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Delivering the "living drug": T cell immunotherapy

Abstract

Background: Cancer is one of the most lethal diseases worldwide. Traditional approaches such as chemotherapy have toxic side effects. New therapies on the rise are more target specific. One such therapy, immunotherapy, has become increasingly attractive in the field. However, to ensure the modulated and controlled manipulation of the immune system, delivery methods for drugs cells and biomaterials must be developed.

Methods: In this review, we analyze the literature to discuss the recent advances in T cell immunotherapy as well as four delivery technologies that address the issues of safety and efficacy associated with this treatment.

Summary: We conclude that the CAR-T approach could be a step towards overcoming the inaccessibility of poorly vascularized tumors and the evasion mechanisms of tumor cells. Delivery methods such as surface conjugated nanoparticles, DNA nanocarriers, scaffolds and artificial antigen-presenting cells aim for a more tumor-targeted approach rather than a systemic one, making this therapy applicable in the clinic.

Introduction

Despite a century of scientific advancement, cancer remains one of the most lethal and challenging diseases worldwide. Tumorigenic cells arise from the accumulation of mutations that collectively result in the acquisition of two cellular properties: the ability to grow and divide in defiance of normal cellular restraints and the capacity to colonize territories normally inhabited by different cell types. To this day, surgery, chemotherapy and radiation predominate as the main treatments for cancer. While surgery is aimed at eliminating local tumor masses, both chemotherapy and radiation therapy operate via non tumor-specific mechanisms that often result in off-target toxicity. Furthermore, chemotherapy seems to have reached a developmental plateau. (1) These concerns have led scientists to seek alternative therapies that could potentially replace or be used in combination with chemotherapy to optimize outcomes and minimize toxic side effects. (2)

Immunotherapy has become an increasingly attractive clinical strategy over the past decade, shifting the paradigm of cancer therapy from a chemical to a biological approach. The fundaments of immunotherapy come from our better understanding of the regulatory mechanisms by which the immune system keeps malignant cells in check. According to the *stranger-danger* model, the immune system not only recognizes foreign entities but also altered-self. However, malignant cells often evolve strategies to evade immunosurveillance and become resilient. Immunotherapy aims to counteract this by strengthening and restoring the ability of the immune system to fight cancer.

Despite being a promising strategy for the cure of cancer, immunotherapies face challenges related to its safety and efficacy. Safety concerns come from the serious adverse effects engendered by these therapeutics: off-target effects and autoimmunity. Efficacy concerns originate from the lack of response to treatment in some patients, likely due to the phenotypic and morphological heterogeneity of tumor cells. (3) Reduction of adverse off-target effects can be achieved by targeting the tumor with higher specificity; efficacy for all patients can be increased by developing a more patient-specific approach that addresses the issue of cancer heterogeneity. To achieve such a controlled and patients-specific immunomodulation, scientists have developed novel delivery technologies for immunotherapy. (2)

The most popular immunotherapies currently used are monoclonal antibodies, non-specific immunotherapies with interferons/interleukins, virus therapy, and T cell therapy. Monoclonal antibodies are used to detect and tag a cancer protein to make cancer cells visible to the immune system or as checkpoint inhibitors of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) to unleash the immune system. Interferon/interleukin immunotherapies help with the activation and proliferation of immune cells that fight cancer. Virus therapy consists of a genetically modified virus injected into the tumor to kill the cells. The antigens released from the dead tumor cells trigger the immune system which can then target all tumor cells displaying these antigens. (4)

In this review, we will discuss in detail one type of immunotherapy: T cell immunotherapy, as well as four delivery technologies that address the issues of safety and efficacy to improve the implementation of this treatment.

