been proven to be a successful immunologic target for B-cell malignancies with immunotoxin approach (FitzGerald et al., 2011). Therefore, this could also be a possible target for CAR T-cell therapy. Haso et al. demonstrated that the ratio of CD22 expression was 6485 to 54878 molecules per cell versus 14112 to 56946 molecules per cell for CD19 expression in 111 patients with B-cell ALL, and that all patients were CD22+. The results showed antileukemic activity of CD22 CAR T-cells *in vitro* and *in vivo* experiments, and the activity of CD22 CAR T-cells was comparable to that of CD19 CAR T-cells. Therefore, CD22 CAR T-cells hold therapeutic value for children and adults with ALL (Haso et al., 2012).

Because all clinical trials were very promising, the US Food and Drug Administration (FDA) has approved Kymriah (tisagenlecleucel) as the first CAR T-cell therapy in 2017. This therapy is CAR T-cell therapy that targets CD19, and is used for treatment of patients under 25 years old with relapsed or refractory B-cell ALL (Novartis, 2017). Updated analysis of the ELIANA trial, Kymriah showed 82% remission rates in pediatric patients with relapsed or refractory ALL, and 62% of the patients were relapse-free after 24 months (Maude et al., 2018).

Applications in myeloid leukemias

There are no cell surface antigens that are exclusively present on leukemic myeloid cells and not on HSCs in every patient (Kenderian et al., 2015). There are a few markers, but there are quite patient specific. Perna et al. and de Boer et al., reported different plasma membrane proteins that were aberrant expressed in AML patients. Perna et al. found 24 markers that were aberrant expressed in AML samples, of which six had low levels of expression on normal HSCs. These are TNFRSF1B, ADGRE2, CCR1, CD96, CD70 and LILRB2 (Perna et al., 2017). De Boer et al., compared markers on AML samples and CD34+ normal bone marrow cells, which are enriched for HSCs. This study showed that CD25, CD82, CD97, CD123, FLT3, IL1RAP and TIM3 were aberrant expressed in leukemic samples, but not in HSCs (de Boer et al., 2018). However, each patient seems to express a different set of aberrant markers. Consequently, there is no general CAR T-cell therapy for AML patients, and CAR T-cell therapy for AML tends to be destructive for the bone marrow (Kenderian et al., 2015). Because of the heterogeneity across patients, it could be that CAR T-cells targeting markers that seemed to be widely expressed in patients, may not be a good target in other patients. This will result in hematopoietic damage. Clinical trials targeting several antigens show this. To illustrate, CD33 was thought to be a marker only present on normal and leukemic myeloid cells, and not on HSCs. CD33 is a membrane-bound molecule expressed on healthy mature and immature myeloid cells, but also on 90% of AML tumor cells (Bernstein, 2002). One clinical study involving one patient with refractory and relapsed AML received anti-CD33 CAR T-cells. *In vitro*

experiments showed anti-leukemic activity of these CAR T-cells. *In vivo* experiments showed that AML cells were decreased after two weeks. However, the disease began progressing again nine weeks after treatment. On top of that, the patient experienced pancytopenia, which is a deficiency of all cellular blood components (Wang et al., 2015). This could be a result of targeting an antigen that is also present on the HSCs due to heterogeneity across patients or a consequence of hyper-activated T-cells. Similar results were found for anti-CD123 CAR T-cell therapy (Kenderian et al., 2015).

Another target for AML could be C-type lectin-like molecule-1 (CLL1). CLL1 is an antigen that is expressed on the myeloid lineage. Data on the expression of CLL1 on malignant cells in AML patients differ, ranging from maximally 40% (de Boer et al., 2018) to 90% (Laborda et al., 2017). CLL1 is not expressed on CD34+CD38- HSCs. In addition, it is present on chemotherapy-resistant leukemic stem cells which are likely to be responsible for relapse. This makes it a promising antigen for AML. Laborda et al. compared anti-CLL1 CAR T-cells to anti-CD123 CAR T-cell activity. They showed that CLL1 was indeed absent on HSCs. *In vivo* experiments in xenograft models with HL60 cells showed that second generation anti-CLL1 CAR T-cells eradicate tumors by day 90, while normally animals of this model die within 3 weeks (Figure 9). A comparison to anti-CD123 CARs showed that these CAR T-cells did attack healthy HSCs. A disadvantage is that the HL60 cell line is artificial and do not contain leukemic stem cells. Therefore, they are not representative for the response towards real leukemic stem cells (Laborda et al., 2017).

