CAR T NKR-2: Leveraging the Breadth of Innate Immunity

White Paper

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Introduction

Our immune system rapidly and effectively attempts to destroy invading pathogens and cells under stress, to defend the body from disease. It employs a carefully orchestrated interaction of cells and molecules to identify and eliminate viruses and bacteria in the blood and also normal cells of the body that have been infected by these pathogens. This ability to eradicate abnormal cells is also highly relevant to cancer. Tumors arise through mutations of normal cells that allow them to overcome their intrinsic growth control mechanisms and begin to exhibit uncontrolled growth. These tumors continue to develop where either the primary tumor or metastases emanating from the primary tumor eventually lead to the early demise of the patient.

The mainstay of cancer treatment today continues to include surgery, chemotherapy, and radiotherapy. While surgery can effectively cure the patient if the tumor is captured at a sufficiently early stage, for the majority, surgery is not feasible due to the inaccessibility of the tumor or metastatic disease. Chemotherapy and radiotherapy effectively depend upon the rapid growth of the tumor cells. However, the genetic instability associated with tumors enables the tumor itself to rapidly develop resistance mechanisms resulting in the patient generally relapsing at a time after initial response to therapy.

Whilst the concept of exploiting the immune system to target cancer is not new, having originated with Coley’s Toxins over 130 years ago, the means to effectively engage the immune system and drive consistent clinical responses has only developed more recently largely through the development of monoclonal antibodies 20 years ago, and more recently with the advent of checkpoint inhibitors.

Consequently, while there has been an improvement in patient survival over the last few decades [1], cancer continues to be the second leading cause of death according to the Center for Disease Control in the United States. Though a new class of therapies, chimeric
antigen receptors T cells (CAR-T), showed impressive clinical results and is about to be available for patients with different B-Cell lymphomas and leukemias, there will remain a major unmet need to develop therapies that may synergize with current approaches or provide new paradigms that can drive improvements in patient survival for all cancers, particularly for patients with solid tumors which have proved more challenging for cell-based treatments to penetrate.

*Cancer Immunotherapy – Monoclonal antibodies and beyond*

Over the last 20 years, cancer immunotherapy has risen to prominence to reach mainstream acceptance in large part through the clinical impact of monoclonal antibodies. Immunotherapy aims to develop approaches that simultaneously enhance immunity while preventing local immune suppression. While extensive studies investigating cytokines and vaccines to induce tumor-specific immunity have met with some limited success in the clinical setting, monoclonal antibody technology has defined the rise of cancer immunotherapy. FDA approval of blockbuster antibodies including rituximab, trastuzumab, and cetuximab, brought antibody technology from the academic to industrial setting and are now regarded as part of the standard of care for different cancers. Whilst these antibodies may involve receptor blocking, they generally also carry immune modulatory functions. More recently, antibodies targeting ‘immune checkpoints’, or key pathways that can effectively switch off the body’s immune response, have been employed [2]. A recent series of antibodies that block the immune checkpoint including nivolumab and pembrolizumab can release local immune cells from their suppressed state resulting in impressive clinical responses across several cancer indications.

While antibodies can potentially activate the local immune response within the patient, the issue remains that most tumors are primarily derived from the body’s own cells, referred to as ‘self’. These ‘self’ cells underwent some alterations to form the tumor cells which retain most of their ‘normal’ characteristics and acquire a relatively small proportion of ‘different’ or
‘new’ characteristics. Checkpoint inhibitor specific antibodies can release endogenous anti-tumor responses; however, the immune system is strongly regulated to prevent recognition of ‘self’; thus, there may not be tumor-specific immune cells present within any given individual, that can deliver a therapeutic impact upon the tumor.

**CAR T-Cell Therapy – ‘providing what the patient lacks’**

Many antibodies, vaccines and cytokines are dependent upon releasing or activating the immune system resident within the patient, and the success of these therapies is therefore entirely dependent upon the anti-tumor specificity of the patient’s immune repertoire, which is mainly based on the generation of a T-cell immune response.