T Cell Immunotherapy

Immunotherapy brief historical review

Even though immunotherapy only gained popularity in the last decade, it has its roots back in the 19th century. The success of vaccination and the idea that weakened pathogens could provide protective immunity fuelled scientific discovery at the time. Medical scientists became optimistic that the acquired ability to manipulate the immune system could be used to treat cancer. Dr. William B. Coley was the first to attempt such an endeavor. Coley dug deep into the literature and came across 47 cases that portrayed a strange phenomenon: the unexpected remission of incurable neoplastic malignancies following a streptococcal dermal infection, erysipelas. Reluctant, he injected patients with heat-killed erysipelas causing agent and achieved long-term cure for many of them. Yet, despite Coley's success, the lack of understanding of his results at the time doomed his strategy. (5) His findings lingered in the dark until modern immunotherapy resurfaced a century later with the theory of cancer immunosurveillance by Brunet and Thomas. According to this theory, lymphocytes act as sentinels to identify transformed cells. (6) Yet, it wasn't until the discovery of interleukin-2 (IL-2) in 1976 that immunotherapy truly spread it wings. Other therapeutics began to rise: in 2010, cancer vaccine sipuleucel-T (cell based vaccine to treat prostate cancer) was approved by the U.S. Food and Drug Administration (FDA), followed by Ipilimumab, a monoclonal antibody against CTLA-4, in 2011. Along with CTLA-4, PD-1 and PD-1L inhibitors are a group of checkpoint inhibitors that act on the programmed cell death ligand; they entered clinical trials in 2006. (7) Finally, the first T cell therapy with chimeric antigen receptor (CAR)-T cells was the breakthrough of 2013 and a turning point in cancer immunotherapy. (2)

T cell immunity and tumor evasion

To fight cancer through immunotherapy, it is crucial to first understand how the immune system operates. The body's immune system has developed its own biological mechanisms to detect and eliminate potentially tumorigenic cells. However, tumors often outsmart the immunoregulatory processes that destroy them and become resilient. In this section, we will primarily focus on T cell mechanisms against altered-self cells and how they get evaded.

The tumor micro environment (TME) enables a three-phase cancer progression in the context of immunity: elimination, equilibrium, and escape. (8) The first phase is characterized by host immunosurveillance: the ability of the immune system to distinguish self from altered self. Professional antigen presenting cells (pAPCs) such as dendritic cells (DCs) engulf tumor cells and present tumor-specific antigens to naïve T cells in an major histocompatibility complex (MHC)-restricted manner. T cells with specificity to the tumor antigens get activated, expand, differentiate and become the effectors of cell-mediated adaptive immunity. (9) The two main T cell lineages, CD4+ and CD8+, operate through different mechanisms. CD8+ T cells recognize specific antigens presented on MHC class I at the surface of tumor cells and interact through Fas-FasL to kill the cells via perforin and granzyme. CD4+ T cells are less well understood in the context of cancer immunity, but some lineages are known to act through inhibition of angiogenesis and eosinophil recruitment. (10)

In the second phase, the tumor cells that have escaped the initial immune attack can neither expand nor be eradicated and therefore cohabitate in a state of equilibrium with the effector T cells. During this long-lasting phase, the tumor does what it knows best: it rapidly mutates until it acquires resistance abilities. These include the downregulation of their MHC class I, without which CD8+ T cells cannot target tumor cells, (9) and the cytolytic ability to kill CD4+ T cells. (10) In a Darwinian-selection fashion, new tumor variants arise with acquired abilities to fight the immune system and progress into the escape phase. During this final and critical phase, tumor cells escape the state of equilibrium and expand uncontrollably under the impotence of the immune system.

Engineered T Cells immunotherapy: CAR-T Cells

In an attempt to strengthen cell-mediated immunity in the fight against the newly weaponized tumorigenic cells, several immunotherapeutic approaches have been developed, most of which involve small molecules such as checkpoint inhibitors and bigger protein complexes such as monoclonal antibodies. Yet perhaps the most revolutionary immunotherapeutic invention was the CAR-T cell. The uniqueness of this therapy compared to those involving molecules comes from the fact that cells are capable of intelligent sensing-response behaviors, making their manipulation far more complex. (11)

