CURRENT CAR THERAPIES USING T AND NK CELLS AND COMPARING THEIR ADVANTAGES AND LIMITATIONS

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Abstract

Recent developments in immunotherapy against cancer have been very promising. One such therapy is the integration of chimeric antigen receptor (CAR) constructs in T or NK cells. These CAR T and NK cells are engineered to be highly tumour-targeted and are therefore very promising compared to other non-specific therapies. Although proven to be very efficient, CAR T cells have been shown to have a high risk of inducing severe side effects. CAR NK cell therapy seems to be a safer option, as they have a considerably lower risk of causing these side effects. Due to a high risk of developing Graft Versus Host Disease (GVHD), CAR T cells are primarily obtained from the blood of the patient itself (autologous product). In contrast, because of their substantial lower risk of GVHD, NK cells can be derived from a number of different allogeneic sources and with that the possibility of creating 'off-the-shelf' CAR NK cell therapies arises. Moreover, CAR NK cells are able to keep the cytotoxic activity naturally occurring in NK cells and can therefore function in both a CAR-dependent and -independent manner, potentially leading to higher efficiency. Altogether, next to the use of potent autologous CAR T cells in certain tumour types, allogeneic 'off-the-shelf' CAR NK cells might become more desirable with less side effects.

Introduction

Since it became clear that the immune system plays a vital role in the fight against cancer [1], therapies inducing or enhancing the immune response against the tumour cells have been developed. In the last decade, breakthroughs have led to the development of new and the improvement of existing immunotherapies. These therapies include strategies involving antibodies, cytokines, gene therapy, checkpoint inhibitors, and cellular immunotherapy [2,3].

Cellular immunotherapy, based on the administration of immune cells to the patient [4], can be a specific and effective way of treatment [5]. Active therapy, such as dendritic cell (DC) vaccines, stimulates antitumour activity of the immune system. For dendritic cell vaccinations, the DCs are loaded with tumour-derived antigens and subsequently injected into the lymph nodes of the patient. Here, the DCs present the antigens to the T cells and activate an immune response against the tumour. Other injection routes may include intradermal (in the skin) and intravenous (in the veins). In passive therapy, immune cells that have an antitumour effectivity themselves, such as adoptive transfer of T or NK cells, are used. This is also known as adoptive cellular therapy [4,6]. In this therapy immune cells are expanded and modified in vitro , giving them antitumour activity, and transferred into the patient [6]. T cells are most frequently used for this type of therapy, although NK cells are now also upcoming [7]. There are three types of adoptive cell therapies, including tumour-infiltrating lymphocytes (TILs), genetically engineered T-cell receptors (TCRs), and chimeric antigen receptor (CAR) T and NK cells [5]. TILs are (non-genetically) lymphocytes, derived from modified tumour tissue, that are able to infiltrate the tumour and exert their antitumour activity from within [5,8]. CAR and TCRs are based on genetically engineered T and NK cells, that obtain specificity against tumour antigens and are therefore highly effective [9,10].

In this review, these CAR therapies, possible with both T cells and NK cells, will be discussed and compared.

CAR T cell therapy

Chimeric antigen receptors (CARs) give the immune effector cells their tumour-specificity [11,12]. The cells used in this type of therapy are typically autologous T cells, which are cells from the patient itself. CAR receptors are chimeric because both the antigen-binding and the T cell-activating functions are combined into one single receptor [12]. The antigen-binding domain, a single-chain variable fragment (scFv), is derived from an antibody that is known to target a tumour associated antigen [13] and thus varies between therapies for different patients. The scFVs are linked, via a transmembrane region, to an intracellular signalling domain of the CD3ζ chain, that exerts the activating functions. For second-generation CAR T cells, these receptors also include costimulatory molecules such as CD28 or 4-1BB [14,15] (Figure 1). The incorporation of the costimulatory CD28 domain has been shown to give the CAR T cell enhanced antitumour activity, as compared to the first generation CAR T cells [16].