T-cells are a major part of the adaptive immune response and are effectively the cavalry of the immune system. They consist of the CD8+ cytotoxic T-cells and the CD4+ helper T-cells, both of which broadly function to kill target cells and produce cytokines that orchestrate an immune response. They can also generate immunological ‘memory’ for a future response against the previously encountered pathogenic agent, and magnify the T-cell response against the target. The innate immune system also comprises, among other entities, Natural killer (NK) cells and macrophages which can rapidly respond to an infection using evolutionarily older and generic systems. These cells are the foot soldiers that can mount a rapid attack but do not generate memory. Thus, NK cells and macrophages can deliver a short, immediate response that is then magnified by the arrival of T-cells.

In the tumor environment, both CD4 and CD8 T-cells can be seen along with macrophages, NK cells and other immune cell types. However, these immune cells are generally suppressed. The tumor itself expends a significant level of effort to escape immune recognition by producing messenger molecules that can shut off the immune response, exploiting immune checkpoints and downregulating proteins that are essential for T-cells to recognize the tumor. T-cells require the presence of cell surface proteins called Major
Histocompatibility Complex (MHC) to which they bind, thus identifying the individual cell as healthy or abnormal. If abnormal, the T-cell recognizes this and is activated to thereby eliminate the abnormal cell. However, there is strong evidence that tumors can down-regulate MHC, thus, even if the T-cell is relieved from checkpoint inhibition, the tumor may effectively be invisible to the T-cell, thereby avoiding elimination.

To address this issue, Zelig Eshhar, working at the Weizmann Institute in Israel, conceived the idea of engineering a T-cell to bypass MHC downregulation. His concept was to express a fusion protein consisting of a portion of an antibody with a portion of the T-cell activating receptor. This engineered T-cell could use the targeting specificity of the antibody to activate the T-cell against a particular cell surface protein, thereby avoiding MHC restriction and tumor immune surveillance. This engineering approach has developed over the intervening 20 years to the clinical concept that is today known as CAR (Chimeric Antigen Receptor) technology. [3-5]

The CAR today comes in many flavors but the underlying concept remains unchanged. Most CARs consist of an extra-cellular target binding domain (most commonly a single chain variable fragment, scFv, derived from a monoclonal antibody) that is fused to a transmembrane anchor by an intervening extracellular spacer, and an intracellular domain (see figure 1 below).
The intracellular domain is fused to the other end of the transmembrane domain and consists of at least one signalling domain that transmits the signal for T-cell activation. In the earliest versions of the CAR, a single signalling domain, typically derived from CD3ζ, was used, but this first-generation receptor appears sub-optimal in terms of driving T-cell activation and, most likely, clinical responses. Second generation CARs have been engineered with an additional co-stimulatory signalling domain, thereby enabling the single binding event of the CAR to drive full T-cell activation. Subsequent CAR generations have been created as have further iterations of the basic CAR approach. [6,7] However, it is the second-generation CAR that is currently most advanced in clinical development, with multiple companies currently striving for licensing of the CD19 CAR T cell therapy for B-cell malignancies.

**The challenges that reside beyond CD19 CAR T cell therapy**

The CD19 CAR T cell approach has seen early clinical success through high levels of clinical responses observed in patients with highly advanced B-cell leukemia and lymphoma [8,9]. However, the same paradigm using CARs specific for tumors other than B-cell malignancies have yet to deliver anywhere
near the same level of clinical success. Moreover, while the CD19 CAR T cell approach is delivering an effective initial clinical response in most patients, there is evidence of CD19 negative tumor variants developing in patients receiving the treatment, suggesting that antigen-loss, that causes the tumor cell variants to escape immune detection, could be an emerging issue. [10, 11]

To reach a broader scope of cancers and avoid the development of tumor variants, tumor cell targeting is a critical factor. The specificity of the monoclonal antibodies from which scFv are typically derived, enables CAR to be precisely targeted to a specific antigen (molecule on tumor cells recognized by cells of the immune system). For B-cell malignancies, CD19 may be the optimal target antigen since it is expressed broadly on B-cell tumors and the concomitant loss of normal B-cells can be routinely managed as a side-effect of the treatment. The issue for non-B cell tumors is the availability of a suitable target antigen. Several are being tested but in many cases, the target may also reside on healthy tissues that could therefore be targeted by the high affinity scFv directing the activity of the CAR. In cases where the target is expressed on an important healthy cell, such as a cell of the lung or heart, significant toxicity could result. Additionally, the single target specificity of the scFv itself is undoubtedly leading to the strong selection of antigen-escape tumor cell variants. This means identifying further targets on the antigen-loss variants that can then subsequently be targeted by other CAR approaches.
**CAR T NKR-2: A unique CAR on the block**