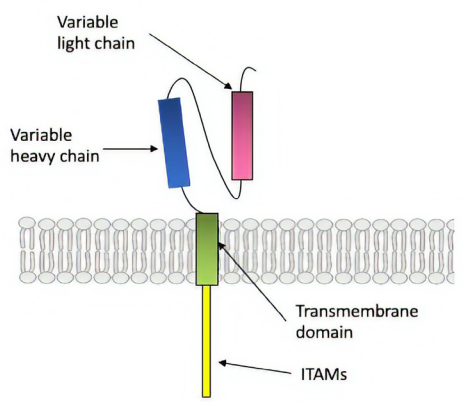

Figure 1. The structure of the Chimeric Antigen Receptor (CAR). On the extracellular side the CAR has the variable domain which consists of a light chain and a heavy chain, which is connected to the transmembrane domain which in turn is connected to the intracellular which has signaling capability such as the immunoreceptor tyrosine-based motif (ITAM).

To make CAR-T cells, T cells are initially isolated from the blood of either the patient (autologous) or a healthy donor (allogenic) through the process of leukocyte apheresis. T cells proliferation is then induced via IL-2 and anti-CD3 antibody. The expanded T cell population is genetically engineered to express a CAR of interest (Figure 1) using the CRISPR/Cas9 mechanism to knock out inhibitory genes such as the T cell receptor (TCR). (12) Viral vector transfection is then used for the expression of the CAR. After the CAR-T cell population has been expanded to the desired numbers, patient undergoes lymphodepletion chemotherapy to destroy and prevent the original T cells to compete with the incoming CAR-T cells for resources. Finally, the CAR-T cells are delivered to the patient. (13)

CAR-T cells offer many advantages over native T cells. For instance, CAR targeting is human leukocyte antigen (HLA) independent, which means antigen recognition in the context of MHC is no longer required (Figure 2). This prevents the evasion of tumors that have downregulated MHCI. HLA-independent recognition also overcomes the issue of MHC allorecognition: CAR-T cells don't need to be specific to the patient's HLA expression profile. Furthermore, unlike native T cells which can only respond to one antigen-MHC complex, CAR-T cells can be engineered to respond to a broad array of targets and to differentiate into various effector subtypes such as CD4+, CD8+ or memory cells. (11)

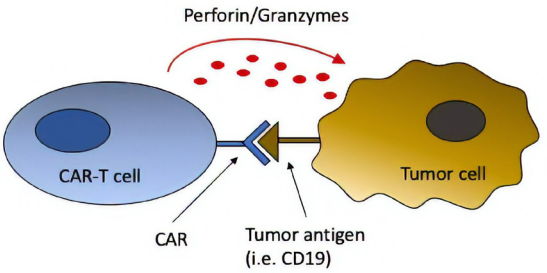

Figure 2. The mechanism of action of CD8+ CAR-T cells. CAR-T cells can target cells independently of HLA, upon which they release perforin and granzyme which induces target cell death.

CAR-T cell therapy was first approved by the FDA in 2017 for the treatment of acute lymphoblastic leukemia. CAR-T cells were engineered to target CD19, a molecule highly expressed in both normal and malignant B cells. The therapy was a success as more than 80% of patients entered remission with few treatable on-target/off-tumor side effects such as B cell aplasia. (14) Despite the optimism for T cell therapies, their rise remains tempered by safety and efficacy concerns. First, with respect to safety, T cell immunotherapy can cause serious side effects: cytokine release syndrome (CSR) and neurological toxicity. Second, with respect to efficacy, the infused cells are unable to conquer solid tumors and do not persist in the TME for extended time periods, resulting in relapse. (15) These hurdles may be overcome by the emerging delivery technologies that will be discussed in the following section.

Delivery technologies for T Cell Immunotherapy

Surface conjugated surface nanoparticles

In their paper, *Therapeutic cell engineering with surface-conjugated synthetic nanoparticles*, (16) Matthias T. Stephan and his colleagues describe a strategy to enhance cell therapy via adjuvant-loaded nanoparticles conjugated to the T cell surface. It has been observed that CAR-T cells often fail to persist and require the systemic administration of adjuvant drugs. Drug administration via the systemic route is not targeted to their site of action, the TME, and therefore higher doses must be used which can lead to global toxic side effects. The authors argue that CAR-T cell conjugated-nanoparticles would provide a more targeted, effective and safe pseudo-autocrine drug stimulation.

