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REVIEW ARTICLE

Combination of Pre-existing and New Strategies to Improve CAR-T Cells' Efficiency

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ABSTRACT: The recent success of CAR-T cell therapy has revolutionized the treatment of cancer. However, its application is currently limited to blood cancers like B-cell leukemia or lymphoma. Major challenges are yet to be resolved before extending it to treat solid tumors, like on-target off-tumor effects, tumor infiltration, immunosuppressive micro-environment, etc. CAR-T cells are down-regulated by mechanisms similar to those suppressing autologous T-cell function. Through genetic deletions, we can eliminate these factors. An interesting factor affecting the CAR-T cells is the lactate-mediated GPR81 receptor. Lactate has been found to play an important role in tumor survival. It carries out functions like metastasis, angiogenesis, and tumor resistance. In addition to current modifications to T-cells required for the therapy, including other cells like dendritic cells for their crucial role in presenting antigens to T-cells and T-cell maturation can aid in tackling problems like lack of identification of tumor antigens. Our technique heavily relies on multiple genetic deletions made possible by CRISPR-Cas9-mediated multiplexing. It is necessary to be able to control the effect of CAR-T cells externally via ON and OFF switches. New advancements have made dose-dependent regulation of CAR-T cells possible instead of complete initiation or termination of their effect.

KEYWORDS: Biomedical and Health Sciences, Immunology, CAR-T cell therapy, Immunosuppression, Dendritic cells.

Introduction

CAR-T cell therapy is a type of adoptive T-cell immunotherapy (ACT). ACT generally requires the extraction of autologous T-cells and the improvement of their tumor detection and eradication mechanisms.[1] T-lymphocytes from the body are engineered to express artificial receptors targeting cancer cells. These receptors are called chimeric antigen receptors.[2] Chimeric antigen receptors influence the T-cells in three aspects: specificity, function, and metabolism.[3]

The first generation of CAR-T cells was developed by Zelig Eshhar and Gideon Gross in 1989-1993. The first-generation CAR-T cells consisted of a scFv unit combined with CD3ζ. This was responsible for activating them, however, the presence of a single signal was not sufficient for a complete activation. In murine models, these cells dampened the tumor growth and showed anti-tumor activity.[4] However, they were soon realized to be ineffective as they could not produce enough co-stimulatory signals to produce a clinically effective response.[5]

The second generation of CAR-T cells created ensured the two signals: Engagement of TCR with MHC (major histocompatibility complex) and costimulatory signal by CD28 required for their activation. Dual-signaling meant that CAR-T cells could proliferate and function on encountering tumor antigens.[3] This co-stimulatory pathway is integral for the functioning of CAR-T cells. Upon impeding the CD28 signaling pathway with the CD28 agonists a suppressed immune response and in some cases, antigen-specific tolerance was observed.[5,6] One of the most crucial roles played by CD28 which is majorly responsible for the success of the second-generation CAR-T cells is its ability to activate cytokine genes. It has control over one of the key cytokines, IL-2 which controls pivotal functions of T-cells like growth, maturation etc. It is observed that in the absence of this CD28 co-stimulation, IL-2 levels decrease.[7] When a chimeric receptor combining CD3ζ and CD28 was used, activation and co-stimulatory signal, improved antigen-dependent proliferation, IL-2 production, and tumor lysis were observed *in vitro*.[4,8]

The third-generation CAR-T cells have two costimulatory domains, CD28 and 4-1BB.[5] It is based on the assertion that CD28 might pace up the expansion of T-cells and quicker cancer killing and 4-1BB may provide longer-lasting T-cells and protect from relapse. The combination of them resulted in better expansion and life of T-cells. Pre-clinical models demonstrate that inclusion of CD28 and 4-1BB resulted in a combined effect of the endo-domains resulting in improved expansion and survival of CAR-T cells.[7] The fourth generation of CAR-T cells are called TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing). They were created to produce sufficient pro-stimulatory cytokine(s) and then transfer it to the tumor site.[5] It was also observed that delivery through CAR-T cells of transgenic IL-18 and IL-12 led to an extensive response against the tumor. This result was not attained by co-administration of the cytokines in tumor-infected mice.[9-11] These cells are controlled by 6xNFAT-responsive elements and the IL-2 minimal promoter. Upon activation of CAR through CD3-ZAP70 signaling supplemented by co-stimulation induces NFAT activation leading to the aimed outcome i.e. release of the transgenic cytokine through NFAT/IL-2 minimal promoter.[1]