The challenges met by CD19 CAR-T led to the investigation of ‘natural’ receptors to target CAR T cells and, specifically, the use of the NK cell foot soldier to aid the T-cell cavalry in targeting and eliminating tumors.

**Combining infantry with the cavalry to tackle hematological and solid tumors**

Professor Charles Sentman working at Dartmouth College, USA, exploited the generic targeting strategies of NK cells to direct T-cells to the target tumor cells. The strategy was to combine the rapid response of the NK cell with the potency and immunological memory generating power of the T-cell. This involved fusion of an NK cell surface receptor, Natural Killer Group 2D (NKG2D) protein, to the CD3ζ cytoplasmic domain of the T-cell receptor, to form a novel CAR. The NKG2D portion of the CAR in this context provides target specificity, while the cytoplasmic CD3ζ region initiates the T-cell activation signal when the CAR binds to its target. Thus, a T-cell engineered with this NKG2D-CD3ζ CAR (hereafter termed CAR T NKR-2) activates its effector functions when the NKG2D portion of the CAR binds target ligands present on the surface of stressed cells. [12]

At first glance (see figure 2), this construction resembles a basic first generation CAR design and thus likely to be sub-optimal in terms of activating a T-cell. However, NKG2D has natural binding partners that stabilize the expression of the protein, like the DAP10 protein that upon engagement of NKG2D with cognate ligand activates a signal similar to the co-stimulatory signal derived from the CD28 protein in classical CARs. Thus, the NKR-2 CAR initiates both signal 1 and signal 2 upon ligand binding in a manner resembling that of a second-generation CAR. [12]

NKG2D is a receptor expressed mainly on NK cells and naturally involved in the protection of the host from infections and cancer, through recognition of its so-called NKG2D ligands at the surface of cancer cells.
An obvious question is why not use NK cells for the CAR T NKR-2 approach since these cells are those where the NKG2D protein generally is expressed and functional. There are two fundamental reasons why using the NKR-2 CAR in T-cells makes sense. First, the NKG2D protein is one of a myriad of receptors that function within the NK cell. Some of these are activators but these are balanced out by inhibitory receptors. Thus, over-expressing the NKR-2 receptor in NK cells may expose the CAR to these inhibitory receptors and could blunt the activation of the CAR. Second, engineered T-cell therapy is at a more advanced stage than engineered NK cell therapy in terms of clinical delivery and efficacy. While NK cell therapy is in clinical development, most studies to date have used a NK cell line (NK92), and even short term culture causes primary NK cells to become activated NK cells that lose their capacity to discriminate based upon MHC expression illustrating the current difficulties of working with primary NK cells. [12]

In addition, CAR T NKR-2 employs a fully human construct, which minimizes a graft-versus host reaction that could be seen with humanized constructs that are modified to be very
similar to the naturally occurring human proteins. Using a fully human CAR T NKR-2 product also enables multiple dosing that could increase treatment efficiency.

A truly broad CAR T cell therapy potentially targeting 80% of cancer indications

As mentioned earlier, there are growing reports of CD19 negative tumor variants arising after the effective eradication of CD19 positive leukemia by CD19 CAR T therapy. The challenge is now to develop a CAR strategy that enables the effective targeting of multiple targets on a tumor cell, thereby reducing the risk of target-negative tumor variants. One approach being explored is relatively binary and involves the selection and testing of other CARs in parallel with the CD19 CAR, such as CD20. Other engineering approaches involve expressing multiple binding domains to direct a single CAR to several targets.

