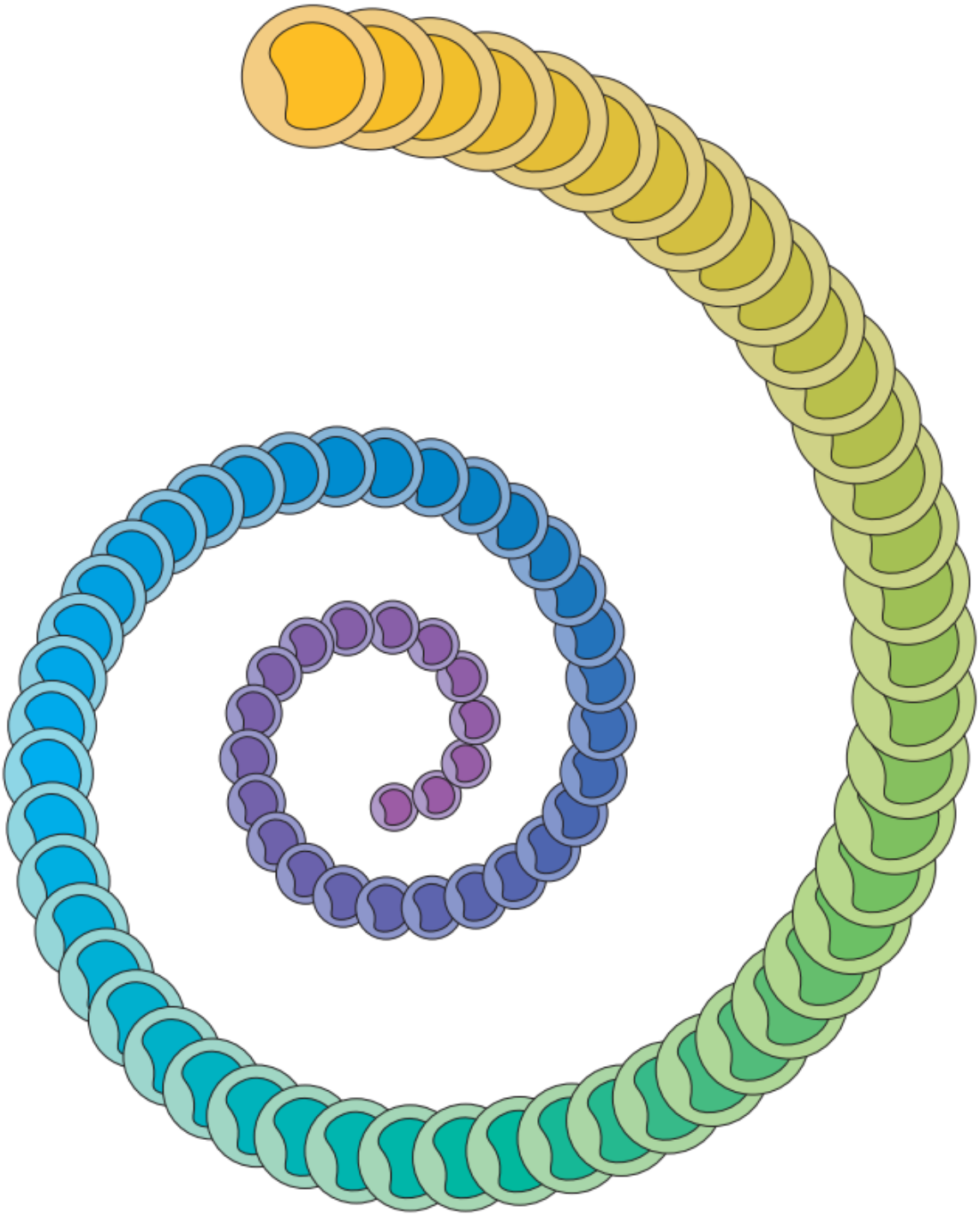
A Solid Foundation for Immunotherapy

the importance of T cell subsets in CAR-T cell therapy for B-cell acute lymphoblastic leukemia

KASPER MELCHERS



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Abstract

B-cell acute lymphoblastic leukemia (B-ALL) is the most common form of childhood cancer worldwide. Chimeric antigen receptor T cell (CAR-T) immunotherapy is a promising new therapy for B-ALL, however achieving long term remission is still a challenge. One important aspect to the efficacy of CAR-T cell therapy is the quality of the patient derived T cells used to make CAR-T cells. By nature T cells are a highly variable cell type containing many different subsets and differentiation statuses. Consequently the T cells that are used to produce CAR-T cells can vary significantly from patient to patient. The presence of less differentiated memory T cell subsets, T memory stem cells and central memory T cells, in the final CAR-T product are associated with a more durable antitumor response than their further differentiated counterparts. Furthermore, while both CD4+ and CD8+ CAR-T cells are capable of antitumor activity separately, they have a synergistic effect when combined into a 1:1 ratio. Preliminary clinical studies using a defined 1:1 ratio of CD4+ and CD8+ CAR-T cells have produced promising results but still lack in long term efficacy. Both a T helper cell type 1 and type 2 CAR-T cell response is needed for effective B-ALL clearance and patients who relapse often lack a type 2 response. Lastly, regulatory T cells in the CAR-T product have been shown to be associated with a worse prognosis for B-ALL patients but also lower levels of cytokine release syndrome. A large variety of methods for promoting beneficial T cell subsets during CAR-T production for B-ALL are being explored, many of which have produced promising results. Optimization of T cell subsets in CAR-T cell therapy for B-ALL is a promising avenue for future research to help improve treatment of patients.

Plain language summary

B-cell acute lymphoblastic leukemia (B-ALL) is the most common form of childhood cancer worldwide. In this type of cancer immune cells, called B-cells, start dividing uncontrollably, making the patient severely ill and eventually causing death if untreated. Treatments for B-ALL have improved in the last decades, including the emergence of a new type of therapy: immunotherapy. In the type of immunotherapy that is used against B-ALL immune cells called T cells are taken from the patients blood. T cells are able to kill cancer cells under normal circumstances but in B-ALL patients this process is not effective enough. To improve the effectiveness, T cells from the patient are genetically modified to be able to better recognize the cancer cells and kill them when they come in contact with them. These genetically modified T cells are called chimeric antigen receptor T cells (CAR-T). After genetic modification the CAR-T cells are injected back into the patient again where they can kill the cancer cells and potentially cure the patient. However this treatment is not perfect, within 12 months after treatment 50% of the patients who received CAR-T cell therapy have a relapse of B-ALL.

One of the factors that is important for CAR-T cell therapy to work well are the T cells that are taken from the patient. Many different subsets of T cells exist and what type of T cells are taken from the patient can therefore vary significantly. In the last decade research has been done to look into which T cell subsets are most effective as CAR-T cells. T cells change as they divide, with further generations being less able to divide themselves but having more killing potential. This process is called differentiation. In general less differentiated T cells that can still divide a lot are more effective when turned into CAR-T cells then the further differentiated T cells. Once fully differentiated a T cell can either become a T helper cell that focusses on stimulating other immune cells or a cytotoxic T cell that focusses on killing foreign cells. What type of cell a T cell will become once fully differentiated is pre-determined when the T cell is still ‘young’. The CAR-T cell product injected into the patient will be more effective if it contains a 1:1 mix of cells that will later become helpers and killers. Furthermore different types of T helper cells exist of which both type 1 and type 2 have shown to be important for the effectiveness of CAR-T therapy. Many different methods of promoting beneficial T cell subsets during the production of CAR-T cells are being explored. Through this research B-ALL patients treated with CAR-T cell therapy in the future will have a better chance of survival.

Chapter 1 | Introduction

1.1 | General introduction and relevance

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer worldwide (1). On average, 115 patients are diagnosed with ALL in the Netherlands annually. In the last few decades a concerted effort has been made to improve existing and develop new therapies for ALL. In the Netherlands this has led to increased five-year survival rate from 80% in 1990-1994 to 91% in 2010-2015. Especially remarkable is the increased five-year survival rate for infants under the age of 1 from 27% in 1990-1999 to 66% in 2000-2015 (2). Roughly 86% of ALL cases originate from a B-cell precursor and are aptly named 'B-cell acute lymphoblastic leukemia' (B-ALL) (2).

