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Introduction to Chimeric Antigen Receptor (CAR) engineered immune cells

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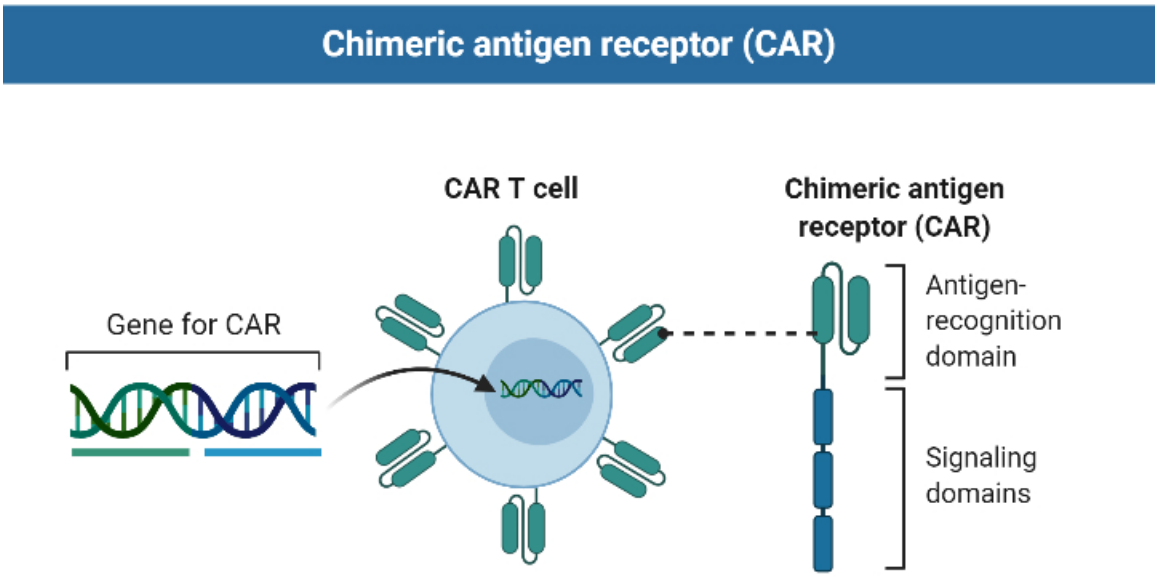
In 2013 the journal Science hailed "Immunotherapy" as the breakthrough of the year [1]. Two crucial advances were highlighted. The first was the use of antibodies to block negative signals on T lymphocytes (called "checkpoint inhibitors") and the second was the use of gene vectors to engineer the expression of a new protein on the surface of T lymphocytes, specifically a Chimeric Antigen Receptor or CAR.

The immune system recognizes foreign cells and proteins that it perceives as dangerous through either cell-mediated immunity, which is the domain of T lymphocytes (T cells) or through the production of soluble proteins that recognize unique structures on foreign pathogens, called antibodies, by B lymphocytes (B cells). Antibodies can be produced in large quantities and stored as lyophilized proteins. The ability to do so was created by the technological breakthrough of monoclonal antibody production, where a single B cell can be immortalized by fusion to a myeloma cell line, and the monoclonal antibody producing cell (now called a hybridoma) can be grown in essentially limitless quantities, and antibody collected and stored for later use as a therapy. As this technology advanced, it was discovered that by crossing species boundaries, an antibody could be made to essentially every human cell surface protein. Two such proteins on the surface of the T cells, CTLA-4 and PD-1, serve as negative signals during normal immune regulation. Tumors are known to subvert this system by expressing counter-ligands, such as PD-L1, and thus instruct T cells in the vicinity to down-modulate or turn off their activity. The requirement for effective use of antibody-based therapy for these negative signals (called checkpoints) is the presence of a pre-existing T cell that can recognize the leukemia or tumor. The explosion in the clinical application of "checkpoint blockade therapy" (e.g. Ipilimumab, Nivolumab, Atezolizumab) attests to the fact that some tumors do create T cell immunity, and that the introduction of a checkpoint inhibitor can have great therapeutic effect.