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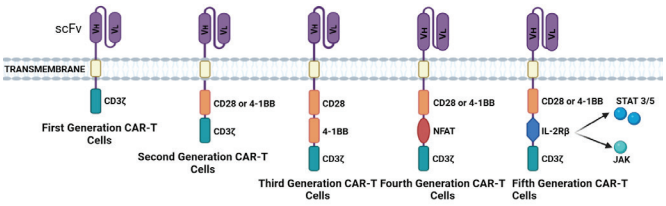

Figure 1: Structure of the five generations of CARs.

When CAR-T cells attack the tumor site, their effect is suppressed and, in some cases, nullified due to the immunosuppressive mechanisms of the tumor cells. Tumor immunosuppressive mechanisms include angiogenesis, cytokines like transforming growth factor-beta (TGF-β) and Interleukin-10; metabolites like tryptophan, etc.;[12] however, any treatment exploiting any of these key factors present at the tumor site would have to disrupt immunosuppressive metabolism individually for each tumor cell which makes it even less viable to produce results due to their rapid proliferative nature.

It is difficult to develop a method to target any of the immunosuppressive factors which include a variety of cytokines, metabolites, environmental conditions like pH and hypoxia, and pro-tumor T-cells like Tregs, etc. The problem often seen in cancer treatment even after successfully targeting any of the key factors, is the **neoplastic control of immunosuppression through a large number of factors and mechanisms** instead of a few, due to which discernible results involving better engagement of the treatment with the neoplasm and a decrease in the rate of cancer progression are not seen and in many cases. This can be understood by an example: One of the immunosuppressive factors of the TME is hypoxia. Creating CAR-T cells resisting the hypoxic conditions can be one potential idea to create the next generation CAR-T cells, however, it must be noted that hypoxia is associated with lactate induction.[12, 13] This lactate can interact with the GPR81 receptor to produce immunosuppressive results against the CAR-T cells. The tumor cells adapt to the targeting of any specific aforementioned factor and formulate new ways to proliferate. One important mechanism by which tumor cells resist the effect of approved and mainstream treatment methods like chemotherapy is via DNA repair mechanisms. Platinum and alkylating agent-based-treatment is associated with ERCC1 over-expression, one of the key genes of Nucleotide Excision Repair. This results in suboptimal results for cisplatin-based treatment of advanced non-small cell lung cancer (NSCLC).[14, 15] Drug delivery is also a major challenge in treating cancer. Methotrexate is a chemotherapy agent and an antimetabolite whose function is reduced as the acidic pH of the TME can inhibit its active transport.[15, 16]

CAR-T cell therapy is novel in a way that we can engineer the body's own T-cells (autologous) (or allogeneic too) to resist the immunosuppressive mechanisms. These mechanisms may require only tumor site-specific genes to function or may require genes that correspond to both cancer cells and T-cells to generate a response suppressing T-cell function. For instance, GPR81 is expressed in immune cells too which may be activated by lactate produced in the TME. GPR81 is, in fact, expressed in dendritic cells and macrophages in the colon. As a result of its presence, inflammation, and immune tolerance are negatively affected in this region.[17]

Discussion

Strategy:

Several proteins have been identified as key players in the immunosuppressive tumor microenvironment. Some of these proteins are also present inside and outside the T-cell. When CAR-T cells are induced in the body several mechanisms might obliterate or suppress the indicators or checkpoints which must be present in the tumor cells for the T-cells to distinguish them from other cells, for example, antigens. This is a major challenge affecting not only CAR-T cells but non-engineered T-cells produced in the body too. We propose the addition of engineered dendritic cells which will aid T-cells in their growth, and maturation as well as the function of detecting tumor antigens through effective tumor presentation.