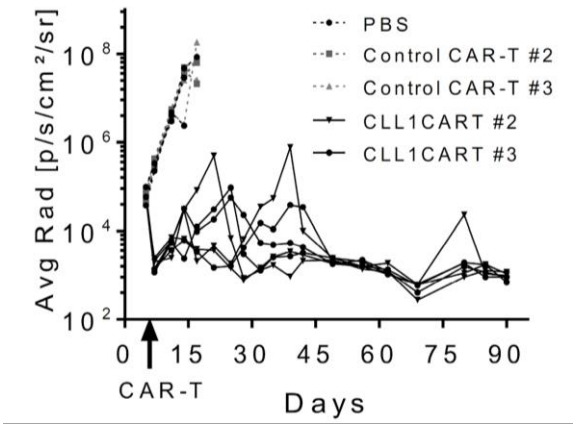

Figure 9: Anti-tumor activity of anti-CLL CAR T-cells in a xenograft model with HL60 cells, plotted as individual mice. tumor decreases after induction of the treatment with CLL1 CAR T-cells (Laborda et al., 2017).

To overcome the problem of targeting HSCs or hyper-activated T-cells, CAR T-cells were adjusted. To illustrate, Cummins et al., generated “biodegradable” mRNA anti-CD123 CAR T-cells in patients with refractory or relapsed AML. This would only target the myeloid cell lineage and not the HSCs. It resulted in no vascular, neurological, or hematological toxicities. Although these CAR T-cells were safe, they did not decrease the tumor, so the efficacy of this approach was limited (Cummins et al., 2017). Another strategy is T-cell deletion after CD123 CAR T-cell treatment. Results showed that CD123 CAR T-cell persistence of four weeks caused sustained leukemia eradication. Treatment with alemtuzumab (anti-CD52) and rituximab (anti-CD20) after four weeks of CAR T-cell therapy completely eliminated the administered CAR T-cells in human AML xenograft models (Tasian et al., 2015). Tashiro et al. generated the same anti-CLL1 CAR

T-cells as Laborda et al., but added an inducible caspase-9 suicide gene system to control T-cell activity. By administering a chemical inducer of dimerization (CID), which triggers apoptosis in caspase 9 anti CLL1 CAR T-cells, the cytotoxic activity in normal mature myeloid cells can be reversed (Tashiro et al., 2017). This is an interesting approach, and could be extended to other CAR T-cells.

Because of the heterogeneity across AML patients, an anti-tag CARs is an interesting approach. Anti-tag CARS are universal immune receptors, that use a standard scFv-based CAR receptor. These CARS recognize a small peptide or molecule tag as antigen. To illustrate, anti-FITC CAR T-cells are FITC-labelled CAR T-cells (Minutolo et al., 2019). These could be generated to recognize AML LSC antigens, which were reported by Perna et al. and de Boer et al., such as IL1RAP (Perna et al., 2017; de Boer et al., 2018).

Compared to ALL, CAR T-cell therapy for AML lags behind in practice. There are two main reasons for this. First, CAR T-cells should not target HSCs, to prevent the damage of healthy stem cells. Hematopoiesis, which is very essential process in the human body, cannot occur anymore when HSCs are damaged. The second challenge is the absence of a universal antigen in AML LSCs. CAR T-cell therapies would then be very patient specific, which would make this therapy more time and money consuming. As a result of these problems, there are no FDA approved compounds for CAR T-cell therapy in AML yet. However, there Mustang Bio is conducting phase I/II clinical trials with MB-102, which is a CD123 CAR, for patients with AML. The FDA granted orphan drug designation to MB-102 for patients with AML in 2019 (Rudzki & Wolf, 2020).