However, checkpoint inhibitors are not universally successful, and may not result in the permanent cure of a malignancy in most cases. In this case, cellular immunity must be activated in another way. When properly activated, T cells not only recognize and bind to foreign or dangerous cells or virally-infected cells, they kill them. While highly effective against bacterial and viral pathogens, T lymphocytes often do not recognize leukemia, lymphoma, or solid cancers such as breast cancer. Although these cells carry genetic mutations, they are still seen as "self" by the immune system. Careful study of the molecular mechanisms of T cell activation and how T cells kill foreign or infected cells led to key insights as to the minimal signals required to mediate cell-mediated killing. But how could we engineer a T cell such that is can now "see" a cancer cell? This is where the "chimeric" part of "chimeric antigen receptor" comes in to play. A chimeric antigen receptor is not a normal protein, but a hybrid created by splicing the portion of an antibody molecule that can recognize foreign proteins to the transmembrane and intracellular signaling domains of proteins that are known to activate T cells [2]. Thus, a CAR is a "recombinant" protein that has an extracellular or outward facing antibody-like binding domain, and a combination of intracellular signaling domains that activate T cells to expand, produce cytokines (soluble protein mediators) that activate other immune cells, and if they happen to contact a cell expressing the protein to which the antibody-like portion of the receptor was derived, to kill that cell. Since a CAR is an engineered synthetic protein, the DNA encoding this protein has to be introduced by means of a gene vector. To this point in time, lentiviral gene vectors have most commonly been used. CAR-engineered T cells are often referred to by means of the specific antigen or protein they recognize. For example, the protein "CD19" is highly

expressed on B-cell lineage leukemias and lymphomas. If a T cell has been transduced (the DNA encoding the CAR has been introduced into the genome of the T cell) with a CAR that features an extracellular binding domain specific for CD19, it is then referred to a as a CD19 CAR-T cell product. The first commercial products approved for the therapy of B cell leukemia or lymphoma (tisagenlecleucel or Kymriah® and axicabtagene ciloleucel, Yecarta®, and lisocabtagene maraleucel, BREYANZI®) are all CD19 CAR-T cells. We will now explore the isolation and expansion of T cells to the number required for effective therapy, the molecular structure of CAR-T, and some of the primary safety considerations for their use.



To date all CAR-T cell therapies are generated "ex vivo" -meaning outside the body, and are from "autologous" material -meaning a patient receives back their own engineered T cells. Current work is seeking to minimize the time required to create CAR-T ex vivo and perhaps to one day allow "in vivo" -meaning by direct injection of a gene vector into the body- generation of the engineered T cell product. Material generated from a single source or donor, and then expanded to doses suitable to many would be termed an "allogeneic" product. In addition to the challenges of immune rejection (as is the case in organ transplantation – the foreign tissue here being the allogeneic lymphocyte) an allogeneic immune cell product must not be reactive to the host that receives that product (this is commonly seen in bone marrow transplantation where "graft versus host" disease can be seen). Advancing cell therapies by these means will be discussed in further presentations. All current process are created ex vivo, using autologous cells.

To create a CAR-T cell, a cell source, a means to activate and culture those cells, a gene vector to transduce those cells, and a medication used to enhance the engraftment of that product all must be in place [3]. Most CAR-T products are initiated with an "apheresis" product. This is a procedure, requiring a dedicated medical device, whereby blood is withdrawn from a patient, the white blood cells removed (containing the T cells) and the red blood cells returned. This allows approximately 4 blood donations worth of T cells to be collected. The CAR-T field started with an apheresis product because it is routinely available at major medical centers and there was no risk of having insufficient starting material. As the field is rapidly advancing, the requirement for apheresis may be

supplanted in the future by a single blood draw. This step alone would decrease the cost of the procedure. Once T cells are purified from the apheresis product, they are stimulated in the presence of protein growth hormones (cytokines). The isolation, culture, and stimulation steps all require sterile enclosed steps that must be carried out by trained personnel. The growth media for T cells is commercially available and most media are now chemically defined and free of animal products. The stimulation of T cells is carried out using beads that have been coated with antibodies that recognize two specific proteins on the T cell surface (CD3- associated with the T cell receptor for antigen, TCR, and CD28, called a co-stimulation molecule). In some cases, these beads can be removed magnetically during the process, or they are biodegradable and consumed during the culture process. The cytokines, called interleukins (IL) are also available commercially and can be used as a single additive, in the case of IL-2, or in various combinations of IL-7, IL-15, and IL-21.