Another strategy can be to selectively pick genes present in T-cells that correspond to immunosuppressive genes present in tumors. Tumor sites may downregulate T-cells by the tumor environment with an altered ratio of intracellular to extracellular lactate,[18] or directly stimulate immune cells to release inhibitory and other down-regulatory signals. This demonstrates the possible potential of elimination of a key gene present in both tumor cells and T-cells like GPR81 which binds to lactate in improving CAR-T cell's efficiency. While drug development and further engineering are a gradual process, I hypothesize that with the current level of gene editing techniques, we can **modify CAR-T cells to make them unresponsive to the immunosuppressive mechanisms requiring a T-cell counterpart.**

CAR-T cell therapy has a benefit over drug-based treatments as T-cells can be extracted out of the body making it easier to modify them instead of treatments based on the administration of a drug in the body, which faces the numerous challenges of drug delivery to the tumor site and drug resistance as well.

Combination Therapy involving CAR-T cells and Importance of Mode of Administration:

Tisagenlecleucel is the first US FDA-approved CAR-T cell therapy treatment. It is generally used for patients with B-cell precursor acute lymphoblastic leukemia (B-ALL) and large B-cell lymphoma. The second FDA-approved CAR-T cell treatment is axicabtagene ciloleucel which is recommended for the treatment of adult patients with recurrent large B-cell lymphoma or those unresponsive to other therapy.[19]

Overall, CAR-T cell therapy's current application and approval are limited to blood cancers. This is for several reasons/factors which can be broadly categorized into (1) inefficiency in targeting, (2) effectively identifying tumor antigens, and (3) the oppressive character of the tumor microenvironment or the survival in the tumor [20] due to a range of unfavorable factors including acidic environment, angiogenesis, another immunosuppressive signaling, etc. The first shortcoming, inefficiency in targeting was observed when an on-target off-tumor action

of CAR-T cells resulted in major cytotoxicity when a metastatic colorectal patient (mCRC) patient received HER2 CAR-T cells [21] and a neuroblastoma patient was treated with GD2 CAR-T cells.[20, 21] It shows the contrast in the effectiveness of CAR-T cells in the treatment of blood cancers and solid cancers.

Currently, CAR-T cells are not sufficiently equipped to pass through these barriers present in solid tumors, leading us to the idea of combination therapy. If a combination can weaken the immunosuppressive barriers then it can pave the way for T-cells to reach the tumor site and with further modifications suffer minimal damage upon reaching the tumor. There are many instances where CAR-T cells have benefitted from the combination and reduced the limitations for which CAR-T cells need to be potentially further engineered as discussed earlier. CARs are unique because they bind to the tumor antigen independent of the major histocompatibility complex resulting in better T-cell activation and powerful anti-tumor responses.[22, 23] According to the review by Xu *et al.*, certain therapeutic agents can upregulate mannose-6-phosphate receptors on tumor cells, increasing granzyme B's (released by cytotoxic T lymphocytes (CTL)) ability to permeate tumor cells, enhancing tumor cell response to immunotherapy through autophagy-dependent mechanisms.[24-27] Chemotherapeutic agents, such as taxanes (docetaxel and paclitaxel) and vinca alkaloids (vinorelbine and vinblastine), aid significantly in tumor cell recognition by increasing exposure to calreticulin and eliminating tumor cells, thereby discharging a large volume of tumor antigens.[27, 28] This can benefit antigen-presenting cells like dendritic cells to present these antigens to CAR-T cells, hypothesizing a combination of these chemotherapeutic agents along with CAR-T cell therapy and DC vaccines.