Besides improvement in traditional cancer therapies such as chemotherapy, the increased survival rates for ALL are also partly attributable to the introduction of a new kind of therapy, namely immunotherapy. A big breakthrough in immunotherapy for B-ALL came when in 2003 Dr. Sadelain and colleagues published a paper showing genetically modified T cells that can kill leukemia cells in mice by targeting the CD19 surface protein (3). These T cells were modified to produce chimeric antigen receptors (CARs). CARs are engineered receptors that consist of three domains. An ectodomain that is composed of a single-chain fragment variant. This single-chain fragment variant is derived from an antibody and able to bind to a specific protein, in this case human CD19. A transmembrane domain which localizes the CAR to the surface of the T cells. And lastly an endodomain that starts a signaling cascade in the T cell when the ectodomain binds its target (4). **See Figure 1**. Since then, the structure of CARs has been improved upon multiple times, mainly in the signaling of the endodomain, but the basic concept remains the same (4). By taking T cells from a B-ALL patient and genetically modifying them outside of the body to produce CARs, chimeric antigen receptor T cells can be created (CAR-T cells). These CAR-T cells can then be injected back into the body again where they will seek out any cells with CD19 on its surface. CD19 is a B-cell marker that is expressed during all phases of B-cell development and on all mature B-cells. This makes it a suitable target for immunotherapy since it will be present on B-ALL cells regardless of where in the B-cell development the initial cancer cell originated (3). When a CAR-T cell binds the CD19 of a B-ALL cell the signaling cascade, started by the CAR endodomain, will drive the CAR-T cell to eliminate the cancer cell (3).

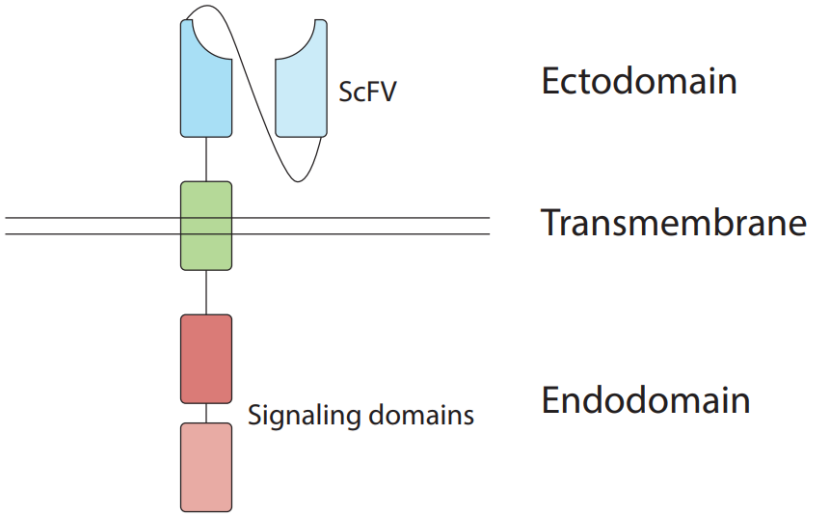

Figure 1: Model of a Chimeric antigen receptor. *A CAR consists of three domains, an ectodomain (Blue), a transmembrane region (green) and an endodomain (Red)*

Currently there are five CAR-T cell therapies approved by the American Food and Drug administration. Only one of these therapies, Tisagenlecleucel (trade name of Kymriah^{TM}), is indicated for use in pediatric B-ALL patients (5). In phase 2 trials, Tisagenlecleucel in combination with lymphodepletion has proven to be effective in the short term, leading to an overall remission rate of 81% in 3 months in a group of 75 patients for whom other cancer treatments had proven ineffective (6). However in the long term the therapy is still lacking. In the same phase 2 trial the researchers noted an event-free survival rate of 73% at 6 months and only 50% after 12 months (6). Real-world follow up studies found similar results with one noting an event-free survival rate of 62% after 6 months and 50% after 12 months in a cohort of 184 B-ALL patients (7).

The reason why for some patients CAR-T cell therapy leads to long term remission while other patients are non-responders or relapse is still unclear. There are likely many factors at play, one of which are the patient derived T cells that are used to make CAR-T cells. Patient derived T cells can differ between patients for a multitude of reasons. By nature T cells are a highly variable cell type containing many different subsets and differentiation statuses. The balance of these subsets can be influenced by internal factors such as (epi)genetics as well as external factors such as the treatments the patient received before CAR-T therapy; the production process of the CAR-T cells; and the environment. By understanding which T cells subsets in the final CAR-T cell product are beneficial and which are detrimental to long-term remission we can optimize future CAR-T cell therapies. The goal of this paper is therefore to determine what is known so far about the role of different types of T cells in CAR-T cell therapy for B-ALL as well as explore what methods could be used to promote these T cell types in future CAR-T cell therapies.

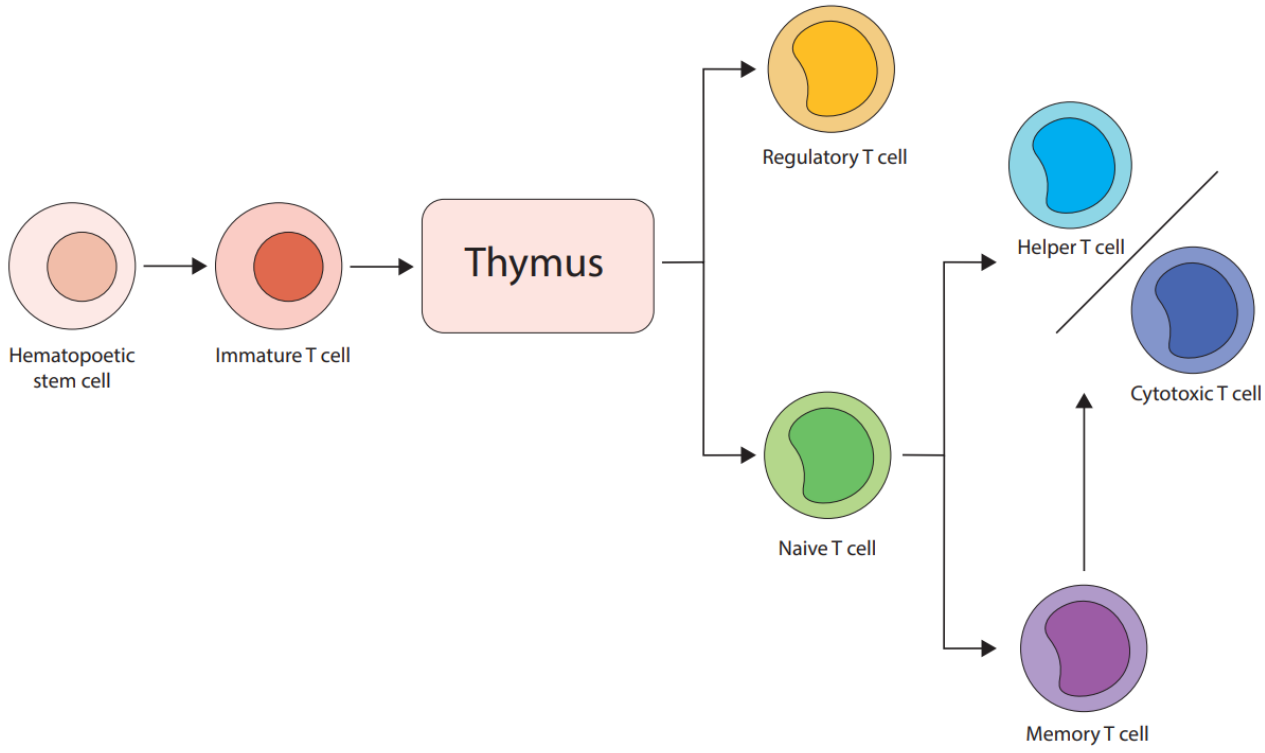

Figure 2: Model of T cell differentiation. *Hematopoietic stem cells differentiate into immature T cells that travel to the thymus. Here selection takes place, and a T cell emerges either as a naïve T cell or a regulatory T cell. Naïve T cells become effector T cells and memory T cells once activated. Memory T cells can later be activated, triggering them to differentiate into effector T cells.*