They initially designed liposome-like synthetic nanoparticles coated with thiol-reactive maleimide headgroups (T cells contain high amounts of free thiols at their surface). They incubated the nanoparticles with the T cells to allow the two to covalently bind via maleimide-thiol coupling. According to the results, coupling was non-toxic and only blocked 17.2% of the cell surface thiol groups. Furthermore, unlike lipid-coated polymers which get removed in washing steps, maleimide-linked particles remained bound to cells.

They then determined the maximum number of particles that could be linked to the T cell surface without jeopardizing cellular functions. They assessed CD8+ key functions: cell killing via cell surface interactions, proliferation, cytokine release, transmission across endothelial tissue, and tissue homing. According to their results, up to ~100 nanoparticles could be conjugated without affecting CAR-T cell proliferation, killing of target cells or cytokine release. Furthermore, they showed that the CAR-T cells carrying 100 nanoparticles had the same transmission efficiency through endothelial layers as native cells. However, following migration the cells only retained ~83% of the nanoparticles initially conjugated. Finally, they assessed tumor homing capacities of nanoparticle-conjugated T cells compared to unmodified T cells with the specificity to the same EL4 tumor antigen. No difference was observed.

In their next experiment, Stephan and his colleagues tested if the adjuvant-drug containing nanoparticles could enhance their carrier T cell action. They encapsulated cytokines known to promote T cell proliferation and effector functions: IL-15 and IL-21. The cytokines had a greater proliferative effect when loaded onto the T cells compared to systemically infused cytokines. Nanoparticle-conjugated T cells also displayed a more proliferative behavior than unmodified cells and persisted for longer after the contraction period. Treatment with nanoparticle–conjugated T cells completely eradicated the tumor, whereas treatment with unmodified T cells and/or systemic cytokine infusion lead to lower survival rates. These experiments were done in mouse models.

In conclusion, this study reveals the advantage cell carriers have over inanimate molecules in delivering a drug to tissues with difficult access. The challenge of pleiotropic activity and toxicity of drugs required for the proper function of CAR-T cells can be overcome by encapsulating the drug in cell-conjugated nanoparticles. This technique limits toxic side-effects, requires lower drug doses and improves T cell functions such as proliferation and homing due to enhance paracrine secretion of IL-15, without interfering with normal cellular activities.

DNA nanocarriers for *in situ* T cell engineering

In their paper, *In situ programming of leukemia-specific T cells using synthetic DNA nanocarriers*, (17) Tyrel T. Smith et. al describe a strategy to improve the application of T cell immunotherapy by engineering T cells in vivo via gene therapy. An obstacle to the implementation of personalized CAR-T cells is the high costs and complicated manufacturing procedures associated with their production. To solve this, Smith and his colleagues propose a DNA-carrier nanotechnology that can efficiently introduce the CAR gene into native T cells and reprogram them *in vivo*.

The first step was to design nanocarriers that could induce CAR expression in T cells. The carriers must effectively be taken up by the T cell and import the CAR DNA into the nucleus. Endocytosis was achieved by coupling anti-CD3e f(ab')2 to the surfaces of nanoparticles. CAR DNA nuclear import was facilitated by integrating microtubule-associated sequences (MTAS) and nuclear localization signal (NLS) into the nanoparticle. To achieve CAR expression by the host's gene expression machinery, the authors designed two plasmids, one containing the CAR cassette flanked by piggyBac transposable elements and the other containing the sequence encoding piggyBac transposase. The transposon machinery enables the integration of vectors into chromosomes.