An alternative is offered by CAR T NKR-2 and exploits the natural specificity of NKG2D to bind eight different ligands commonly expressed on the surface of stressed cells including tumor cells. The ligands bound by NKG2D are MHC like proteins that do not function like MHC molecules that bind the T-cell, but have evolutionarily evolved to act as markers of stressed cells. Stressed cells are those that may be infected by pathogenic bacteria or viruses, endure metabolic stress or caused by abnormal events such as untoward up-regulation of DNA repair systems. The latter is an event common in tumor cells where genetic instability requires near constant DNA repair activity. Thus, most tumor cells tend to express these stress proteins commonly on their cell surface and can provide a handle upon which the NKR-2 cells can target and eliminate tumor cells.

The eight ligands consist of MIC-A, MIC-B and the ULBP’s 1-6. The RNA encoding these ligands are present within many tissues but the protein is only selectively expressed due to secondary regulatory mechanisms [13]. In this manner, the proteins can be rapidly expressed should the individual cell encounter a stressful situation without the delay caused by initiating transcription of silent genes.
Accordingly, ULBP transcripts in humans appear to be widely expressed in healthy adult tissues (i.e. kidney, prostate, uterus, tonsil, and lymph node) [14] but no ULBP1-3 protein expression was reported in control normal tissues [15–18]. Similarly, immunohistochemistry showed that most human tissues (heart, brain, liver, thyroid, lung, skin, kidney, placenta, adrenal gland, tonsil, and spleen) do not express MICA, but gastric and glandular epithelial cells [19, 20] and podocytes within the renal glomeruli [21] do express MICA, although subsequent studies nevertheless determined that most MIC-positive normal gut epithelial cells expressed the protein intracellularly, rather than at the cell surface [22].

In contrast, many tumor cell lines and primary tumors from diverse tissue origins express NKG2D ligands (see Table 1), making them an excellent target for cancer therapy [24, 27]. However, NKG2D ligands on primary tumor isolates are heterogeneous with respect to the ligands found and amounts expressed, and ligand expression can also vary with tumor progression [28–32].
Interestingly, animal studies have shown endothelial cells from the tumor vasculature to express NKG2D ligands [67,68], and may be recognized by NKG2D-specific effector cells even when the tumor cells themselves did not express the ligands [68]. Similarly, immunosuppressive regulatory T cells (T-reg)s [69,70] and myeloid-derived suppressor cells (MDSCs) [69–71] isolated from the tumor microenvironment (TME) of tumor-bearing mice, but not from naïve mice or from healthy tissues, express NKG2D ligands and may be targeted by NKG2D-specific CAR T NKR-2 effector cells [69–73]. Overall, these data support the idea that NKG2D can be used to target a wide range of tumor types, through recognition of 8 ligands from 2 main families, reducing the probability of clonal selection of target-negative tumor cells during the treatment, and also targeting their supportive immunosuppressive and vasculature-associated cells of the tumor microenvironment.