1.2 | T cell differentiation

To understand what role T cell subsets play in CAR-T cell therapy it is first important to have an overview of how T cells differentiate in humans. Firstly, immature T cells are produced in the bone marrow by hematopoietic stem cells. These immature T cells travel to the thymus where they are selected based on their antigen recognition and become mature T cells. T cells that recognize foreign antigens leave the thymus as naïve T cells (T_N) while T cells that recognize self-antigens can sometimes become regulatory T cells (T_{reg}). Once a naïve T cell is activated by an antigen presenting cell they undergo proliferative expansion, during this process the naïve T cells differentiate into memory T cells and effector T cells (T_{EF}) (8,9). Memory T cells patrol the body to become activated again in case of antigen recognition. Once activated memory T cells can differentiate into T_{EF}. T cells express either CD4 or CD8 on their surface, this expression is determined during maturation in the thymus. CD4+ T_{EF} are classified as helper T cells (T_H) while the CD8+ T_{EF} cells are classified as cytotoxic T cells (T_C). T_H cells are classically involved in signaling other immune cells by producing cytokines. Based on which cytokines they produce and what type of immune response they stimulate they can be further subdivided into different subsets, namely T_H1, T_H2, T_H9, T_H17 or T_H22. As their name suggests T_C are typically involved in killing cells that present antigens they recognize trough their T cell receptor. This includes cancer cells but also cells infected with a virus or other pathogen. **See Figure 2**

1.3 | CAR-T cell production

Promotion of beneficial T cell subsets can in part be achieved during CAR-T cell production. Once a patient is deemed eligible for CAR-T cell therapy peripheral blood mononuclear cells are taken from their blood through apheresis. In a sterile environment the T cells are then stimulated to activate and expand. This is classically done with the use of anti-CD3 and anti-CD28 antibody coated beads (CD3/CD28 beads) and optionally one or more cytokines. During this expansion phase a lentiviral vector (or other genetic modification method) is added to the T cells which contains the genetic instructions to produce the CAR. T cells transfected with this vector will become CAR-T cells. After a few days the T cells are transfused back into the patient where the CAR-T cells can bind and eliminate the B-ALL cells. Manufacture time can vary depending on how the CAR-T cells are produced but typically the whole process takes around 14 days. **See Figure 3.**

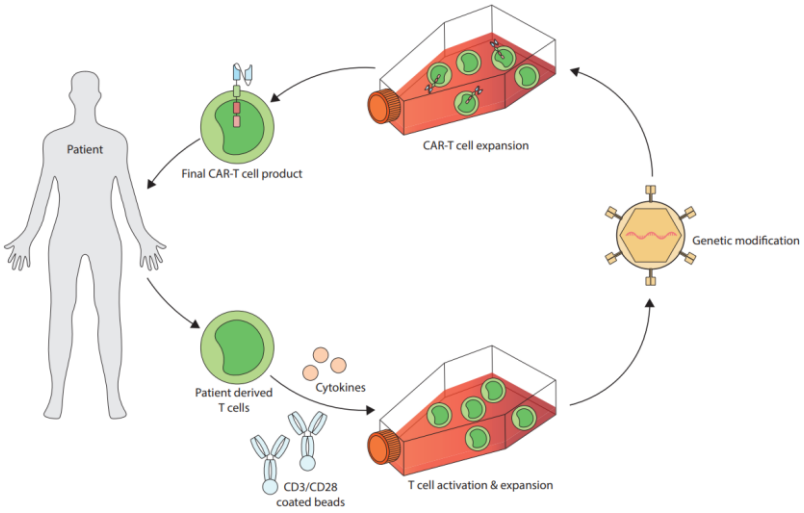

Figure 3: Model of CAR-T cell production. *T cells are taken from a patient and subsequently stimulated using CD3/CD28 beads and optionally one or more cytokines. Afterwards the T cells expand and are genetically modified to produce a CAR. The cells are then allowed to expand further after which they are injected back into the patient again.*

Chapter 2 | Different T cell subsets and their effect on CAR-T cell therapy for B-ALL

2.1 | Naïve and memory T cells

Memory T cells are not a homogenous cell group as was once thought. When memory T cells differentiate into T_{EF} they progressively lose their stemness and proliferative potential while gaining effector functions and cytotoxicity. Based on these properties and the expression of surface markers, three distinct groups of memory T cells have been identified. The least differentiated group of memory T cells are called T memory stem cells (T_{SCM}), slightly more differentiated cells are called central memory T cells (T_{CM}) and the most differentiated memory T cell subset are called effector memory T cells (T_{EM}). Lastly the T_{EM} terminally differentiate into effector T cells (T_{EF}). These cells lack any proliferative potential but have the most effector functions. Naïve T cells have the highest capacity for self-renewal but do not exhibit any effector functions **See Figure 4** (8,9). In theory all memory T cell subsets could have their own specific benefits to CAR-T cell therapy. The subsets with more proliferative potential could lead to better long-term persistence, thereby preventing relapses. Conversely, further differentiated memory subsets might be able to dispatch cancer cells more effectively due to their higher cytotoxic activity, thereby clearing the cancer quicker in the primary response. Which memory T cell subset is the most effective in practice remains under contention, but various efforts have been made in the last decade to shed light on this subject.

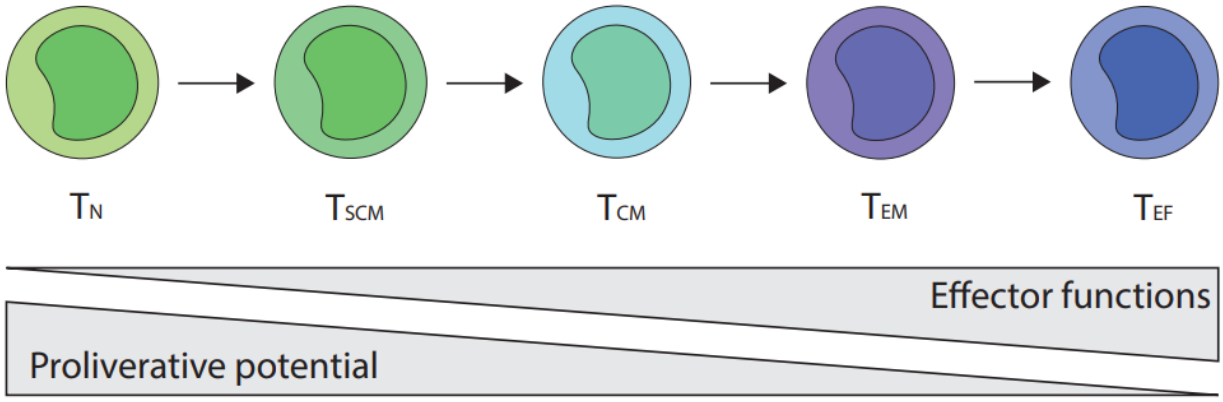

Figure 4: Model of memory T cell subsets. *Memory T cells gradually differentiate into effector T cells. During this process they lose proliferative potential but gain effector functions. T_{SCM} are the least differentiated memory T cell, TC_M are the next step and T_{EM} are the most differentiated memory T cell. Lastly the T_{EM} cells terminally differentiate into T_{EF} cells.*