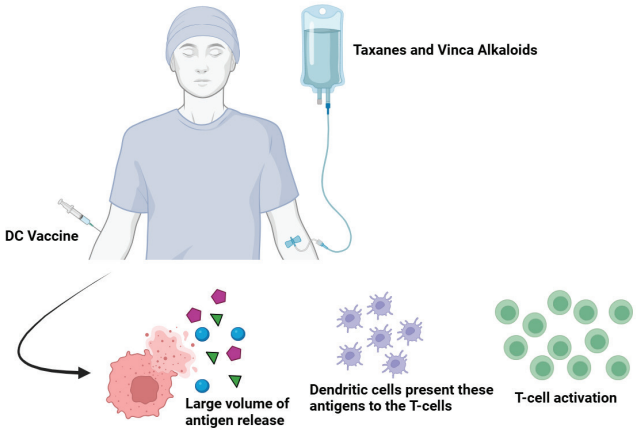

Figure 2: Possible combination of taxanes and vinca alkaloids, DC vaccine, and CAR-T cells.

Some chemotherapeutic agents (doxorubicin, fluorouracil, gemcitabine, cyclophosphamide, and docetaxel) can specifically target immunosuppressive cells (regulatory T cells and myeloid-derived suppressor cells) which will support the antitumor immunotherapy.[29] Combination immunotherapy with CAR-T cells and checkpoint blockade can be considered as a potential treatment as it supports: 1. CAR-T cells, which provide the infiltrate, and 2. PD-1/PD-L1 blockade, which can ensure the continuous function of T cells.[30] One study in which 11 mesothelioma patients preconditioned with cyclophosphamide when administered with mesothelin targeted CAR-T cells and doses of anti-PD-1agent resulted in a 72% response rate and complete metabolic responses in two patients.[30, 31] Its effectiveness can be understood by comparing it with another study, in which CAR-T meso cells enhanced by cyclophosphamide although left their evidence of interaction with tumor cells with their DNA detected in the tumor, human anti-chimeric antibody (HACA) detected in the blood, they were well-tolerated, showed limited effectiveness in terms of clinical response.[32]

Solid tumors are different from hematological neoplasms and their differences can be broadly categorized into: antigen heterogeneity, heavy tumor burden, solid tumors are present at specific sites in the body, and have an oppressive microenvironment and immunosuppressive mechanisms.

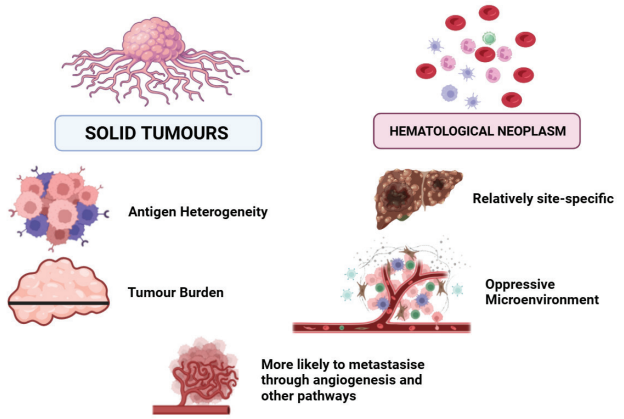

Figure 3: Additional challenges posed by solid tumors in comparison to hematological neoplasm.

The primary issue is improving tumor infiltration and trafficking as no amount of improved T-cell competency would make a difference until the cells are able to reach the tumor site. Grosser *et al.*,[33] employed regional administration of CAR-T cells to augment T cell infiltration into the tumor, which promoted early intratumoral accumulation and enhanced antitumoral efficacy in a clinically relevant model of mesothelioma. Furthermore, they demonstrated that regional administration resulted in the systemic circulation of CAR T cells that retain their functional activity, establishing a long-term systemic presence capable of eradicating metastatic tumor sites. In their phase I clinical trial in patients with pleural mesothelioma and breast and lung primaries metastatic to the pleura (NCT02414269), they demonstrated that intrapleural administration results in circulating CAR T cells detectable in the systemic circulation up to several months following a single infusion. This strategy can be possibly adapted to ensure the longevity of the T-cells and ensure a response in inaccessible sites.