Usually, the T cells are taken from the patient self, modified, and transferred back into the patient [12,15]. Besides killing cancerous cells, the CAR T cells are also able to promote immune surveillance, preventing the tumour from reoccurring [11]. Another major advantage of using CAR T cell therapies, as opposed to other immune therapies, is the fact that CAR T cells can operate independently of major histocompatibility complex (MHC) recognition [15,17]. This way, the therapy is not affected by possible immune evasion exerted by the tumour cells, through downregulation of human leukocyte antigen (HLA) class I molecules or a defective processing capability of antigens [17].

In the best-studied CAR T cell therapy, the T cells recognise the CD19 antigen. CD19 is mainly expressed on the surface of B lymphocytes and is essential for the intracellular signalling of the B cells [11,18]. The fact that the CD19 antigen is expressed on most B cell malignancies, but not on hematopoietic stem cells or other tissues, makes these antigens excellent targets against lymphomas and leukaemias. These include chronic lymphocytic leukaemia and acute lymphoblastic leukaemia (ALL), with a low risk of side effects [10,18]. However, since

CD19 is not required for survival, the tumour cells are able to escape recognition by the T cells by means of CD19 loss or downregulation [18].

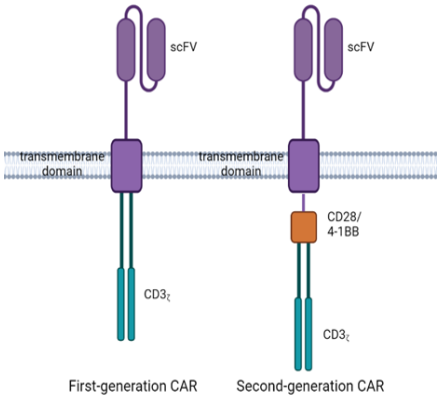

Figure 1: *Structure of first- and second-generation of chimeric antigen receptor constructs. The first-generation includes a scFV that is connected via a transmembrane domain to an intracellular signalling CD3ζ chain. For the second-generation CAR, there is an extra incorporation of a CD28 or 4-1BB domain in between the transmembrane domain and the CD3ζ chain.*

Universal T-cell therapies

As mentioned before, the T cells for this therapy are generally taken from the patient itself, modified, and infused back [12,17]. Although this makes the T cells patient-specific and therefore very efficient with a low chance of rejection, the production of these cells is extremely time-consuming and a highly skilled process. Moreover, cancer patients might develop immunodeficiency or lymphocytopenia after receiving chemotherapy, resulting in insufficient numbers of T cells. To solve these problems, universal 'off-the-shelf' CAR T cells have recently been developed [19,20]. These universal T cells are produced by genetically editing allogeneic T cells from a healthy donor in such a way that they can be used for multiple other patients [20]. However, the HLA expressed on allogeneic T cells might be recognised as foreign, leading to immune reactions against these T cells and thus rejection or even graft-versus-host disease (GVHD) [21].

Side effects

Unfortunately, CAR T cell therapies have been known to cause some severe side effects. These may include cytokine release syndrome (CRS), graft-versus-host disease (GVHD), tumour lysis syndrome (TLS), and immune effector cell-associated neurotoxicity syndrome (ICANS) [12].

CRS is a systemic inflammatory response caused by an increase in the secretion of cytokines, such as IL-1 and IL-6 [22]. The highly proliferative CAR T cells secrete more granulocyte-macrophage colony-stimulating factor (GM-CSF), which has been found to cause oversecretion of cytokines by monocytes and macrophages. By knocking out the GM-CSF production using CRISPR-Cas gene editing, the CRS risk was reduced without affecting the antitumour activity [23]. As a treatment, tocilizumab, which is an IL-6 receptor antagonist, has been shown in clinical trials to effectively work against CRS, without affecting the CAR T cell efficiency [24,25]. In 2017, the FDA approved the use of this drug for the treatment of severe CRS induced by CAR T cell therapy [26].