A recent study compared bulk sorted T cells (CD4+/CD8+) to T cells sorted for $T_{N/SCM}$ surface markers (CD4+/CD8+ & CD62L+ & CD45RA+) from matched healthy donors. After sorting, the cells were cultured and transfected with a CD19 CAR. The two CAR-products were cultured with a variety of different CD19+ tumor cells for a period of four days. Against all cancer types the CAR- $T_{N/SCM}$ performed less than their unsorted counterparts, displaying a slightly reduced degranulation capability and cytotoxic potential while having the same proliferative potential. Conversely, when the CAR products were tested in a HSPC-humanized mouse model that were injected with NALM-6 leukemic cells the CAR- $T_{N/SCM}$ cells were able to create a more durable antitumor response and had higher expansion rates. The CAR- $T_{N/SCM}$ gave rise to at least ten times more T cells after being rechallenged with a second injection of NALM-6 cells. Despite these high expansion rates, The CAR-

$T_{N/SCM}$ were significantly less likely to induce cytokine release syndrome (CRS) when compared to the bulk sorted CAR-T cells. As 33% of the mice treated with bulk sorted CAR-T cells developed grade 4 CRS while none of the CAR- $T_{N/SCM}$ treated mice developed this level of CRS. Additionally, when analyzing the T cells in the peripheral blood of the mice 14 days after administration of the CAR-T therapy the researchers found significant differences in the T cell subsets present. In both the blood of the bulk CAR-T cell and the CAR- $T_{N/SCM}$ treated mice the most common T cell subset was T_{EM} with 71.9% and 64.1% respectively. For the bulk CAR-T treated mice the remaining T cells present in the blood were mainly T_{EF} cells with 21.9% while for CAR- $T_{N/SCM}$ treated mice this was T_{CM} with 27.8% (10). In conclusion, CAR-T cells produced from T_N and T_{SCM} cells are slightly less effective in vitro but in a in vivo leukemic mice model they expand better, are less toxic and give rise to more T_{CM} cells after infusion.

These results were corroborated in humans by multiple studies that retrospectively analyzed the T cells used to produce CD19 CAR-T cell therapies for pediatric B-ALL patients. They found that patients who suffered a CD19+ relapse had a significantly lower proportion of T_{SCM} and T_{CM} cells among their isolated T cells (11–13). Furthermore, these T cell subsets were also less prevalent in relapsed patients in the final CAR-T cell product produced from their T cells. And when exposing this CAR-T cell product to CD19+ cells in vitro, the cells of the relapsed patients also gave rise to less T_{SCM} and T_{CM} cells compared to patients who went into complete remission (11). While the T_{EM} and T_{EF} were found to be more metabolically and cytotoxically active than their less differentiated counterparts this was counteracted by a similar increase in apoptotic signaling pathways. The affinity towards programmed cell death of these subsets could explain why they do not contribute to CAR-T cell persistence and thus long-term remission (12).

While it seems clear that T_{SCM} and T_{CM} cells are clearly beneficial to CAR-T cell therapy the role of T_N is more complicated when it comes to human studies. One paper found that a higher proportion of T_N in the apheresis product of B-ALL patients was related to long term remission (12). While another study found higher levels of T_N in the final CAR-T product of CD19+ relapsed patients with ~14% compared to ~10% in patients who stayed in remission. Though this difference was just shy of significant (P=0.5476) (11). It has also been found that higher levels of T_N in the apheresis product lead to higher fold expansion of T cells in the CAR-T production process (14). Interestingly, yet another group expanded healthy donor T cells sorted per subset for 7 days using CD3/CD28 beads. They found that sorted T_N gave rise to 22% T_{SCM} cells and 78% T_{CM} cells. In comparison, sorted T_{SCM} cells gave rise to 24% T_{CM} and 76% T_{EM} cells when stimulated (15). These studies indicate a model where T_N present in the apheresis product give rise to beneficial early memory cells in the CAR-T cell product. The presence of T_N in the CAR-T product might be detrimental because it suggests inadequate stimulation of these cells, thereby not allowing them to differentiate into the beneficial subsets T_{SCM} and T_{CM} before infusion. However it is also good to note that the data in the last study seems overly optimistic with T_N not giving rise to any T_{EM}. Some limitations of this experiment may be the lack of interaction between different T cell subsets, the use of healthy donor T cells and an unrepresentative stimulation. These results may therefore not reflect how T_N behave in the production of CAR-T products from patients, and more research is needed to confirm this model of T_N.

2.2 | CD4+ and CD8+ T cells

As mentioned earlier T cells can also be subdivided in another manner, namely based on their expression of either CD4 or CD8. Since expression of either CD4 or CD8 is already determined during maturation of the T cell, all memory T cell subsets (T_N, T_{SCM}, T_{CM} and T_{EM}) can be further classified as either CD4+ or CD8+.

When compared in vitro, single cultures of CD4+ CAR-T cells show superior proliferation and activation levels compared to CD8+ CAR-T cells. Conversely, the lytic activity of the CD8+ CAR-T cells may be higher than that of the CD4+ CAR-T cells, although this result remains disputed in the literature (16,17). Furthermore, CD4+ CAR-T cells are better at activating monocytes. While this may seem positive at first it was also noted that this resulted in the higher release of monocyte derived cytokines. This hints that CD4+ CAR-T cells are an important factor in the development of CRS (16).

To test the difference between CD4+ and CD8+ T cells in vitro, researchers sorted T cells from patients with B-cell malignancies based on CD4 or CD8 expression. They then used these different subsets to produce CD19 CAR-T (4-1BB) cells and then individually tested their efficacy against CD19 expressing cancer cells. They confirmed that the CD4+ T cells were less cytolytic but produced more cytokines than their CD8+ counterparts. Next they sorted peripheral blood mononuclear cells from healthy patients based both on their CD4/CD8 expression and their memory subset ($T_{N/SCM}$, T_{CM} & T_{EM}) and made CAR-T cells from all the different subsets. They then tested the different CAR-T cell therapies in a leukemic mouse model (NSG mice injected with Raji tumor cells). They found that for CD4+ CAR-T cells the cells derived from T_N/T_{SCM} and T_{CM} subsets conferred longer survival to the mice than cells derived from the T_{EM} subset. The result was the same for CD8+ positive T cells except that the T_{CM} cells performed slightly better than the T_N/T_{SCM} cells. However, neither CD4+ nor CD8+ cells were as effective separately as they were combined in a 1:1 ratio. A combination of CD8+ T_{CM} and CD4+ T_N/T_{SCM} were especially effective in clearing the tumor cells. This efficacy correlated with a greater proliferation of the CD8+ cells, possibly caused by the cytokines produced by CD4+ T_N/T_{SCM}. Illustrating this efficacy, all mice treated with unsorted CAR-T cells, only CD4+ T_N or only CD8+ T_{CM} had died 8 weeks after tumor injection. In comparison 16+ weeks after tumor injection all mice treated with a 1:1 ratio of CD4+ T_N/T_{SCM} and CD8+ T_{CM} were still alive (18).

Following these positive results, a clinical trial was performed where in B-ALL patients received CAR-T cell therapy with a defined 1:1 ratio of CD4+ and CD8+ cells (19,20). The patients were given a combination of unsorted CD4+ T cells and either unsorted or T_{CM} CD8+ T cells depending on availability of the cells in their apheresis product. In the short term this therapy proved incredibly effective, achieving an initial remission rate of 93%. However, after 3 months there was only a ~40% chance of event-free survival, reducing even down to <20% after 12 months. Some of the patients were also given the chemotherapy drug fludarabine for lymphodepletion prior to the CAR-T cell therapy. This is similar to how the patients receiving Tisagenlecleucel were pre-treated. For them the long-term event-free survival was considerably better with ~90% after 3 months and ~65% after 12 months (compared to 81% and 50% for Tisagenlecleucel) (19,20). Another clinical trial also testing a 1:1 ratio of CD4+ and CD8+ cells in children and young adults with ALL found similar results. Combined with lymphodepletion their therapy resulted in ~75% event-free survival after 3 months and ~50% after 12 months (21). These results show that CAR-T cell therapies of a defined CD4+:CD8+ ratio are effective, but still struggle when it comes to long-term remission. The reason why CD4+:CD8+ ratios have an impact on the effectiveness of CAR-T cell therapy is not yet fully known. One possible cause for this effect could be the different effector subsets these cells give rise to. In the next chapter we will explore the role of these cells in CAR-T cell therapy for B-ALL