Immunosuppressive Mechanisms:

- ***Lactate Receptor GPR81:***

Lactate has been hypothesized to be a signaling molecule by binding to the lactate-receptor GPR81/HCAR1. GPR81 was

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discovered in the adipocyte membrane.[17, 34] GPR81's presence extends beyond adipose tissue to many types of cells, including muscle cells, immune cells, tumor cells, and the cells of the nervous system.[17, 35] The GPR81/lactate combination vests lactate with additional signaling capabilities like suppressing lipolysis,[6] regulating the immune checkpoint pathway,[17] and modulation of cellular DNA repair through upregulation of DNA repair proteins, such as BRCA1, nibrin, and DNA-PKcs which can provide resistance to cancer cells from chemotherapies [17, 36] and other various other pro-tumor functions.

One such function of the lactate/GPR81 pathway is immunosuppression. According to a study, [37] GPR81 had a key role in the induction of immune regulatory genes (IL-1o, retinoic acid, and IDO) with suppression of the expression of inflammatory cytokines in DCs and macrophages. It was also found that GPR81 is activated by lactate in its physiological concentration range of 1-20 mM.[38] Moreover, to substantiate the importance of GPR81, genetic deletion of GPR81 in mice resulted in a reduced number of Tr1, Foxp3^{+}, and Tregs with a concomitant increase in the frequencies of Th1/Th17 cells in the colon. The imbalance in T-cell subsets was due to increased expression of inflammatory cytokines by colonic APCs (Antigen Presenting Cells) that drive Th1/Th17 cell differentiation.[37] These Th1 cells are subsets of CD^{4+} cells. CTLs are the major effectors of tumor cell lysis and are supported in this capacity by TH1 substantiating the need to knock out GPR81.[39] To substantiate the significance of GPR81, it was observed that GPR81 deletion in two breast cancer models led to inhibition of cancer growth due to the presence of an increased number of tumor-infiltrating T-cells and MHCII^{hi}.[17, 40]

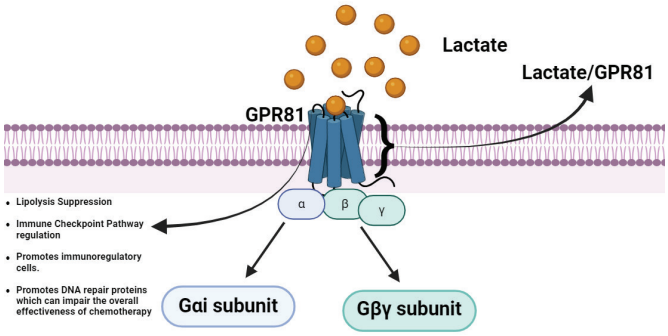

Figure 4: GPR81 receptor bound to lactate.

According to Brown *et al.* not only in cancer cells but also in stromal cells of the tumor is pivotal for tumor growth, and, specifically, GPR81 expressed in antigen-presenting dendritic cells may be key to cancer cell immune evasion.[41, 42] Upregulation of GPR81 in dendritic cells leads to decreased secretion of proinflammatory IL-6 and IL-12, downregulation of MHC, and consequently decreased tumor antigen presentation and activation of T cells, which all are associated with immune evasion at the immune cell level.[42]

- ***Transforming Growth Factor (TGF-β):***

TGF-β is often recognized as a pro-tumor cytokine. Some evidence supporting this belief can be seen in the fact that it inhibits the activation and functioning of the NK cells and **deletion of the TGF-β receptor subunit** improved mTOR activity and the cytotoxic functions in response to IL-15.[43] Not only is it responsible for dysregulating the function of anti-tumor immune cells but also activates cells like (Foxp3)$^{+}$ Tregs, MDSCs and CAFs which supposedly have a pro-tumor function and are one of the key players of the immunosuppressive mechanism.