GVHD is the result of an immune reaction against foreign cells. This may occur in two ways after the infusion of allogeneic T cells. The existing immune system of the recipient may form a response against the infused CAR T cells, or the allogenic CAR T cells may form an immune response against the antigens expressed on the recipient's tissue cells. With the use of second-generation CAR T cells, more specifically those including the 4-1BB co-stimulatory domain, there is a higher risk for GVHD [27].

TLS has been observed after infusion of anti-CD19 CAR T cells [20]. It occurs after a large amount of cancer cells die within a short period of time, leading to the release of nucleic acids, proteins, and electrolytes, such as phosphate and potassium [26]. Thir sudden increase in blood and tissue concentration of electrolytes may cause severe toxic effects, including renal insufficiencies and cardiac arrythmias [28]. TLS generally more frequently occurs in hematopoietic cancers, although it might also occur after treatment of solid tumours [29].

ICANS is a neurotoxicity that might be life-threatening and occurs between 20 to 70% of patients treated with CAR T cell therapy [29]. Typically, ICANS correlates with CRS severity, although it may also occur in absence of CRS [24,30]. Patients with severe ICANS show an increased number of T cells and an increased level of IL-6 in their cerebrospinal fluid, due to a blood-brain permeability that leaves this fluid susceptible to cytokine infiltration [24,30,31]. It has been found that the administration of GM-CSF-neutralizing antibodies has a positive effect on reduction of ICANS in mice by decreasing cytokine release. With neutralizing this stimulatory factor, the blood-brain barrier permeability seems to be decreased to a level that is similar to controls that did not receive CAR T cell therapy [30,31].

Thus, although CAR T cell therapies seem highly efficient, there are a lot of side effects that may arise with the use of this type of therapy. Therefore, these therapies should be used with caution to prevent potential side effects as far as possible. In addition, further research is needed to minimize these side effects and optimize CAR T cell therapies.

CAR NK cell therapies

Natural killer (NK) cells belong to the innate immune system and account for 5-15% of human peripheral blood leukocytes [32]. NK cells are functionally similar to T cells and are able to kill target cells through cytotoxic mechanisms [32-34]. However, because NK cells are part of the innate immune system, they do not require activation by antigen-presenting cells (APCs) [33,35]. This gives them an advantage over T cells in research and treatment, as they will not take up as much time in production [36]. Usually, CAR T cells are activated using monoclonal anti-CD3 antibodies and anti-CD28 antibodies in vitro, mimicking stimulation by APCs, before they can be infused into the patient [35,37]. NK-cell function depends on stimulation of activating or inhibitory signals generated by germline-encoded receptors [32,36,38]. MHC class I molecules, expressed on the surface of normal healthy cells, act as an inhibiting ligand for NK cells [34], giving the NK cells their self-tolerance. Killer cell immunoglobulin-like receptors (KIR) are receptors that are able to recognise these MHC class I molecules [39]. An example of an activating receptor is the NKG2D receptor, which is one of the best-studied activating receptors. This receptor can recognise stress-induced ligands expressed on damaged, transformed, or abnormal cells. Here, ligand-receptor interaction, and the lack of MHC class I expression, will lead to NK activation, subsequent cytokine and chemokine release, and eventually lysis of the stressed cell [40,41]. This receptor-dependent activation gives the NK cells an advantage over T cells in immunotherapy. Since the NK cells are not dependent on antigen recognition, NK cells are able to respond independently of tumour-antigens, resulting in continuous antitumour effectivity even if the tumour downregulates antigen expression [36,42]. Moreover, in the rare event that tumour cells downregulate the expression of

certain MHC molecules in order to escape T cell recognition [39,43], they will become more susceptible to NK response because of reduced KIR-mediated inhibition [32,43].