2.3 | Effector T cells

T_H1 and T_H2 cells

As stated earlier T_H cells are the fully differentiated state of CD4+ cells and are classically involved in signaling and activating other immune cells. However, while this is the main function of T_H cells under normal conditions, CAR-T_H cells have been shown to be able to directly kill tumor cells (17). Based on what type of immune response they stimulate and which cytokines they produce different types of T_H cells can be classified. Of these subsets type 1 T helper cells (T_H1) and type 2 T helper cells (T_H2) are the most researched when it comes to CAR-T cell therapy for B-ALL. By retrospective single cell transcriptional profiling of the final CAR-T products from B-ALL patients researchers were able to compare the immune profiles of activated CAR-T cells between relapsed and long-term remission patients. They found a significant up-regulation of T_H2 associated cytokines in long term remission patients, namely IL-4, IL-5, and IL-13. In contrast, no differences were found in expression of T_H1 related genes. These results suggest that both a type 1 and type 2 response is needed for long term remission of B-ALL and that patients who relapse often lack a type 2 response. These results were further corroborated by the researchers in an additional patient cohort. Here they found an increased number of T_H2 CAR-T cells among the long-term remission patients as well as higher functional strength of IL-5 in a multiplexed secretomic assay compared to the relapsed patients (11). Interestingly another paper found that activated CAR-T cells show both a T_H1 as well as a T_H2 response in the same cell (17). This could suggest that the lack of a T_H2 response is rather caused by (epi)genetic differences between patients rather than the lack of a certain T cell subset in the patient material.

T_H9, T_H17, and T_H22 cells

Research into the other T_H subsets as CAR-T cells against B-ALL is still lacking. Singular efforts have been made to investigate T_H9 and T_H17 cells while no research about T_H22 cells in this context has been published as of writing. T_H9 cells are characterized by the promotion of a type 2 immune response and the secretion of IL-9, 10, and 21 (22). To evaluate the functionality of this T cell subset as CAR-T cells one study produced CAR-T cells under two varying culture conditions. One group was expanded to mainly favor T_H1 cells (CD3/CD28 beads & IL-2) while the other group was cultured to promote T_H9 cells (CD3/CD28 beads, IL-4, TGF-β, anti-IFN-γ & IL-2). After producing CD19 CAR-T cells from the cultures and confirming that the T_H9 cultured cells did indeed produce significantly more IL-9 the subsets were compared in several functional assays. The T_H9 polarized cells were phenotypically distinct from the T_H1 polarized cells, containing mostly T_{CM} cells after culture compared to the T_H1 polarized cells having mostly T_{EM} cells. As a result the CAR-T cells produced from T_H9 polarized cells fared better in an in vivo leukemic mouse model (NSG mice injected with NALM6). Resulting in ~60 percent survival after 63 days compared to the mice treated with T_H1 polarized cells which had al died after 49 days (23).

T_H17 are typically involved in mucosal defense and are characterized by the secretion of IL-17. T_H17 cells present a small fraction of the T cells taken from patients and are hard to generate in vitro. Though in a recently published article, researchers managed to produce CAR-T T_H17 cells by adding the C3aR domain to the 3' end of a 4-1BB CAR. C3aR is a receptor that recognizes the complement fragment C3a. C3a is an important signaling molecule for activating both innate immune responses as well as T cell immune responses. The additional signaling domain had a profound effect on the T cell subsets present in the final product. Increasing the amount of CD8+ T_{CM} cells but also increasing the amount of T_{EM} cells and decreasing the number of $T_{N/SCM}$ cells present in both CD4+ and CD8+ compartments. This further differentiated phenotype is typically less effective as a CAR-T therapy, but this may be offset by the impact the signaling domain had on the T_{EF} cell compartment. Here the

researchers found that their novel CAR increased the amount of T_H17 cells from 1.92% to 3.24% in the final product while decreasing the amount of T_{regs} from 5.24% to 2.86% (24). When comparing the classic 4-1BB CAR-T cells to the novel BB-ζ-C3aR CAR-T cells researchers found the T cells with the C3aR domain were better at killing NALM6, Raji, and K562-CD19 leukemic cells in vitro. The BB-ζ-C3aR CAR-T cells were also more effective in a in vivo mouse model (NCG mice injected with NALM6), allowing the mice to live on average ~20 more days (24).

While research into both T_H9 and T_H17 seems promising, it is important to note that research into these two T cells subsets as CAR-T cells against B-ALL is still sparce. In both the studies mentioned, these T_H cells subsets were preferentially stimulated but still only represented a fraction of the final CAR-T product. In the study where T_H9 cells were promoted this also led to the promotion of T_{CM} cells as compared to the T_H1 culture. More T_{CM} cells being present is a significant confounder. The effectiveness of the T_H9 polarized culture in vivo might primarily te cause by the larger amount of T_{CM} cells as opposed to T_H9 cells. In this aspect T_H17 cells seem more promising as they were more effective in spite of a further differentiated phenotype in the final CAR-T product compared to the control. However, no follow-up studies looking into factors related to long term remission of B-ALL after CAR-T therapy have found any of these T_H subsets to play a significant role (11). To determine their true function more research is needed in isolated settings where their effectiveness cannot be confounded by the presence of other T cell subsets. Though it is questionable of T_H9, T_H17 and T_H22 are biologically relevant to CAR-T cell therapy at all as they only represent small niches of T cell subsets in patients.

Regulatory T cells

T_{regs} are a unique T cell subset in that they have the ability to downregulate the immune response when activated and could therefore potentially be detrimental to successful immunotherapy. Indeed researchers have found CAR-T cells with T_{reg} signatures circulating in patients undergoing CAR-T cell therapy, and patients with high levels of these cells had a worse prognosis (25,26). It was later confirmed that high T_{reg} levels in the CAR-T cell product before injection were also related to worse disease outcomes for the patients (26,27). However, T_{reg} in CAR-T cell therapy may also have some positive effects on therapy as researchers have also noted lower levels of CRS and neurotoxicity in patients with higher levels of T_{reg} CAR-T cells (25).

Cytotoxic T cells

T_C are the fully differentiated state of CD8+ T cells. Though CD8+ cells have been shown to be important to the function of CAR-T cell therapy, research into this effector subset is lacking. While T_C CAR-T cells often show high cytotoxic potential post-infusion, this benefit is counteracted by the fact that these T cells are prone to exhaustion eventually resulting in apoptosis (12,28). To further explore if any T_C subsets could be effective in CAR-T cell therapy researchers compared the pre-infusion CAR-T cells to the post-infusion CAR-T cells in B-ALL patients with a technique called lineage tracking. Post-infusion samples were taken from the patients at week 1-4, week 8, and month 3 and 6. By sequencing the unique T cell receptors present on the CAR T cells in the samples the differentiation of different CAR T cell subsets could be tracked over time. By doing this they were able to identify a subset of T cells in the pre-infusion product that in the patients gave rise to T_C that were cytotoxic but importantly had a low expression of the exhaustion-associated transcription factor TOX. These unique pre-infusion cells, characterized by the surface phenotype (TIGIT+, $CD62L^{lo}$, CD27-) demonstrate that there are in fact some niche T_C subsets that are be beneficial to CAR-T cell therapy (28).

2.4 | Chapter summary

This chapter explored different T cell subsets and their efficacy as CAR-T cells in the therapy for B-ALL patients. In general less differentiated memory T cell subsets are associated with a more durable antitumor response than their more differentiated counterparts. Especially the presence of T_{SCM} and T_{CM} in the final CAR-T cell product seem indicators for a durable response. Furthermore, while both CD4+ and CD8+ CAR-T cells are capable of antitumor activity separately, they have a synergistic effect when combined into a 1:1 ratio. Preliminary clinical studies using a defined 1:1 ratio of CD4+ and CD8+ CAR-T cells have produced promising results but still lack in long term efficacy. Additionally, different T_{EF} subsets were discussed. Both a T_H1 and T_H2 response is needed for effective tumor clearance and patients who relapse often lack a T_H2 response. Some research has been done into T_H9 and T_H17 CAR-T cells in the context of B-ALL and while preliminary results seem promising more data is needed to evaluate the true function of the T_H subsets. Moreover, T_C have proven to be short lived to be effective CAR-T cells but a niche, less exhaustion prone, subset of these cells may yet be promising. Lastly, T_{regs} have shown to be associated with a worse prognosis for B-ALL patients but also lower levels of CRS.