A review by WanJun Chen,[44] discusses the implication of TGF-β expression in various CD4+ and CD8+ T cells. CAR-T cell therapy mainly uses effector CD4+ and CD8+ T cells. TGF-β mainly suppresses the effector CD8+ T cells in two ways: (1) by inhibiting expression of effector molecules like granzyme A and B, perforin, Fas ligand, and IFN-γ or (2) by not directly dysregulating the effector CD8+ T cells but instead, through Treg-derived cell membrane-bound TGF-β and by excluding CD8+ T cells from tumor infiltration. According to research by Park *et al.*, PD-1, which is a cell-surface receptor that interacts with its ligands PD-L1 and PD-L2 can result in inhibition of T cell effector function. TGF-β1 improves antigen-induced PD-1 expression independent of Smad-2 and dependent on Smad-3 transcriptional activation in T-cells *in vitro*.[45] In research by Li *et al.*, a strain of floxed TGF-βRII mice (RII) were crossed with CD4-Cre transgenic mice (4cre);[46, 47] histological examinations showed heavy infiltration of leukocytes into multiple tissues including stomach, lung, liver, pancreatic islets, and thyroid gland of 4cre-RII/RII but not those of 4-cre-RII/+ mice.

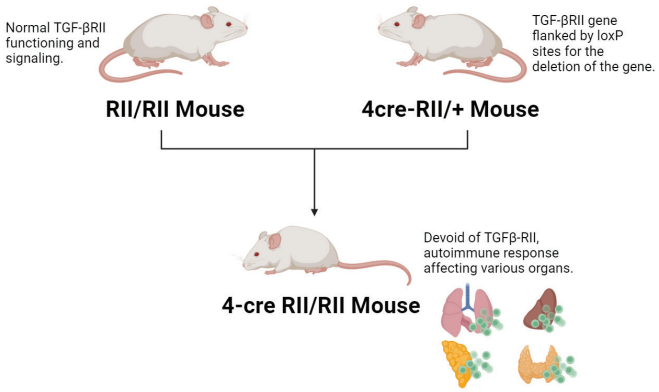

Figure 5: Autoimmune response shown by mouse devoid of TGF-βRII, emphasizing its immunoregulatory role.

- ***Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4):***

CTLA-4 is often associated with the CD28 receptor as they both are homologous receptors and are expressed by both CD4+ and CD8+ T-cells. Together they positively (by CD28) and negatively (by CTLA-4) regulate T-cell function. They both interact with CD80 dimer and CD86 monomer. Both ligands are present on APCs like dendritic cells, however, targeting them would not only mean inhibition of CTLA-4 but also CD28 which showed a dampened immune response associated with decreased CD28 costimulation when knocked out of mice.[48, 49] CTLA-4 is largely present in FoxP3+ Treg cells or activated T-cells.[48] In preclinical studies, blockade of CTLA-4 leads to inhibition of cancer cell lines like lymphomas, col-

orectal, renal, and fibrosarcoma [50] and activation of 'human tumor-specific CTLs'.[12, 51]

However, there are some serious downsides to targeting this receptor, according to Ise *et al.*, a lethal autoimmune response is observed in CTLA-4 knockout mice which resulted in the release of 'self-reactive' T-cells.[48, 52-54] The trials have also shown a wide spectrum of immune-related adverse events (irAEs) occurring in 60% to 65% of the patients. These events commonly affect the skin, gastrointestinal tract, and endocrine organs [36] and additionally also affect most of the body's other organs, including the liver, eyes, nervous system, lungs, heart, kidneys, and joints.[48, 55-73]

The aforementioned section talks about the GPR81 receptor, TGF-β cytokine, and CTLA-4 protein. These are some key components of the tumor microenvironment responsible for immunosuppression. They are also present in the T-cells, dendritic cells, macrophages, Natural Killers (NK), etc. Their presence in the TME is not solely responsible for the immunosuppression but their presence in/on immune cells also plays an important role in attenuation the activity of these cells. Recent advancements have led us to modern gene-editing techniques out of which CRISPR-Cas9 stands out. It is with these gene-editing techniques that we can knock out these genes from the CAR-T cells and potentially include other genetically modified cells like dendritic cells, natural killers (NK), and macrophages as a part of the treatment. With the deletion of these receptors, cytokines, and proteins we can achieve an increased level of immune response. However, we should keep in mind the side effects knocking out of some of these components might have on the overall metabolism and life-cycle of the immune cell, for instance, the lethal autoimmune response caused by inhibition of TGF-β and other effects on the overall functioning of the body.