Fewer side effects

CAR NK cells, although modified, still possess their natural cytotoxicity and functionality, which means that CAR NK cells are able to target tumour cells in both a CAR-dependent and -independent manner [44-46]. With this, the NK cells produce cytokines and chemokines to induce cytotoxicity against the tumour cells, while also reducing risks of relapse in patients due to CAR-dependent mechanisms [38]. This would already be a big advantage over the use of CAR T cells, however, NK cells could be even engineered to have a non-killing functionality of the CAR. Instead, they would use the CAR to promote natural NK target recognition and with that activation of its CAR-independent cytotoxicity [32]. This leads to less on-target/off-tumour toxicity, where normal healthy cells that express the same antigens are recognised by the CAR but will not induce a natural NK cell response [32,46]. Because NK cells possess a different cytokine profile, the risk of a CAR NK cell recipient developing CRS is significantly lower than that of CAR T cell recipients, or even completely absent [42,47]. This is largely because the main driving factors in the induction of CRS are the cytokines IL-1 and IL-6 [22,48], which are mostly secreted by T cells and only rarely by NK cells [34,42,48]. ICANS may be correlated to CRS severity. In many articles, no cases of neurotoxicity, including ICANS, after receiving CAR NK cells have been reported so far, which corresponds to the low risk of CRS with NK cells [48-50].

Lastly, also GVHD seemed to be less prominent with the use of CAR NK cells, despite some mismatches in HLA molecules between donors and recipients [47,51]. NK cells are even able to suppress GVHD because they inhibit alloreactive T cells, which are T cells that form a response against allogeneic MHC peptides, without causing GVHD themselves [51,52]. This reduced risk of GVHD gives rise to the opportunity for the development of 'off-the-shelf' CAR NK cells [48,50], which can be obtained from numerous sources other than autologous [53]. Using CRISPR-Cas techniques, obtaining NK cells that do not express KIRs on their surface has been shown to be possible [50]. This lack of KIRs will make the NK cells HLA genotype-independent, meaning that any donor/recipient match can be made, without causing unwanted rejection effects. This makes NK cells a better fit for 'off-the-shelf' CAR therapy than T cells, which cannot become HLA genotype-independent [50].

Sources

NK cells for CAR-mediated immunotherapy can be obtained from various sources, as illustrated in fig. 2, including cell lines (NK92 cell line), umbilical cord blood (UCB), peripheral blood (PB), and induced pluripotent stem cells (iPSCs) [54-56].

Of the existing NK cell lines, NK92 seems to be the only one that has shown strong antitumour activity [55-57]. They have been extensively used as a source for CAR NK cells because they can expand indefinitely in vitro, and do not show to be affected by multiple freezing rounds [32]. Also, the NK92 cells have little to no expression of inhibitory KIRs or CD16 on their surface [44,48]. Altogether makes these cells perfect for the production of 'off-the-shelf' CAR NK cells. However, since the NK92 cell line is derived from a cancer patient with non-Hodgkin lymphoma [54,58], there are some limitations. The NK92 cells will require irradiation before they can be infused in the patient [32,56], which potentially can be lethal to the individual cells, creating a loss of proliferation of the cells in vivo. Yet, this irradiation is necessary in order to reduce the risk of tumourigenicity [56,59].

NK cells can also be isolated from umbilical cord blood (UCB). These cells have a low expression of CD16 [48,56,59] and are relatively easy to collect as they constitute up to 30% of UCB [59,60]. Moreover, there are only a few T cells present in UCB and most are immature, resulting in a minimal risk of GVHD [60,61]. However, the quantity of cells in UCB is limited, as it contains 10- to 100-fold fewer nucleated