To test the functionality of the designed nanoparticle in the production of leukemia-specific CAR-T cells, the authors conducted a series of *in vitro* experiments on mouse splenocytes. They first confirmed by flow cytometry that CD3-targeted nanoparticles effectively bound T cells with few off-targets. They then observed by confocal imaging that particles were rapidly internalized into the cytoplasm. Shortly after administration, leukemia-specific receptors were detected on the surface of T cells. They demonstrated the advantage of NLS and MTAS containing nanoparticles compared to negative controls by measuring nuclear transfection rates. Finally, piggyBac transposons sustained high levels of CAR gene expression over a longer period of time compared to nanoparticles that lacked the transposon machinery.

The next step was to further examine CD3-mediated targeting of T cells as well as the efficacy of cell-reprogramming mechanisms, this time *in vivo* with mice. They obtained the same results for CD3+ T cell targeting and the few off-target effects decreased over the course of treatment. Furthermore, they demonstrated that targeted nanoparticles effectively localized to lymphatic organs (spleen lymph nodes and bone marrow) as opposed to the non-targeted ones which localized to the liver. Subsequently, they tested for *in vivo* toxicities. This time, they used a prostate-targeting CAR to ensure that the observed effects originated from the nanoparticles themselves and not the DNA editing activity. Cell counts and blood profiles were normal; inflammatory cytokine levels increased minimally. *In vivo* data also evidenced the need for co-delivery of the transposon machinery to achieve efficient expansion of CAR-T cells as well as tumor eradication. Finally, to assess the efficacy of cancer treatment with DNA-carrying nanoparticles compared to conventional therapy, the authors treated a group of mice following conventional CAR-T cell therapy protocol, described in section 2.3. The results showed that survival rates were slightly higher in the conventional therapy group, but overall very similar in both approaches.

In conclusion, the use of nanoparticles to reprogram gene expression *in vivo* is an attractive alternative to the costly and labor-expensive T cell therapy, with comparable results as the latter. Nonetheless, some challenges have yet to be overcome. First, the recurring issue of solid tumors remains a concern as their accessibility is limited. Second, with respect to clinical translation, the safety issues of off-target gene transfer must be further addressed. For instance, the inclusion of a T cell-specific promoter upstream of the CAR sequence could ensure that despite there being off-target integration, CAR is only expressed in T cells.

Biomaterial based implants for engineered T cell delivery

In a study by Tyrel T. Smith et. al, a possible solution to the recurrent hurdle of solid tumors is put forward. (18) Solid tumors have been the lingering nightmare of T cell immunotherapies for two reasons. First, solid tumors create an immunosuppressive environment that impedes normal T cell function. Second, solid tumors are highly heterogeneous and include cells that lack CAR-targeted antigens. As a solution, the authors proposed a biopolymer scaffold comprised of CAR-T cells and co-stimulator stimulator of interferon genes (STING) agonist. They hypothesized two functions: the physical delivery of ex-vivo engineered CAR-T cells and the STING induction of native T cells with different tumor antigen specificities as CAR-T cells. The latter would help overcome the obstacle of tumor heterogeneity.

In their experiments, the authors used pancreatic cancer mouse models. They first designed a CAR specific to RAE1, a pancreatic tumor antigen. They then manufactured a bioactive scaffold using polymerized alginate and macromolecules important for migration and stimulation of T cells. The scaffolds were porous matrices made from ultrapure sodium alginate powder and were delivered to the animals through surgery. Initially, they tested the T cell expansion and capacity to clear pancreatic tumor in two conditions: direct injection of CAR-T cells into the tumor, and direct implantation of CAR-T cell scaffold onto the tumor. In the first group, cells did not persist and only temporarily delayed cancer progression. In contrast, robust CAR-T cell proliferation and reduced tumor growth was observed for the second group. Nevertheless, neither group achieved complete clearance and RAE1 negative tumor cell variants emerged, resulting in tumor evasion.