Table 1 Expression of NKG2D ligands on human tumor cells

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Expressed NKG2D ligand</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>28-67% MICA/B, 9-20% ULBP1-3</td>
<td>[30–36]</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>0-75% MICA/B, 16-50% ULBP1, 4-64% ULBP2, 16-100% ULBP3</td>
<td>[29–31, 34, 35, 37–47]</td>
</tr>
<tr>
<td>Bladder Carcinoma</td>
<td>70% MICA</td>
<td>[48, 49]</td>
</tr>
<tr>
<td>Brain cancer</td>
<td>90% MICA, MICB and ULBP1-3</td>
<td>[18, 50, 51]</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>35-100% MICA/B, ULBP 1-5</td>
<td>[20, 52–55]</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>20% MICA, ULBP2</td>
<td>[56, 57]</td>
</tr>
<tr>
<td>Chronic Lymphocytic Leukemia</td>
<td>0-85% MICA/B, 10-20% ULBP1-3</td>
<td>[31, 35, 38, 59]</td>
</tr>
<tr>
<td>Chronic Myeloid Leukemia</td>
<td>28-100% MICA/B, 12-20% ULBP1-3</td>
<td>[31, 35, 60, 61]</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>80-100% MICA/B, ULBP 1-5</td>
<td>[20, 62, 63]</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>40-100% MICA/B, ULBP2</td>
<td>[64–66]</td>
</tr>
<tr>
<td>Hepatocellular Carcinoma</td>
<td>60-100% MICA,</td>
<td>[17, 67–69]</td>
</tr>
<tr>
<td>Head and neck squamous cell carcinoma</td>
<td>100% MICA/B (7/7 cell lines)</td>
<td>[70, 71]</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>28-44% MICA/B, 12-20% ULBP1-3</td>
<td>[33, 35, 36, 72–79]</td>
</tr>
<tr>
<td>Melanoma</td>
<td>50% MICA/B</td>
<td>[26, 80–82]</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td>10-60% MICA, 0-34% ULBP1-3</td>
<td>[28, 83–88]</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>86% MICA/B, ULBP1-3</td>
<td>[89]</td>
</tr>
<tr>
<td>Non-small-cell lung carcinoma</td>
<td>20-30% MICA/B, ULBP1-3</td>
<td>[20, 90–92]</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>50-97% MICA/B, ULBP1-5</td>
<td>[20, 27, 93–95]</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>68-89.3% MICA/B</td>
<td>[96–98]</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>75-95% MICA/B, sMICA/B</td>
<td>[20, 99]</td>
</tr>
<tr>
<td>Renal Cell Carcinoma</td>
<td>&gt; 95% MICA/B</td>
<td>[20, 61, 100, 101]</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>100% MICA/B, ULBP 1-3</td>
<td>[102, 103]</td>
</tr>
</tbody>
</table>
Unique mode of action, targeting tumors from different angles

Professor Sentman’s pre-clinical studies using mouse T-cells armed with CAR T NKR-2 showed very high efficacy in a series of established solid and hematological models achieving complete eradication of tumors in many models. His series of experiments in multiple cancer indications such as leukemia, myeloma, colorectal cancer, melanoma, and ovarian cancer [12], concluded the following on the characteristics of NKR-2:

• NKR-2 T cells could drive an effective anti-tumor response in the absence of pre-conditioning chemotherapy or with only low-grade lymphodepletion. Pre-conditioning (lymphodepletion) is considered essential for the clear majority of current clinical CAR T approaches including CD19 CAR T cell therapy, to avoid rejection of the engineered cells, but is associated with enhanced toxicity or graft rejection when donor cells from another person are used due to their increased persistence [111-113]. Indeed, experiments using sub-optimal dosing of CAR T NKR-2 cells imply that pre-conditioning has no beneficial effect on the in vivo anti-tumor activity of CAR T NKR-2 cells [114]. This is a major difference between CAR T NKR 2 and current CAR-T therapies, with potentially important consequences in terms of patient safety, and length of hospitalization.

• Optimal response was achieved using multiple doses of CAR T NKR-2 cells administered over the course of a few weeks, without issues related to activation of the immune system, due to short persistence of the injected cells and use of an autologous (human) construct, hence enabling improved efficacy [106,115]. The standard CAR T cell therapy approach involves giving a single or split dose soon after pre-conditioning to exploit the subsequent short window of opportunity for enhanced CAR T cell engraftment. The progression of tumor-infiltrating T-cells and CAR T-Cells to hypo-functionality is a limitation to solid cancers, successfully overcome by serial T-cell infusions that resulted in a near-doubling of animal survival without overt toxicities [116-118]. Hence, even though
it might involve multiple administrations over a few weeks, CAR T NKR-2 is likely to generate a longer lasting response than the standard CAR T therapies.

- Investigation of tumors in animals receiving CAR T NKR-2 cells showed a dramatic impact upon the immune suppressive tumor microenvironment including a startling reduction in the number of T-reg and MDSCs expressing NKG2D ligands and an apparent influx of pro-inflammatory 'M1' like myeloid cells [106, 107, 119]. These observations, which are not reported in relation to other CAR T therapies, are indicative of a re-modelling of the tumor microenvironment putatively towards that of a less immune suppressive character even without the addition of other external compounds such as checkpoint inhibitors that inhibit tumor cells’ defense mechanisms.