Once a T cell is in culture, stimulated, and expanding, it is primed for the introduction of genetic material encoding the CAR. In one of the strange twists of nature and science, a highly-engineered, non-replicative form of HIV (human immunodeficiency virus) is the best current means to engineer (introduce genetic material) T cells [4]. The safety of this process has been long-established, as the virus is re-edited into multiple genetic units to create a gene-delivery system that cannot replicate on its own[5]. HIV is a "retrovirus" and retroviruses derived from murine sources have been similarly engineered (the term often used is "gutted" as numerous genes required for replication have been removed). If the genetic vector is from sequences derived from HIV it is termed a lentiviral vector (LV). If the genetic vector is from sequences derived from another retrovirus it is termed a retroviral vector (RV). The term "vector" is used instead of "virus" because these portions of the viral genome cannot make new copies of themselves, i.e. replicate. Thus, the DNA encoding the CAR is inserted into the genome of the T cell, but it does not exit or reassemble, as a virus would. We know from clinical experience that LV are a safest approach. Although the data stems from a tragic source, the millions of people infected with HIV, HIV does not cause leukemia. Moreover, the safety of LV has been established in numerous clinical trials and several approved products. Other approaches to transducing T cells include "electroporation" wherein an electric field is used to punch holes in the T cell membrane and genetic material and an enzyme to cut and paste this material into the T cell genome is used. Electroporation has the advantage of not requiring the production of a LV or RV, however it has not proven to be less costly or safer. Specialized devices are still required, and while the process of cutting and pasting genetic material is relatively safe, the true risks have yet to be established. The current approach is to use the Crispr-Cas9 protein system or sequence specific "zinc-finger nucleases." Other viral vectors, such as Adenoviral vectors, and other nucleases are being developed. To date, the relative ease and standardization possible with LV production, has left this as the standard to which other techniques are compared.

Now that a T cell has been isolated, activated, transduced with a LV encoding a CAR, and expanded to the target dose (number of T cells per kilogram body weight), it is ready to be formulated for infusion. In the simplest sense this means harvesting the T cells by centrifugation (creating a cell pellet), removing the growth media, and exchanging it for a solution suitable for intravenous (i.v.) infusion. During this process (CAR-T cell harvest), both supernatant and cells are retained for sterility and other quality assays. As these cells are being produced under clinical good manufacturing processes (cGMP), and this process is regulated by government health authorities (the Food and Drug Administration, FDA, in the United States), CAR-T therapy requires a specified set of protocols to assure that a product safe for infusion has been generated.

Finally, we return to the patient. CAR-T cells are given to patients with serious medical conditions. In oncology, clinical trials have demonstrated that CAR-T cell therapy requires pre-treatment with

chemotherapeutic agents that make "space" for the incoming cells. These agents (cyclophosphamide and fludarbine), decrease the native or residual T cells in the patient and allow for the infused CAR-T cells to expand and execute their therapeutic effect. Pre-treatment doses of these agents are far below those used for the actual chemotherapy of cancer, but have not been effectively replaced with another approach. We do not know if pre-treatment is required for infectious disease applications, such as HIV, where the CAR-T itself protects T cells that would be targets of viral infection.

Once infused, CAR-T cells migrate to sites of disease, release cytokines and other factors that activate other immune cells, and then kill the cancer target. These released factors have powerful effects throughout the body. The aches, malaise, and fever we experience when we are infected with the flu are not due to the viral infection itself but are due to these very same factors. In CAR-T therapy, so many of these factors are produced at the same time that sometimes they can become life-threatening. This state of generalized CAR-T immune activation is called "cytokine release syndrome, CRS." While CRS was a large concern in the early days of CAR-T therapy, the wider availability of agents to counter CRS (like anti-IL6 receptor antibody, Tocilizumab), and broader medical experience, has led to therapeutic protocols where CRS is often averted before it becomes severe. A much rarer condition has been seen called ICANS (immune effector cell-associated neurotoxicity). ICANS presents as a rapid onset of potentially fatal cerebral edema. The cause and prevention of this complication is still being investigated [6]. The history of CAR-T cell therapeutic development has been rapid, but it is based on decades of experience gleaned from bone marrow transplantation and other forms of adoptive cell therapy. The steady progress in these fields over the last few decades created a foundation of experience and set of technologies that allowed CAR-T cell therapy to rapidly develop. And we can be assured that further innovation and innovation will make CAR-T therapy highly safe, more effective and less expensive.

Once, a cure for HIV was thought impossible. But our studies of long-term non-progressors, and the complete cure of the Berlin patient, Timothy Ray Brown, changed our thinking [7]. I was at the Pediatric Oncology Branch of the National Cancer Institute when the first four patients were treated with CD19 CAR-T cell therapy. These patients were entirely chemo-refractory, meaning that all our current medicines had failed to make a dent in their disease. Three of these patients are long-term survivors because they received CAR-T cell therapy [8]. We are in a revolutionary time in medicine. What seemed unimaginable, is now a series of approved products. We must continue to press on so that the technological innovations and the decrease in cost to manufacture CAR-T, make CAR-T therapy available to all patients who need these life-saving therapies.

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