Since the CAR-T cells that targeted single antigens did not halt tumor progression, the authors refined their strategy. The natural recognition of

various tumor antigens relies on the proper priming of T cells by DCs, a mechanism that is impaired in cancer. To recruit and stimulate DCs, the authors added STING agonist cyclic di–GMP (cdGMP) to the scaffold. They assessed DC activity by measuring their CD86 and MHC class II expression profiles, which are indicative of DCs' activated phenotype. The cdGMP-CAR-T cell scaffold treatment group presented higher numbers of activated DCs than the group stimulated by only CAR-T cell scaffolds. The next step was to visualize the localization and magnitude of T cell activation in the same two treatment conditions: CAR-T cell scaffold treatment and cdGMP-CAR-T cell scaffold treatment. They created mice carrying a transgenic gene composed of nuclear factor of activated T cell (NFAT) linked to a luciferase reporter gene. In the CAR-T cell scaffold condition luminescence was detected in the tumor area but not as strongly as in the cdGMP/CAR-T cell scaffold condition. In the latter, other organs such as the spleen and lymph nodes showed induction of native T cells.

Finally, the authors evaluated the host anti-tumor immunity in metastases and the side effects on pancreatic functions. The most effective results were again seen in the cdGMP-CAR-T cell scaffold condition. When they re-challenged the cured mice with pancreatic tumor cells to further test for global anti-tumor immunity in lung metastases, no tumor formation was seen. On the downside, decreased serum amylase and lower lipase activity were indicative of defective pancreatic exocrine function.

All in all, this delivery technique provides a solution to solid tumors that do not respond to conventional T cell therapies. Moreover, targeted delivery of adjuvant STING reduces off target exposure and requires lower drug dosage. However, in the case of pancreatic cancer, some adverse effects should be carefully watched for in clinical trials. Furthermore, the implant-to-tumor size ratio in mice could account for some of the findings in this study that might not be reproducible in human tumors.

Artificial antigen-presenting cells

Artificial antigen-presenting cells (aAPCs) constitute a promising platform for the stimulation and amplification of T cell responses. T cells are typically activated by signals 1 (survival) and 2 (proliferation) through cell surface interactions with APCs. The T cell-APC interaction induces the formation of an immune synapse characterized by the clustering of TCRs. Previous data have shown that conventional spherical micron-sized aAPCs function efficiently in vitro but not in vivo. Based on previously observed advantages of nanoparticles and benefits of ellipsoid over spherical particles, Randall A. Meyer and his colleagues developed a nanoellipsoidal aAPC model that could overcome the limitations of conventional aAPCs immunotherapy. This approach is described in their study *Biodegradable Nanoellipsoidal Artificial Antigen Presenting Cells for Antigen Specific T-Cell Activation*. (19)

They first synthesized PLGA nanoparticles by emulsion and PVA film stretch to obtain spherical and ellipsoid shapes of different curvatures. Then, to turn the ellipsoid nanoparticles into aAPCs, they conjugated peptide-MHC-IgG dimers and anti-CD28 antibody to the PLGA polymer. They verified particle stability by incubating the artificial cells in physiological conditions. The optimal particle/antigen dosages and curvature of aAPCs were determined by titration and carboxyfluorescein succinimidyl ester (CFSE) dilution analysis. 2-fold ellipsoid particles with the highest particle/antigen ratios were the most efficient at stimulating CD8+ T cells and surpassed their spherical counterparts.

The first feature they studied was nanoparticle non-specific cell uptake in ellipsoid nanoscale aAPCs (naAPCs) compared to spherical naAPCs. They used two different models of uptake: by macrophages and by human umbilical vein endothelial cells (HUVEC). *In vitro*, both macrophages and HUVECs preferentially phagocytosed spherical naAPCs. The *in vivo* circulation and half-life of naAPCs was assessed via a biodistribution experiment. Ellipsoid naAPCs moved faster, remained in the periphery for longer, and had a longer half-life than spherical naAPCs.