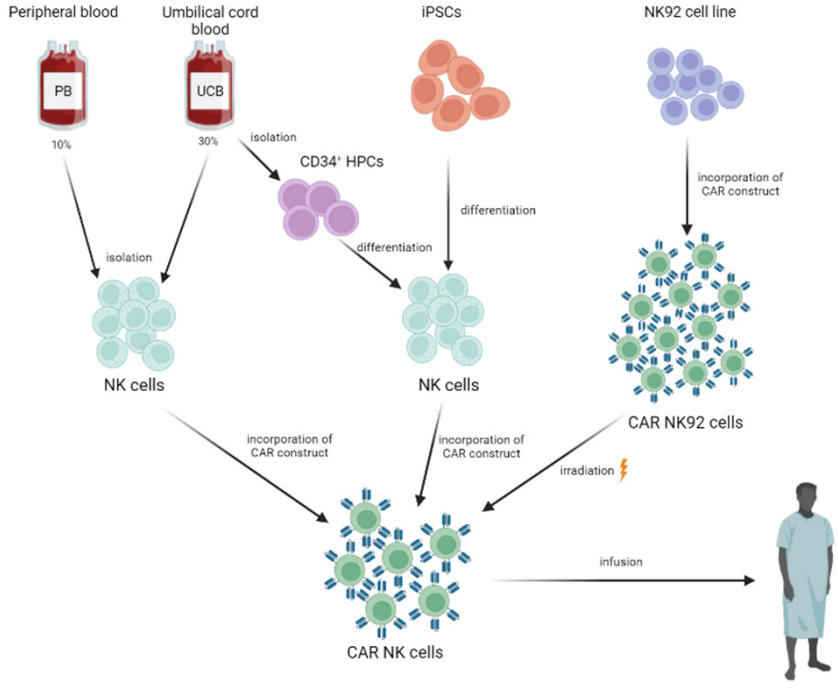

Figure 2: *NK CAR cell sources, including peripheral blood, umbilical cord blood, induced pluripotent stem cells, and NK92 cell line. Of peripheral and umbilical cord blood NK cells can be isolated after which they can be modified unto CAR NK cells. From umbilical cord blood, CD34+ hematopoietic progenitor cells can also be isolated, which can be in turn differentiated into NK cells and subsequently modified to CAR cells. Cells of the NK92 cell line can be directly modified into CAR NK cells, but they have to be irradiated before infusion, leading to a loss of cytotoxicity.*

cells compared to other sources. Moreover, the NK cells obtained from UCB often show an immature phenotype, which has an inferior cytotoxicity [32,62]. CD34+ hematopoietic progenitor cells (HPCs) can also be extracted from UCB, which can serve as an appealing and versatile option for obtaining NK cells [63,64]. These CD34+-derived NK cells have been found to possess higher antitumoural cytotoxicity than NK cells directly isolated from blood [65] and are therefore also an efficient source for CAR NK cells.

In peripheral blood (PB), the amount of NK cells is lower compared to UCM, as they constitute up to only 10%. [48]. The NK cells can be derived from autologous or allogeneic PB, making it a desirable source [66]. Although the same procedure can be applied for the isolation of NK cells from PB and UCB [67], due to the low numbers of NK cells in PB, the isolation is more difficult [56]. Besides, purification is of the highest importance when obtaining NK cells from PBMCs, since remaining B and T cells, which are present in high concentrations in peripheral blood, may cause unwanted side effects [48]. However, approximately 90% of the NK cells isolated from PBMCs have a mature phenotype, giving them a higher cytotoxic activity [32,48,67]. This makes them readily available for therapy and able to be expanded in vivo without any further stimulation [68].

NK cells derived from donor blood are not from a homologous source, making the the development of 'off-the-shelf' CAR therapies more difficult [55,66]. A way to circumvent the dependability on donors is the use of induced pluripotent stem cells (iPSCs) that can be differentiated into NK cells [55]. iPSC-CAR NK cells, expressing NKG2D, showed improved anticancer activity in leukaemia and ovarian cancer [55,69]. Additionally to the CAR construct, other modifications, such as higher or lower expression of certain receptors to further enhance antitumour activity, are easier with iPSCs than with isolated NK cells from blood [69]. Moreover, because the NK cells derived from this source are easily accessible for modification and expansion, 'off-the-shelf' production is readily achieved [70]. Although iPSCs-NK cells seem to be a better fit for CAR therapies due to their numerous advantages, the development of NK cells from this source is a lengthy and expertise-requiring process [38,71].