- Additionally, a strong anti-tumor response was achieved when treating tumor cells that lacked expression of the targets required for CAR T NKR-2 activity [120]. Further investigation indicated that the CAR T NKR-2 cells were also targeting tumor endothelial cells expressing NKG2D ligands [105]. These endothelial cells generate new blood vessels (angiogenesis) that enable the tumor to grow beyond a few millimeters in size to reach a clinically relevant stage. Therefore, the destruction of these endothelial cells by CAR T NKR-2 cells is suggestive of an anti-angiogenic activity. Moreover, targeting of non-neoplastic (non-tumor) cells within the tumor environment provides an additional level of targeting for the systemically infused CAR T NKR-2 cells i.e.; these cells have the potential to engage target and activate before the need to infiltrate into the tumor mass, at least partially overcoming the barrier to standard CAR technology that requires access to targets buried deep within the tumor to undergo activation.

- Several experiments suggested that the CAR T NKR-2 therapy could induce an immunological anti-tumor memory, enabling a potential long-term persistence of the therapy despite short-term persistence of the injected cells. Animals that had eradicated
a tumor could then eradicate a second subsequent challenge with the same tumor. Furthermore, this immune response was tumor specific since cured animals failed to challenge the growth of different tumors. These immune responses occurred in the absence of CAR T NKR-2 – no NKR-2 cells could be detected in these animals, showing that the induced immune responses were the result of CAR T NKR-2 cell therapy but not requiring the long-term persistence of the NKR-2 cells. In confirmation of the absence of long-term persistence, further studies confirmed that NKR-2 cells persist to detectable levels in the mouse up to 10-14 days, strongly suggestive that the NKR-2 therapy has induced immunological anti-tumor memory. [115, 121, 122]

Hence, CAR T NKR-2 functions by a 4-pronged mode of action that includes:

1. Direct tumor cell killing activity
2. Immuno-modulation towards a less suppressive immune function
3. Anti-angiogenesis or prevention of the formation of new blood vessels around the tumor that would support its growth
4. Long-term anti-tumor immunological memory to minimize the chance of relapse

On-target, off-tumor toxicity

With any therapy, there are generally toxicities that mean the risk to benefit analysis around toxicity and clinical response determines whether an individual therapy is likely to progress. For CD19 CAR T cell therapy, the toxicities have proven to be significant and include cytokine release syndrome (CRS) and neurological toxicity besides the undesirable effects of the chemotherapy used to pre-condition the patient prior to receiving the study treatment [123, 124]. However, the extraordinary levels of objective clinical response observed, counter-balance these toxicities.

For CAR T NKR-2 therapy, at this early stage, no toxicities are apparent but there are clearly hypothetical risks that have been considered. The main risk is on-target, off-tumor toxicity.
For antibody-directed CAR T cells, the target is generally a single entity and thus the expression profile of the target can be relatively readily assessed. For CAR T NKR-2, there are eight potential target ligands, which although expressed on most tumors and thought to be largely absent from normal, healthy tissue, are expressed when healthy cells are stressed. Hence, the expression profile of CAR T NKR-2 is a composite involving eight targets and the potential status of the individual cells.

CAR T NKR-2 has demonstrated complete responses and long-term survival in several murine models of both hematologic and solid tumor types, with no evident toxicity [12]. Single administrations of low dose CAR T NKR-2 in humans also displayed an encouraging safety profile, as detailed in the table below.
Table 2: Characteristics differentiating CAR T NKR-2 from classical CD19-CAR T therapies