Applications in lymphomas

The CD19 antigen is expressed during different stages in the differentiation of B-cells, and therefore also present in most B-cell lymphomas. Several clinical trials are executed targeting CD19 in B-cell lymphomas (Avanzi & Brentjens, 2017). For example, a first generation CAR T-cell (19z1) specifically targeting CD19. These CAR T-cells lysed CD19+ human tumor cells *in vitro*. The addition of CD80 and IL-15 expanded T-cells to a functional phenotype *in vivo*. In a xenograft SCID-Beige mice model using a Raji cell line, which is a Burkitt lymphoma cell line, the treatment with 19z1 CAR T-cells resulted in 50% overall long term survival in the mice. Survival was even more increased with repeated CAR T-cell injections (Brentjens et al., 2003). *In vivo* studies with seven human patients with relapsed diffuse large B-cell lymphoma (DLBCL), a form of non-Hodgkin lymphoma, showed that anti-CD19 CAR T-cells were efficient. Five of the seven patients had complete remission, of which four were long-term and did not relapse (Figure 10A). One patient had complete remission but relapsed after 39 months into myelodysplastic syndrome, which is also a hematologic malignancy (Figure 10B). Besides periods of B-cell aplasia, no substantial chronic toxicity occurred (Kochenderfer et al., 2017). In the ZUMA-1 phase I trial, KTE-C19 CAR T-cell therapy was evaluated in patients with DLBCL. Five out of seven patients involved showed response, and four of them achieved complete remission. Regarding safety, severe cytokine release syndrome and neurotoxicity were self-limiting and mostly reversible (Locket et al., 2016). In the ZUMA phase II study, results showed that 76% of the patients achieved a response, with 47% complete remission (Neelapu et al.,

2016). Because of these promising results, the US FDA has approved and Yescarta (axicabtagene ciloleucel) in 2017 and Kymriah in 2018 (tisagenlecleucel) as CAR T-cell therapy for patients with relapsed or refractory non-Hodgkin lymphoma. These CARs both target CD19 on cells (FDA, 2017; Novartis, 2018). Currently, phase III clinical trials (ZUMA-7) are conducted to compare the efficacy of axicabtagene ciloleucel to standard therapy in patients with relapsed or refractory DLBCL. There are no results yet (Gilead Sciences, 2020).

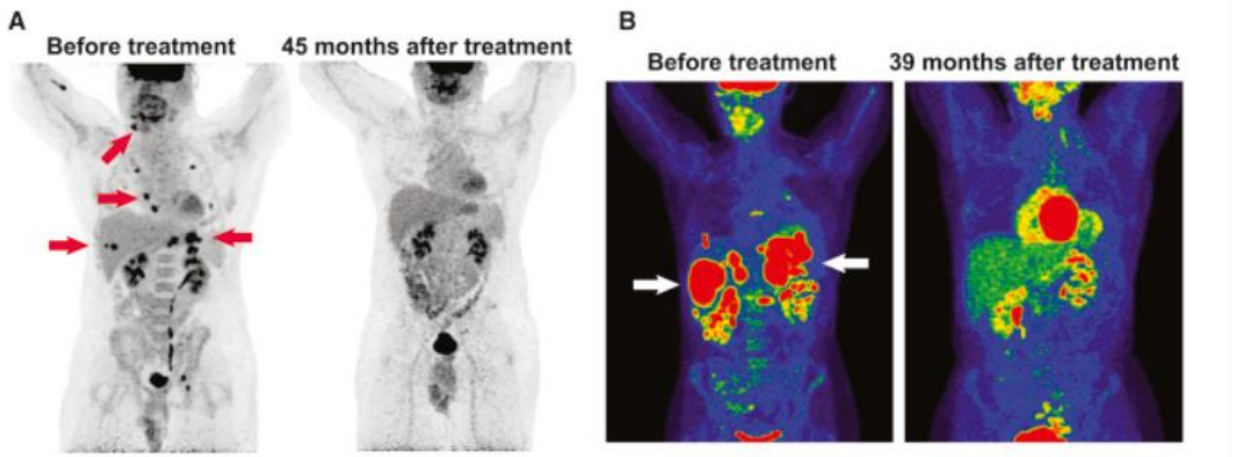

Figure 10: PET imaging showing the results of CAR T-cell therapy in two different patients. 10A: Lymphomas are indicated by the arrows. This patient still obtained complete remission after 45 months after treatment. 10B: Lymphomas are indicated by the arrows. This patient obtained complete remission, but was diagnosed with myelodysplastic syndrome 39 months after treatment (Kochenderfer et al., 2017).