Chapter 3 | Optimizing CAR-T cell therapy trough promotion of beneficial T cell subsets

3.1 | Chemotherapy

Virtually all B-ALL patients will receive one or multiple rounds of chemotherapy before they are considered eligible for CAR-T cell therapy. Since chemotherapy can disrupt the patient's immune system it is important to investigate how the T cell subsets in the patient are affected and what consequences this has for producing CAR-T cells from them.

One study examined the T cell distribution in ALL patients after each cycle of chemotherapy they received. For the purposes of this study patients were divided into high and low risk groups based on their age and white blood cell count at diagnosis (high risk = age 1< or >10 with white blood cell count >50.000/uL). Interestingly, they found that chemotherapy did not significantly affect the ratios of any the T cell subsets in standard risk patients except for T_{CM} cells, which increased in percentage. For high-risk patients, chemotherapy had a different effect, significantly decreasing the percentage of T_N present in their samples (14). The ratio of CD4+ and CD8+ cells were also measured after each cycle of chemotherapy, but no significant trends were identified. These results indicate that for standard risk ALL patients chemotherapy leads to a more favorable T cell subset distribution while for high-risk patients it is detrimental. Next, the researchers tested the expansion capabilities of the T cell samples after each cycle of chemotherapy. They did this by stimulating the T cells with CD3/CD28 beads. They saw that after each cycle of chemotherapy the number of patients whose T cells we able to expand 2-fold or more decreased. Going from about 60% of patients before chemotherapy to only ~20% of patients after 7 cycles of chemotherapy in both standard risk and high-risk groups (14). While this is expected for high-risk patients as they have less T_N it is unexpected for standard risk patients who have a more favorable T cell subset distribution after chemotherapy. This data suggests that chemotherapy also affects the proliferative capacity of T cells independent of what T cell subsets are present. What then causes this decrease in proliferative capacity is unknown, but one possibility future research could look into is the presence of exhaustion markers on T cells after cycles of chemotherapy. It is therefore likely that the best CAR-T products can be produced from patient material acquired before any chemotherapy takes place, regardless of what T cell subsets are present.

3.2 | CAR-T cell stimulation & expansion

After patient material is acquired the next step for CAR-T production is to culture and expand the patients' cells in preparation for transfection with the CAR construct. During this expansion process additional factors can be introduced to influence the ratios of T cell subsets that will be present in the final CAR-T cell product.

Primary stimulation

T cells are typically expanded using beads coated with anti-CD3 and anti-CD28 antibodies. However, other alternatives are also being explored, such as Phytohemagglutinin (PHA) and antigen specific stimulation (29,30). PHA is a lectin that can bind to the glycosylated surface proteins, including T cell receptors thereby crosslinking them. T cells that were activated with either PHA or CD3/CD28 beads and subsequently cultured for 14 days with IL-2, IL-7 and IL-15 showed similar T cell subsets ratios post-expansion. However, the cells activated by PHA did produce a higher percentage of CD8+ $T_{N/SCM}$ as well as a slightly lower percentage of both CD4+ and CD8+ T_{EM} cells. While these minor differences might not be clinically significant PHA activated cells did proliferate significantly more

than bead activated cells (30). Therefore, PHA might make a good alternative for patients whose cells are unable to reach adequate proliferation with classic techniques.

Both CD3/CD28 beads and PHA are nonspecific stimulators of T cells. Nonspecific stimulation has the drawback that it stimulates all T cells in the culture, not just the ones that are expressing a CAR. This can be detrimental since in the CAR-T production process not all T cells are successfully transduced with a CAR and no sorting of the CAR+ cells takes place after transduction. Because of this downside methods for antigen-specific stimulation of CAR-T cells are being explored. One promising method is trough stimulation by membrane vesicles containing CD19. These membrane vesicles are produced from whole cells through blebbing, this has the benefit that the target protein is presented to the CAR in the right orientation and with proper glycosylation. T cells that were first transduced with a CAR and subsequently cultured with either CD19 vesicles or CD3/CD28 beads with IL-2, expanded in different manners. The vesicles stimulated both CD4+ and CD8+ cells equally while the beads preferentially stimulated CD4+ T cells. Resulting in a higher CD4:CD8 ratio after 7 and 14 days of culturing, though this difference was not significant. The frequency of $T_{N/SCM/CM}$ cells was also higher in the vesicle stimulated cells compared to the classically stimulated cells with ~70% vs 40% respectively (29).

Expansion phase additives

Two common cytokines that are added during the expansion phase are IL-7 and IL-15. When comparing T cells expanded using CD3/CD28 beads alone versus beads in addition to IL-7 (25ng/ml) and IL-15 (10ng/ml) there is a significant difference in post-expansion T cells. For both standard and high risk ALL the cytokines lead to more patients being able to reach 5-fold T cell expansion, increasing from ~70% to ~90% and ~30% to ~50% percent respectively. The cytokines also influenced the T cell subsets ratios in the post-expansion product. Leading to ~30% more T_{SCM} cells present in the samples that showed improved expansion after adding the cytokines compared to stimulation with beads alone. Furthermore, samples that still failed to reach adequate expansion levels after adding the cytokines showed ~20% more T_{CM} in relation to only bead stimulated samples. However, in samples that were already able to reach 5-fold expansion without the cytokines there was a potentially undesirable increase of ~5% more T_E cells when IL-7 and IL-15 were introduced (14). In conclusion, the use of IL-7 and IL-15 during the expansion phase of CAR-T production may potentially be beneficial to T cell subsets present post-expansion, however they may also cause T cells that are already able to proliferate without cytokines to become terminally differentiated.

As demonstrated by this, the expansion phase of CAR-T cell production is often a balancing act between adequately stimulating the T cells without inducing terminal differentiation. During the expansion phase of T cells two major signaling pathways are activated, the MAPK and the PI3K/AKT/FOXO pathways. Inhibition of the MAPK pathway has been shown to significantly impair proliferation of T cells in humans. This is not the case for the PI3K/AKT/FOXO axis where inhibition does not seem to compromise proliferation (31). However, inhibition of the PI3K/AKT/FOXO pathway does seem to have influence on the ability of T cells to differentiate. As was illustrated by a research group that proliferated T cells with CD3/CD28 beads and IL-2 in addition to an allosteric AKT inhibitor (AKT inhibitor VIII). The T cells cultured with the AKT inhibitor produced a higher ratio of $T_{N/SCM/CM}$ cells, going from ~60% to ~80% while the amount of T_{EM} cell decreased from ~38% to ~13%. This phenotype was even more pronounced in T cells where FOXO1, the phosphorylation target of AKT, was mutated to be phosphorylation-resistant (31). Moreover, in a leukemic mice model (NSG mice injected with NALM6 leukemic cells) the CAR-T cells cultured with an AKT inhibitor were more effective than conventional CAR-T cells. After 35 days all mice treated with AKT inhibitor CAR-T cells were still alive while those treated with conventionally cultured CAR-T cells had died after day 29

(31). Another study utilized the PI3K inhibitor 'Idelalisib' during the CD3/CD28 bead mediated expansion of T cells from diffuse large B-cell lymphoma patients. They found similar results, producing 54.4% $T_{N/SCM/CM}$ cells in cultures where the PI3K/AKT/FOXO pathway was inhibited compared to 27.4% $T_{N/SCM/CM}$ cells in uninhibited cultures (32). **See Figure 5**.