Next, they sought to evaluate the stimulatory capacity of naAPCs *in vivo* in the context of immunotherapy. To this end, mice were administered either ellipsoid naAPC with CAR-T cells, spherical naAPC with CAR-T cells, or CAR-T cells alone. According to blood analysis carried throughout the experiment, ellipsoid naAPCs elicited a notably higher CAR-T cell proliferation rate than the two other treatments. They also observed a higher number of ellipsoid naAPC in the dissected spleen and lymph nodes.

In conclusion, aAPCs delivery systems offer an attractive alternative to conventional T cell therapies. The conventional aAPCs technology was successful *in vitro* but not *in vivo*. In this experiment, the authors improved the technology by using nanoellipsoid design which not only provides higher efficacy but also reduces unwanted cellular uptake and displays an enhanced biodistribution compared to conventional aAPCs. Nonetheless, other aAPC features such as membrane fluidity and particle rigidity should be further assessed to optimize this delivery system for clinical immunotherapy.

Discussion

Over the past ten years, immunotherapy has taken the lead over traditional cancer treatments such as radiotherapy and chemotherapy. The developing field of immunology has allowed scientists to better understand and hence exploit our immune system for the treatment of cancer which to this day, remains a major challenge for health practitioners. Initially, scientists employed small molecules such as IFNα and IL-2 as well as checkpoint inhibitors for the application of immunotherapy. However, this methodology took a revolutionary turn with the invention of CAR engineered T cells. Often referred to as the "living drug", CAR-T cells adopt and enhance the anti-tumor functions of native T cells, the sentinels of cell-mediated adaptive immunity. This cellular approach tackles some of the hassles associated with poorly vascularized tumors via trafficking mechanisms superior to those of small molecules. As well, and perhaps most importantly, artificial T cells overcome the evasion and immunosuppressive powers tumor cells hold over native T cells.

Nonetheless, to exploit the high potential of CAR-T cells in a clinical setting, scientists must first jump the hoops of safety and efficacy this therapy may have. The immune system is a powerful weapon against cancer, yet it can just as easily turn into a bomb against our own body. Uncontrolled stimulation of the immune system in the hopes of eradicating cancer can result in lethal auto-immune responses and cytokine release syndrome. It is crucial to tailor the immune stimulation to the tumor, reduce off-target effects and minimize potential toxicities. To address these issues, scientists have come up with different delivery techniques for the treatment with CAR-T cells. These include surface conjugated surface nanoparticles, DNA nanocarriers for *in situ* T cell engineering, biomaterial-based implants for engineered T cell delivery and artificial antigen-presenting cells. All of these techniques aim for a more tumor-targeted approach rather than a systemic one, but all therapies target different aspects of tumor progression to enhance treatment efficacy: surface conjugated nanoparticles aim to improve adjuvant drug delivery to the CAR-T cells, DNA nanocarriers render the treatment more accessible and available in the market, CAR-T cell bio-scaffold address the hurdle of solid tumors, and aAPCs fight the immunosuppressive effects of tumors in the evasion phase.

Even though the field of immunotherapy is rapidly progressing, these delivery methods are still at nascent stages and need to be further investigated and optimized to be used in clinical trials. *Ex-vivo* manufacture and expansion remains an arduous process that is both time-consuming and costly. Improving manufacturing methods can greatly increase the applicability of these delivery methods in a clinical setting. With respect to surface conjugated nanoparticles, the release of adjuvant drugs could be extended so that less treatment administrations are required. The DNA carriers, despite being more cost effective, pose a higher risk of off-target gene insertion, an effect that should be addressed perhaps through the inclusion of a T cell specific promoter, (16) or a more targeted vector delivery system. In the treatment with biosynthetic scaffolds, the authors do not specify whether or not the subjects undergo lymphocyte eradication by chemotherapy like in conventional CAR-T cell therapy before administrating the scaffold, which is meant to prevent CAR-T and native T cell competition. In the scaffold approach, the authors aim to both deliver the engineered cells and stimulate the native ones and the interplay between

the two should be further investigated for the implementation of this delivery method. Lastly, design specificities for aAPCs currently under study slowly set the path towards the perfectly shaped, rigid and sized particles to achieve optimal induction of T cell immunity.

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