Another marker on lymphoma cells is CD20. To prevent the escape of CD19 negative lymphoma cells in CAR T-cell therapy, Zah et al., produced CD19/CD20 bispecific CAR T-cells, called OR-gate CARs. These CARs target cells with either CD19 or CD20. This was an effective solution for antigen escape. All CAR T-cells eliminated WT Raji cells 6 days after treatment. At this point, CD19- mutant Raji cells were given to each sample. Both OR-gate and CD20 CAR T-cells efficiently eliminated CD19- targets, while CD19 CAR T-cells failed to target the escape mutants (Zah et al., 2016).

In Hodgkin Lymphomas, CD19 and CD20 are not widely expressed. Thus, CD19 and CD20 are not suitable targets for HLs. Instead, almost all cells in HLs overexpress CD30, which could be a possible target for CAR T-cell therapy (Avanzi & Brentjens, 2017). CD30 is a member of the TNF superfamily and is overexpressed in HRS cells in HL. The expression of this antigen is low in normal tissues, which makes it suitable for CAR T-cell therapy. Anti-CD30 CAR T-cell therapy was efficient in anti-tumor response *in vitro* and *in vivo,* resulting in seven out of eighteen patients involved achieving partial remission, and six achieving stable disease (Wang et al., 2016).

Disadvantages of CAR T-cells

Resistance against CAR T-cells

The major limitation of CAR T-cell therapy is resistance. There are several factors that play a role in resistance. First of all, antigen escape, which is the escape of tumor cells that do not express the antigen to which the CAR T-cell is targeted. On top of that, the tumor cells can mutate into antigen-negative cells over time. These antigen-negative cells are not targeted and attacked by the CAR T-cells, leading to escape of these tumor cells (D'Aloia et al., 2018). Secondly, the tumor microenvironment plays a role in resistance. The tumor microenvironment interferes with the function and infiltration of immune effector cells. To illustrate, tumor cells can upregulate PD-L1 to induce apoptosis in immune effector cells. Finally, the immune function itself plays a role in the resistance against CAR T-cells. Any defect in the immune system will lead to attenuation of the efficacy and increase of resistance (Cheng et al., 2019). This will result in relapses or no respond at all, which both occurred in some patients in clinical trials.

Side effects

When CAR T-cells are used to target hematologic malignancies, various side effects occur, which are driven by cytokines, such as the cytokine release syndrome (CRS), macrophage activation syndrome and hemophagocytic lymphohistiocytosis. After administering CAR T-cells to the patient, the lymphocytes and/or myeloid cells are activated and they will release inflammatory cytokines. CRS is the clinical response to this, and the patients may experience fever, neurological changes, low blood pressure and low oxygen levels in the blood (Zhao et al., 2018). This can result in serious and life-threatening complications, like cardiac dysfunction (Lee et al., 2014). To illustrate, a study by Brentjens et al. also monitored cytokine levels during the whole study. The cytokine levels were indeed elevated and correlated to CAR T-cells therapy (Brentjens et al., 2013). Another side effect is neurotoxicity, which is observed in patients treated with CD19 CAR T-cells. This results in endothelial dysfunction, capillary leakages and blood-brain barrier disruption. Hence, patients experience visual hallucinations, epilepsy, speaking disabilities and seizures (Zhao et al., 2017). The two main components that play a role in CRS and neurotoxicity are IL-1 and IL-6. Blockage of the IL-6 receptor in mouse models with tocilizumab antibodies prevented CRS, but not neurotoxicity. Using IL-1 receptor antagonist anakinra in the same model prevented both CRS and neurotoxicity (Norelli et al., 2018). These findings may contribute to prevention of CRS and neurotoxicity in patients in the future.