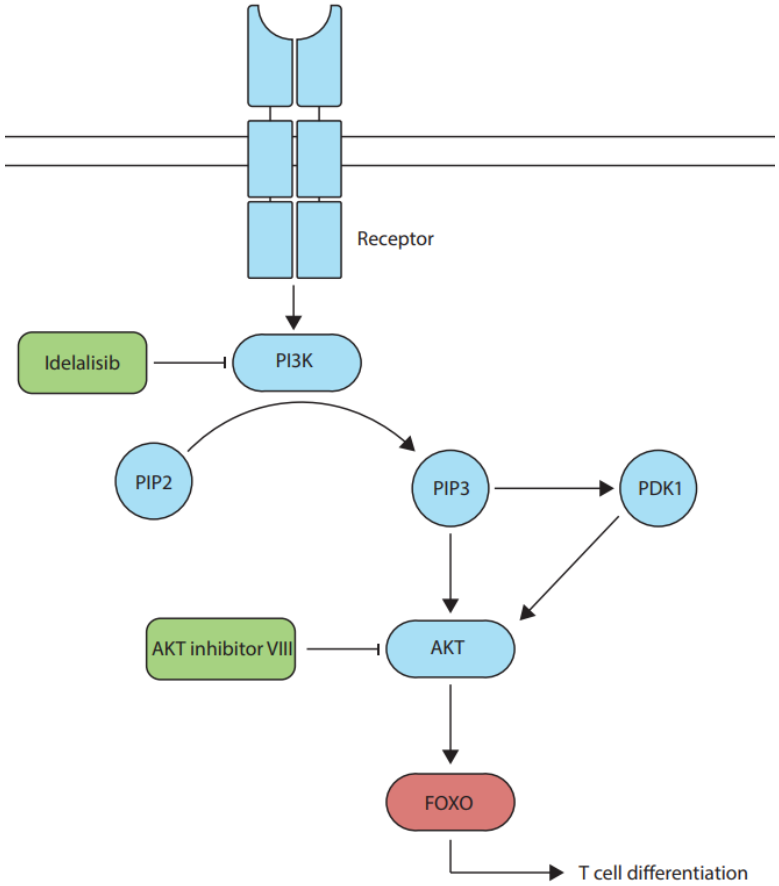

Figure 5: Simplified representation of the PI3K/AKT/FOXO axis. *The PI3K/AKT/FOXO axis is started by a receptor binding to a growth factor, cytokine, etc. The receptor activates PI3K, which starts converting PIP2 into PIP3. PIP3 and PDK1, which is also activated by PIP3, both phosphorylate AKT. AKT can in its turn phosphorylate FOXO (red) which downstream leads to differentiation in T cells. Idelalisib and AKT inhibitor VIII (green) can both inhibit this pathway at different points.*

3.3 | Co-expressed proteins

A challenge with the techniques we have discussed until now is that they can only be administered to the T cells during the expansion phase of CAR-T cell production. Therefore, once the CAR-T cells are administered to the patient there is no more control over what T cell subsets will be present. Another method for manipulating the T cell subsets present in the final CAR-T cell therapy that does not suffer this problem is by introducing proteins into the T cells through genetic manipulation. These proteins can be integrated into the CAR sequence using self-cleaving peptides, thereby becoming separate proteins once translated. Several different proteins have been co-expressed with CARs in the last decade .This chapter will explore some of the most promising ones for the use against B-ALL.

The CD28 receptor is the most important second signal involved in T cell activation while the TIM-3 receptor is an inhibitor of antitumor activity. By combining the extracellular receptor domain of TIM-3 with the intracellular signaling domain of CD28 a switch receptor can be made. This T3/28 switch receptor can convert suppressive signals made by tumor cells into an activating signal for T cells. By

then co-expressing this switch receptor to a 4-1BB CAR a potentially more effective CAR-T cell can be produced. To test the functionality of this switch receptor the CAR-T cells were co-cultured with Namalwa tumor cells for 18 days. Afterwards T3/28 CAR-T cells had higher amounts of T_{CM} compared to 4-1BB CARs (39.22% vs 30.19%) and lower amounts of T_{EF} (15.83% vs 30.52%). The CD4/CD8 ratio also slightly differed in these cells going from 1.1 in 4-1BB CARs to 1.4 in T3/28 CARs (33). This indicates that after CAR-T cell production the T3/28 receptor is able to maintain beneficial subsets of T cells in the CAR-T product.

Another but similar approach is to attach the LRP6 protein to the CAR using a self-cleaving peptide. LRP6 is a protein involved in the Wnt signaling pathway. Overexpression of this protein leads to activation of the Wnt pathway, which has been shown to improve persistence and decrease differentiation of memory T cells. Indeed, when LRP6 was overexpressed in CAR-T cells it increased the percentage of T_{SCM} from ~18% to ~45% and the amount of T_{CM} from 10% to 20% compared to regular CAR-T cells (34). Another study demonstrated that T_{SCM} can be generated in vitro from T_N by activating the Wnt pathway in vitro. By adding the GSK-3β inhibitor, TWS119 to a standard CAR-T cell production protocol they were able to preferentially induce T_{SCM} in the final product among CD8+ cells (35). Indicating that the Wnt pathway is a promising target for promoting early memory cells in CAR-T production.

3.4 | Cell sorting for CAR-T production

Cell sorting is another technique that can be implemented during the CAR-T cell production phase to select for desirable T cell subsets. One research group sorted T cells from ALL patients post-apheresis, selecting all cells that were positive for either CD4 or CD8. Subsequently, the cells were stimulated using IL-7 and IL-15 (12.5 ng/ml each) in addition to an anti-CD3/CD28 conjugated polymeric nanomatrix. Finally, the cells were transduced using a 4-1IBB lentiviral vector to become CAR-T cells. All these steps were done in one culture per patient containing both the CD4+ and CD8+ cells, as opposed to creating separate cultures for these two cell types and then combining them in a 1:1 ratio. With this technique the researchers were able to achieve a ratio of 50.3% CD4+, 38.7% CD8+ (ratio of ~1.3) and 10.2% natural killer cells in their final product. In future studies the natural killer cells could be selected out by also using CD3 as a marker, thereby possibly achieving a CD4:CD8 ratio closer to 1. Moreover, the initial sample which contained a broad variety of T cell subsets including T_{EM} and T_E cells dramatically changed becoming 50% T_{CM} and 46% T_{SCM} cells in the final product (36). The researchers also measured the effector functions of the CAR-T cells by co-culturing them with a CD19+ cell line. The CAR-T cells secreted high levels of GM-CSF, IFN-γ, IL-2 and TNF-α indicating a strong affinity for Th1 type response. The CAR-T cells were only able to produce low concentrations of the Th2 related cytokines IL-5 and IL-10 (36). This expansion method shows promise as it is able to produce a beneficial ratio of CD4/CD8 T cells as well as an early memory phenotype in the final product. Why sorting for CD4+ and CD8+ cells produces an early memory phenotype is not clear. One possibility is that in classical stimulation the other leukocytes in the apheresis product influence the expansion of the T cells. One downfall of this method may be the low Th2 activity, as this has also been shown to be an important factor in obtaining long term remission of B-ALL (11) Another caveat of this study is that only four ALL patients were included. Another study expanded the T cells of 22 ALL patients and five patients with other B-cell malignancies. They used a similar method except they sorted the cells with magnetic bead separation and only cultured for 7-10 days instead of 12 yet they found significantly different results (37). For them this method resulted following averages of T cell subsets in the final product, T_N: 7.71±13.9, T_{SCM}: 5.26±12.0, T_{CM}: 31.01±16.7, T_{EM}:35.11±17.7, and T_E: 4.2±9.5 as well as a CD4:CD8 ratio of 1.61±1.0 (37). Especially remarkable is the higher percentage of T_{EM} cells and lower amount

of T_{SCM} cells, something that could be detrimental to the longevity of CAR-T cell therapy. The researchers also tested the use of IL-2 instead of IL-7/IL-15 and both 4-1BB and CD28 CARs and found that none significantly changed the T cell subsets present. The difference between these two studies illustrates that while cell sorting for CAR-T production is able to improve the final product for some patients more refinement of the method is still needed before it can be applied universally to all patient material and that results may be hard to reproduce.