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Classical CD19-CAR T Therapy</th>
<th>CAR T NKR-2 Therapy</th>
</tr>
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<tbody>
<tr>
<td><strong>Construct</strong></td>
<td>Fragment (scFv) of an antibody engineered on T-cells that recognizes one specific target on tumor cells</td>
<td>NKG2D receptor engineered on T-cells that recognizes a range of targets on tumor cells</td>
</tr>
<tr>
<td></td>
<td>CD3ζ signaling domain</td>
<td>CD3ζ signaling domain</td>
</tr>
<tr>
<td></td>
<td>Requires one or more added co-stimulatory signals</td>
<td>Naturally expressed (DAP-10) co-stimulatory signal already within the cells</td>
</tr>
<tr>
<td><strong>Engineered Cells</strong></td>
<td>Requires <em>in vivo</em> expansion</td>
<td>Only limited <em>in vivo</em> cell expansion</td>
</tr>
<tr>
<td></td>
<td>Long persistence of CAR-T cells after treatment administration</td>
<td>Short persistence of NKR-2 after treatment administration</td>
</tr>
<tr>
<td><strong>Mode of action</strong></td>
<td>Direct anti-tumor killing by CAR T cells</td>
<td>• Direct anti-tumor killing by NKR-2,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Destruction of specific endothelial cells in blood vessels around the tumor (anti-angiogenic effect),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Reduction of immunosuppression in the TME,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Induces long-term anti-tumor immunological memory thus minimizing disease relapse</td>
</tr>
<tr>
<td><strong>Pre-conditioning</strong></td>
<td>Employs pre-conditioning chemotherapy (lymphodepletion)</td>
<td>Lymphodepletion does not appear to be essential from pre-clinical studies.</td>
</tr>
<tr>
<td><strong>Delivery schedule</strong></td>
<td>Need to inject the entire dose within a few days to exploit window provided by pre-conditioning</td>
<td>Can dose over several weeks</td>
</tr>
<tr>
<td></td>
<td>One single/split dose administered during short window period</td>
<td>Multiple injections possible for better efficacy and controlled kinetics</td>
</tr>
<tr>
<td></td>
<td>Multiple dosing may require additional pre-conditioning</td>
<td></td>
</tr>
<tr>
<td><strong>Patient Experience</strong></td>
<td>May need up to 2-week hospitalization period after treatment administration due to pre-conditioning regimen</td>
<td>Administered at out-patient clinics without extensive hospitalization</td>
</tr>
<tr>
<td></td>
<td>Cytokine release syndrome and neurological toxicities common. Long term B-cell aplasia</td>
<td>No obvious toxicities observed as yet at low cell doses. Short term persistence and lack of patient pre-conditioning suggests likelihood of acute CRS is low.</td>
</tr>
<tr>
<td><strong>Potential indications</strong></td>
<td>Targets B-cell leukemias/lymphomas; Limited by the mono-target specificity of the scFv-CAR construct.</td>
<td>Single product applied to blood and solid cancers</td>
</tr>
</tbody>
</table>

TME: tumor microenvironment
Clinical development of CAR T NKR-2

First-in-human single-dose Phase 1 CM-CS1 trial.

These pre-clinical observations supported the initiation of a Phase I clinical trial of NKR-2 cells at the Dana Farber Cancer Institute, (CM-CS1, NCT02203825). This trial focused on the safety evaluation of NKR-2 cells systemically infused as a single dose in patients with hematological tumors (multiple myeloma (MM), myelodysplastic syndrome (MDS), acute myeloid leukemia (AML)) and followed a standard 3 by 3 dose escalation strategy. The starting dose was a highly conservative $1 \times 10^6$ total cells per patient, rising to $3 \times 10^7$ total cells per patient at the highest dose level. The trial was concluded at the end of the highest dose escalation with the preliminary results reported at the American Society of Hematology (ASH) annual general meeting in December 2016. Seven AML and five MM patients were successfully treated with freshly prepared NKR-2 cells at the indicated cell doses. There were no observed dose limiting toxicities nor other adverse events identified that could be related to the NKR-2 cells.

As anticipated from pre-clinical studies, there was no evidence of long term systemic persistence of the NKR-2 cells and no observable shift in cytokine or chemokine profiles in the patient’s peripheral blood over the first 28 days. At the Day 28, there were officially no treatment responses by the criteria set within the trial protocol. However, the pre-clinical model data suggests that the NKR-2 cell mode of action involves the induction of a tumor specific immune response and this is likely to require some time post-infusion. While not definitive, there were some observations classed as ‘unexpected activity’ where several patients had responded to a subsequent therapy to a higher level than that expected. Furthermore, a patient treated for AML at the highest dose level tested ($3 \times 10^7$ total cells per patient), experienced prolonged survival and a restoration in hematological parameters with no subsequent therapy.
NKR-2 cells given as a single infusion in the CM-CS1 trial showed an encouraging safety profile. The low level of dosing and the use of only a single dose as compared to the successful regimens used in the pre-clinical models did not initially suggest that clinical responses would be seen. However, there were some intriguing observations that provided unexpected results, although only for individual patients within an early phase clinical trial.