On top of that, although CAR T-cells are quite specific, the targeted antigens are often also present on healthy non-malignant cells. These cells or tissues are also targeted, which is called on-target/off-tumor toxicity. This happens in for example anti-CD19 CAR T-cell treatments, because CD19 is a marker on both leukemic and healthy B-cells, resulting in B-cell aplasia (Zhao et al., 2018; Brentjens & Curran, 2012).

Additionally, patients enrolled in clinical trials for CAR T-cells were only followed for a short period of time. This limits the assessment of the long term effects of these therapies. These long term effects remain unknown.

Individual engineering

Another disadvantage of CAR T-cells is the time consuming and costly process of CAR T-cell production for each patient. For every patient, the T-cells need to be separated from other cells in blood samples and collected. Afterwards, the T-cells have to be activated by coated beads before attaching them to the chimeric antigen receptor via viral vectors. This process takes about 7 days (National Cancer Institute, 2019). For each patient, the targeting system, gene transfer vector and master cells need to be optimized, which is also time consuming (Whilding & Maher, 2015). A more important problem is the costs that arise from the individual engineering. The price for Kymriah, which was approved for B-cell ALL and non-Hodgkin lymphoma, is $475.000 or €435.000 (Hay & Cheung, 2019).

Future perspectives of CAR T-cells

CAR T-cell therapy is one of the most promising types of immunotherapy and has improved substantially in the last couple of years. However, there are some challenges that need to be conquered, of which increasing efficacy and decreasing resistance are the main challenges. First of all, efficacy of CAR T-cell therapy could be improved by using TRUCKs to specifically targeting leukemia or lymphoma antigens. This fourth generation CAR T-cells seems very promising since the addition of IL-12 could prevent the escape of antigen-negative tumor cells. However, to date, there are no preclinical studies performed in hematologic malignancies using TRUCKs. This is mainly due to the fact that this generation is relatively new and there are several hurdles, such as IL-12 related toxicities and finding the best combination when engineering T-cells (Chmielewski & Abken, 2015). Further research is needed to overcome these hurdles and to improve CAR T-cell therapy.

Additionally, in (pre-)clinical trials not every malignant sample or involved patient responded to CAR T-cell therapy. Knowledge on the molecular mechanisms underlying the response is crucial to develop CAR T-cell therapy into a more efficient therapy for every patient.

Specificity can also be improved for example with the production of bi-specific CAR T-cells, which are CARs that have two antigen recognition parts. Therefore, these CAR T-cells can recognize either the one or the other TAA, attack both of them and eventually kill these cells. As mentioned before, a bispecific anti CD19/CD20 CAR has been developed to target lymphoma cells, which was very effective (Zah et al., 2016). Bi-specific CAR T-cells prevent the escape of antigen-negative tumor cells that occurs when using only ‘normal’ CAR T-cells. Perna et al. also combined CAR pairing to improve the efficacy without increase toxicity. This study found 66 combinational pairing, of which four were examined more in depth. Three out of the four combinations stained positive in 97% of AML samples, and all of the combinations stained <5% of HSCs and T-cells (Perna et al., 2017). Further development of bi-specific CAR T-cells or CAR pairing could be important to prevent antigen escape and relapse of the patients.

On top of that, CAR T-cell therapy in AML is quite hard, because of the heterogeneity of markers (Perna et al., 2017; de Boer et al., 2018). However, generating FITC-tag CARs targeting some of these targets could be a solution for this.

Another hurdle to overcome is the adverse effects, like CRS and on-target/off-tumor effects. A way to do this is with elimination gene systems, which can be activated to eliminate CAR T-cells to regulate cytotoxicity and expansion of T-cells (Zhao et al., 2017). For instance, as mentioned before, Tashiro et al., developed a CAR T-cell with caspase-9 as elimination gene system in an anti-CLL1 CAR T-cell to regulate expansion and cytotoxicity. The results were very promising since the cytotoxicity was prevented in normal mature myeloid cells (Tashiro et al., 2017). Additionally, treatment with IL-6 antibodies or IL-1 antagonists prevented CRS and neurotoxicity in humanized mouse models (Norelli et al., 2018). More insight into both gene elimination systems and using IL-1 antagonists or IL-6 antibodies is needed to expand this knowledge in order to apply it in practice.