3.5 | Chapter summary

In this chapter different methods for promoting beneficial T cell subsets beneficial to CAR-T cell therapy against B-ALL were discussed. Chemotherapy administered to patients before CAR-T therapy has a detrimental effect on the fitness of T cells of the patients. Different methods of expanding T cells during the production of CAR-T cells were discussed. IL-5 and IL-17 can promote expansion of T cells as well as early memory subsets but also cause over differentiation in certain cases. Inhibiting the PI3K/AKT/FOXO axis prevents T cells from differentiating to T_{EM} cells. Conversely, stimulating the Wnt pathway seems to increase the amount of T_{SCM} in the final CAR-T product. Other methods discussed and their effects on T cells subsets are summarized in **Table 1**.

Name	Category	Effect on T cell subsets	Reference
PHA	Primary stimulant	↑ CD8+ $T_{N/SCM,}$ ↓ T_{EM}, ↑ Proliferation	(30)
CD19 membrane vesicles	Primary stimulant	↑ $T_{N/SCM/CM}$ cells, equal CD4+ CD8+ stimulation	(29)
IL-7 + IL-15	Expansion additive	↑ Proliferation, ↑ T_{CM}, ↑ T_{SCM}, (↑ T_E)	(14)
AKT inhibitor VIII	Expansion additive	↑ $T_{N/SCM/CM}$, ↓ T_{EM}	(31)
Idelalisib	Expansion additive	↑ $T_{N/SCM/CM}$, ↓ T_{EM}	(32)
TWS119	Expansion additive	↑ T_{SCM}	(35)
Sorted CD4+/CD8+ cells	Sorting method	Beneficial CD4/CD8 Ratio, ↑ T_{SCM}, ↑ T_{CM}, ↑ T_H1	(36)
C3aR	Modified CAR	↑ CD8+ T_{CM}, ↑T_H17, ↓ T_{REG}, ↑ T_{EM}, ↓ $T_{N/SCM}$	(24)
T3/28	Co-expressed protein	↑ $T_{CM,}$ ↓ T_{EM}	(33)
LRP6	Co-expressed protein	↑ T_{SCM}, ↑ T_{CM}	(34)

Table 1: summary of different methods of influencing T cell subsets during CAR-T cell production. *Potentially adverse effects of these methods are marked in red.*

Chapter 4 | Discussion

B-ALL is the most common form of childhood cancer worldwide (1). While survival has improved in recent decades the current available treatments are still inadequate for a significant number of patients. One of the most promising new therapies for B-ALL is CAR-T immunotherapy. Here T cells from the patient are genetically modified outside of the body to recognize and eliminate tumor cells. However, achieving long term remission is still a challenge, with the currently approved therapy 'Tisagenlecleucel' only conferring a disease-free survival rate of 50% after 12 months (6). While many factors play a role in the efficacy of CAR-T cell therapy, one important aspect is the quality of the patient derived T cells. By nature T cells are a highly variable cell type containing many different subsets and differentiation statuses. Consequently the T cells that are used to produce CAR-T cells can vary significantly from patient to patient. It is therefore important to understand the influence they have on the effectiveness of CAR-T cell therapy. The goal of this paper is to determine what is known so far about the role of different subsets of T cells in CAR-T cell therapy as well as explore what methods could be used to promote these T cell subsets in future CAR-T cell therapies.

One of the most researched T cell subsets in the context of CAR-T cell therapy for B-ALL are the different memory T cell subsets. Many articles have described the benefits of the less differentiated memory cell subsets, T_{SCM} and T_{CM}, to the long- and short-term effectiveness of CAR-T cell therapy (10–13,15). T_N also seem to be beneficial to CAR-T cell therapy though only when present in the apheresis product and not in the final CAR-T product (11–15). While the benefits of CAR-T cells produced from early memory T cells are well described, studies into this subject still face some limitations. Many studies group T_{SCM} and T_N together in analyses. This limits direct comparisons between T_{SCM} and T_{CM} and might make the effects of T_{SCM} go underappreciated. Especially if they are grouped together with T_N in the final CAR-T product where these cells are potentially less effective. Future research could explore what makes T_{SCM} and T_{CM} so effective in CAR-T therapy and what the true role is of T_N. A method for achieving this could be through T cell receptor lineage tracking. With this method the lineages of individual T cells can be tracked by sequencing the unique T cell receptor sequence. By doing this after apheresis, after CAR-T production and several times after injection of the therapy more insight can be gained on what cells early memory T cells give rise to both during CAR-T cell production and in the patient.

Another area of T cell subsets which has seen a lot of attention is the effect of the CD4+ and CD8+ content of the final CAR-T cell product. These cells have been shown to function more effectively when combined in a 1:1 ratio then they do separately. So far a ratio of 1:1 seems standard in the literature, but future research could explore different CD4:CD8 ratios to see if this enhances the potential of CAR-T cell therapy even more. While CAR-T cells of a defined CD4:CD8 ratio were effective in mouse models this effect was less pronounced in humans (18,19). This points to another limitation of research into different CAR-T cell products, the in vivo mouse models. To assess the efficacy of different CAR-T cell products, leukemic mice models are often used. However, in these mouse models survival is often only tracked for a few months. While this can give a good representation of how CAR-T cell products function in the short-term, long-term survival, which is currently the therapy's biggest challenge, is hard to evaluate.

While 1:1 CD4:CD8 ratio products remain an interesting avenue for CAR-T cell products more is needed to improve their long-term efficacy. One possible solution is to take into account both the CD4:CD8 ratio as well as the memory differentiation of a CAR-T product. While previous studies have attempted this, they often struggle to extract adequate amounts of the preferred memory T cell

subsets when also sorting for CD4 and CD8. one possible solution could be to further optimize the culture conditions under which CAR-T cells are created. The use of IL-2 and/or IL-7 and IL-15 is a simple and effective method for stimulating expansion of T cells, but many different methods have been developed to optimize the early memory T cells present in a CAR-T cell product. These methods can, however, sometimes have the drawback of overstimulating T cells thereby creating T_{EM} and T_{EF} cells. The over-differentiation of T cells can be counteracted with the use of inhibitors of the PI3K/AKT/FOXO axis and stimulators of the Wnt pathway (31,32,34). One promising new tactic could therefore be combining the pre-sorting of CD4 and CD8 cells, novel stimulation methods, and inhibition of over differentiation using small molecule inhibitors. With this CAR-T production method it might be possible to create enough early memory T cells of defined CD4:CD8 composition to inject into patients.

While early memory subsets are promising to CAR-T cell therapy some T_{EF} subsets might also be beneficial. It has been described that B-ALL patients who relapse often lack a T_H2 response (11). Promoting this subset in CAR-T therapy might be challenging as stimulating them also means the stimulation of other, potentially less beneficial, T_{EF}. One possible novel approach to achieve a T_H2 response in B-ALL patients is by creating a modified CAR construct that skews T_H cells towards a T_H2 phenotype once activated by a tumor cell. This would allow for the production of an early memory phenotype CAR-T product while still conferring the benefits of a T_H2 response once injected into the patient.

The large variety of factors that play a role in CAR-T cell therapy make it hard to directly compare studies looking at T cell subsets. Stimulations methods that work in one paper may produce widely different results for another group. And T cell subsets that look beneficial in one experiment might not be as effective in a different context. Furthermore, T cell subsets are only one of the elements involved in the effectiveness of CAR-T cell therapy. Other factors such as the design and target of the CAR construct and the pre-therapy lymphodepletion used also have a big impact on the effectiveness of CAR-T cell therapy. It is therefore unlikely that optimization of the T cell subsets alone is enough to provide long term remission for all B-ALL patients receiving CAR-T cell therapy. Nevertheless, optimization of T cell subsets in CAR-T cell therapy has shown potential and is a promising avenue of research to help improve future treatment of B-ALL patients.

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