As of now, the NK92 cell line and peripheral blood NK cells are the most commonly used sources. NK92-derived NK cells have shown higher cytotoxicity levels compared to PB-derived NK cells [70]. However, because NK92 cells require irradiation before infusion, their cytotoxicity is significantly reduced [56,70]. Newer approaches are therefore being developed. Despite their slightly more complicated manufacturing iPSC-derived NK cells will have an advantage over NK cells derived from other sources in the future [71].

Conclusion

In the field of immunotherapy, the development of CAR therapies has been a breakthrough. Here, both CAR T and NK cells have been shownto be effective in targeting and killing tumour cells in various studies. This type of treatment can be highly specific and effective, and is therefore an ideal therapy. However, CAR T cell therapies have a high risk of causing severe side effects, including CRS, GVHD, TLS, and ICANS. These side effects may cause detrimental effects on the patient, in some cases even leading to death [28]. CAR NK cells on the other hand have been associated with a significant lower risk of developing these side effects and are therefore considered a safer option. Moreover, upon the construction of CAR NK cells, the NK cells seem to be able to keep their natural cytotoxic activity, leading to the possibility for CAR NK cells to function in a CAR-dependent and -independent manner. This may result in a higher effectivity in general and also a lower risk of on-target/off-tumour effects.

Another advantage of using NK cells instead of T cells in CAR therapies is the fact that NK cells can be derived from various different sources. T cells most often need to be derived from the peripheral blood of the patient [54] to reduce the risks of GVHD. However, since the patient may have developed immunodeficiency or lymphocytopenia after receiving chemotherapy, there might be insufficient amounts of T cells present, leading to difficulties in the development of a CAR T cell therapy. Because allogeneic CAR NK therapies have a significantly lower risk of GVHD, the sources of NK cells are endless. Currently, various sources are already being used and improved, but also new sources of NK cells are being discovered. An example of such a new source is the NK101 cell line, derived from a patient with an extra-nodal natural killer/T-cell lymphoma, as an improvement on the NK92 cell line [72]. This NK101 cell line has been shown to produce higher levels of pro-inflammatory cytokines and can positively influence leukocyte proliferation.

Also, feeder cell lines, that make expansion of the NK cells possible, are being developed [73]. Because of these various sources, is also possible with CAR NK cells to easily produce 'off-the-shelf' CAR therapies, further increasing the efficiency of the therapies.

Although most of the CAR therapies are being used in hematopoietic cancers, solid tumours may pose as targets as well. However, tumours are able to evade T cell recognition by several mechanisms within the tumour microenvironment, which may explain the often poor results of CAR T cell therapies in solid tumours. On the other hand, CAR NK cells, mainly derived from the NK92 cell line, have proven to be more successful for such solid tumours and can thus also be used to target cancers, such as pancreatic, ovarian, and prostate cancers [50,54]. However, this type of treatment is still in early clinical development and cannot yet be used on patients [74], but it holds a promising future.

Currently, another type of CAR therapy is being developed using macrophages to increase efficiency in treating solid tumours [75,76]. However, it seems that there is a hurdle in the production of CAR macrophages (CAR-Macs) because there is no possibility for expansion. For this, the CAR constructs are being incorporated into iPSCs. These CAR-containing iPSCs will be instructed to differentiate into macrophages, generating CAR-iMac lineages, which thus also contain the CAR construct [77]. Although CAR-Macs have been seen to be effective and therefore a promising therapy, they are still in pre-clinical stages [76]. It is expected that this therapy in the future will become more efficient, although some limitations are likely yet to be uncovered.

All in all, next to autologous CAR T cells, 'off-the-shelf' CAR NK cells may be the more desired therapy in certain tumour types and clinical settings, due to several advantages. These include a higher cytotoxicity of the NK cells against the tumour cells, a lower risk of developing side effects, the possibility of deriving NK cells from various sources, and the possibility of developing 'off-the-shelf' CAR NK cells. However, there are still some limitations linked to this type of therapy. New sources of NK cells are being developed, as well as new CAR therapies using macrophages. Although CAR-Macs are still in very early stages, there seems to be effectiveness and they may pose a promising future therapy.

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