The last remaining challenge is the individual engineering of current CAR T-cells, which is costly. As a result, the price for Kymriah, which was approved for B-cell ALL and non-Hodgkin lymphoma, is $475.000 or €435.000 (Hay & Cheung, 2019). The development of a universal targeting CAR T-cell would be optimal to reduce the costs, but further research into this is needed.

Conclusion and discussion

Leukemia and lymphomas are complex diseases, and the current standard treatment does not achieve the desired results, resulting in low overall survival of patients and serious side effects. In the past years, immunotherapy gained more attention, resulting in big improvements and steps in this field. A specific type of immunotherapy is CAR T-cells, which are specific antigen recognizing parts attached to T-cells, so cancer cells can specifically target the malignant cells. In this thesis, the possibility of CAR T-cells as an alternative treatment for standard cancer therapies to treat different hematological malignancies has been reviewed.

Literature has shown that CD19 is a good target for treating lymphoid leukemia with CAR T-cell therapy. Several studies showed that the overall survival of xenograft models, young and old patients increased after treatment with anti-CD19 CARs. This is also the first approved CAR T-cell therapy by the US FDA. Additionally, anti-CD22 CAR T-cells hold therapeutic value for treating ALL as well. In myeloid leukemia, however, it is harder to target the leukemic cells only. Aberrant expression of certain markers was found on AML LSCs, and these markers were not widely expressed on HSCs. However, these markers are rather patient specific, which makes the development of a general CAR T-cell therapy for AML patients hard. Clinical trials in AML showed that adverse effects occurred in some patients, which is probably due to the heterogeneity in antigens among patients. As a result, HSCs were targeted as well. Nevertheless, some adaptations, like suicide gene systems, could be used to reduce the cytotoxicity to HSCs, but more research is needed for this. In non-Hodgkin lymphomas, CD19 is a suitable target as has been shown *in vitro* and *in vivo* experiments. Patients with DLBCL responded well to treatment with anti-CD19 CARs. Therefore, the US FDA also approved this CAR T-cell therapy as treatment for relapsed or refractory non-Hodgkin lymphoma. Additionally, CD19/CD20 bi-specific CAR T-cells were developed to prevent antigen escape in non-Hodgkin lymphomas. This is a very promising and interesting development, which could be extended to

other targets. Lastly, CD30 was a suitable target for Hodgkin-lymphomas, resulting in stable diseases or partial remission of the included patients.
There are some disadvantages to CAR T-cell therapy. The major disadvantage is resistance, which can be caused by the microenvironment of the tumor, immune system deficits or antigen escape, and will result in a relapse. Another disadvantage is the adverse effects, such as CRS, which occurs due to cytokine production by the activated T-cells. The last disadvantage is individual engineering, which makes this therapy quite expensive. More research would definitely expand the knowledge on these problems, and could help overcome them. There are several questions that remain unanswered, such as why do some patients respond to the treatments, and others not, and more importantly what are the underlying molecular mechanisms of this? Can we use IL-1 antagonists or IL-6 antibodies to prevent serious CRS or neurotoxicity in patients? Can we design a universal CAR to reduce the high costs of this therapy? And lastly, would CAR pairing or bi-specific CARS prevent antigen escape and thus relapse in patients?

Overall, CAR T-cells provide a promising alternative treatment option for both lymphoid leukemia and lymphomas. In myeloid leukemia, it is harder to find a universal suitable target because of heterogeneity of the markers across patients. Of course, there are downsides of this type of therapy, such as resistance, adverse effects and individual engineering. More research and clinical trials need to be conducted to improve efficacy and reduce the disadvantages, which is necessary to develop this promising form of immunotherapy even more. This will expand the therapeutic value of CAR T-cells.

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