The safety profile and clinical observations although at doses far below that thought to be relevant for clinical activity from the mouse models, strongly supported further clinical testing with multiple administrations at higher doses.

*THINK: Phase 1 multi-dose clinical trial.*

The pre-clinical studies carried out by Professor Sentman at Dartmouth College showed the efficacy of CAR T NKR-2 therapy but that this was optimal when multiple doses of CAR T NKR-2 were infused at doses that approximate to around the $1 - 3 \times 10^9$ cells per dose for an 80 Kg patient. To move the clinical development toward this level and frequency of dosing, the THINK trial (NCT03018405) was conceived. The trial strategy involves three doses of CAR T NKR-2 given at two-weekly intervals starting at a $3 \times 10^8$ cell dose escalating to $10^9$ and then $3 \times 10^9$ cells per dose. To deliver these doses, a single leukapheresis or white blood cell collection, will be taken from the patient and used as the source to generate all three doses of CAR T NKR-2, which are then cryopreserved at very low temperatures, and released to the clinical centers when needed. Again, in contrast to most current CAR T cell trials including CD19 CAR T cells, the patients are not necessarily lymphodepleted prior to infusion of the NKR-2 cell product, in line with the mechanisms of action identified in pre-clinical model systems.

Given the broad targeting potential of CAR T NKR-2, the trial encompasses two arms; the first focuses upon hematological tumors following on from the CM-CS1 trial (AML, MM), and the second arm involving exploration in five solid tumor types (triple negative breast cancer,
ovarian, bladder, pancreas and colorectal). The choice of the tumor types was primarily based on the presence of NKG2D ligands on the surface of the tumors and the great unmet medical need. Patients chosen had either relapsed or refractory cancers that were resistant to standard of care therapies. The first dose escalation segment of the trial involves a standard 3 by 3 dose escalation to the maximum tolerated dose (MTD) in each arm. Once the maximum tolerated dose is identified, the trial moves into the second dose expansion segment where 7 different parallel groups of patients, each with a different cancer type, receive the MTD identified in the previous dose escalation segment. The plan for this expansion segment is to recruit up to fourteen patients per indication with the primary intention of further evaluating the safety of CAR T NKR-2 cell therapy at the recommended dose as well as early indicators of clinical activity that could support movement into a future clinical trial to assess treatment response in a controlled manner.

The broad targeting capability of the NKG2D receptor towards 8 different ligands expressed on the surface of various types of cancer cells, allows the THINK trial to encompass a broad range of cancer types, contributing to an undoubtedly large Phase I trial. The key questions relate to individual cancer types and whether the CAR T NKR-2 therapy is safe across all cancers assessed, and whether there are hints of clinical efficacy in specific indications. Hence, the trial is designed as an intensive test of the broad targeting capability of CAR T NKR-2 across seven different solid and hematological cancers.
Conclusion

CAR T NKR-2: A different CAR T cell therapy approach

Here we describe a different CAR-T that replaces the transplant paradigm by a controlled kinetics paradigm, similar to that of a drug. The exploitation of NK cell specificity to target T-cells against a breadth of tumors, provides CAR T NKR-2 with a novel approach. Lack of pre-conditioning, the targeting of tumor endothelium, modulation of the tumor’s immunosuppressive microenvironment, and induction of anti-tumor immunological memory coupled with short term persistence of the injected cells, sets CAR T NKR-2 apart from the CAR T field and importantly, could enable the application of a cell-based immunotherapy to solid cancers. Unlike the gold standard CD19 CAR T, CAR T NKR-2 leverages the power of the patient’s immune system to eradicate tumors and prevent their relapse, representing a paradigm-shift in cell-based immuno-oncology. Of course, this is dependent upon achieving clinical responses in patients with highly advanced, very difficult to treat cancers that require the plurality of mechanisms of action of CAR T NKR-2.

This approach could provide an option that is accessible to more patients (including the elderly), safe, and potent against 80% of all cancers, consequently bringing us closer towards finding a one-cure-fits-all weapon against cancer.
References


upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood 105:3615–3622.