- ***CRISPR/Cas9:***

CRISPR genome editing is arguably the most efficient, effective, precise, and extensible in its application. CRISPR can insert, delete, modulate, silence genes, and do many other things like base editing, live imaging of the genome, targeted manipulation of epigenetic markers, etc. We can also use CRISPR to knock out immunosuppressive receptors from the CAR-T cells.

One of the key moments in the usage of CRISPR for treating neoplasm and related malignancy was shown by Schumann *et al.*[74] The baseline of the methodology adopted by them was the presence of inhibitory ligands such as PD-L1 on tumor cells and surrounding tissue and also its presence on the T-cells where it has been identified as a marker of T-cell exhaustion and a critical regulator of T cell fate and function. Their results were positive with CAR-T cells being able to tackle PD-L1+ tumor cells and possible future implications of its success allowing physicians to achieve similar results by administering fewer cells. One of the prominent cytokines in cancer signaling is TGF-β which is related to Type-1 and Type-2 TGF-β receptors. In another research by Tang *et al.*,[75] TGFBR2 was knocked out (KO) using the CRISPR/Cas9 system. Their research puts forward various functions of TGF-β where TGFBR2 KO could eradicate the pro-tumor effects of TGF-β1 on tumor lysis and cytokine release.

One way of knocking out receptors is by synthesizing CRISPR sgRNA (single guide RNA). Here, we must know the sgRNA sequence of the receptor we are targeting. For instance, for targeting PD-1 we can use the PD-1 sgRNA: 5' GGCCAGGATGGTTCTTAGGT3'.[76, 77] Then we can electroporate the cells, T-cells in this case. A ribonucleoprotein (RNP) compound can be formed by incubating the Cas9 protein and the sgRNA. We can then centrifuge the cells and resuspend them in a buffer. This mixture of RNP complex, resuspended cells, and enhancer is finally electroporated and incubated.[76]

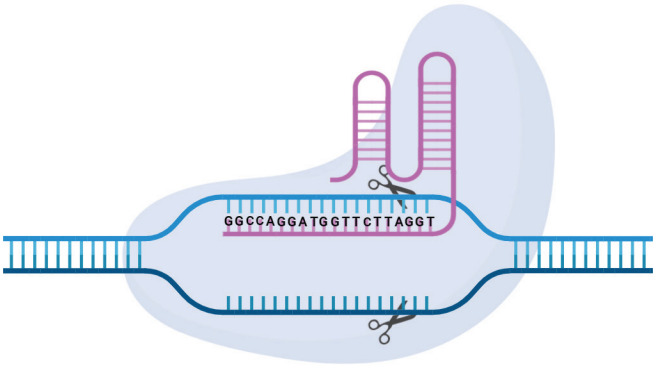

Figure 6: CRISPR sgRNA targeting PD-1.

There are many ways by which we can alter the aforementioned method of editing. For instance, using microinjection to deliver gene editing system cargo is considered the 'gold standard' with very high efficiencies.[78-80] Also, if we try to KO various receptors, the increased molecular weight of the cargo can be a 'limiting factor' which is not so in microinjection.[78] We also have viral vector delivery methods like adeno-associated viruses (AAVs), adenovirus, and lentivirus. AAVs might not be the best choice of delivery here, as it is not the most suitable for multiplexing with a diameter of ~20 nm and lentiviruses or adenovirus might be better if considering specifically viral vector delivery methods.[78] Wang *et al.* [81] designed a library of 73, 151 members, which contained various sgRNAs targeting 7, 114 genes and 100 non-targeting controls which is a significantly high number for an sgRNA library.[78] Lipid nanoparticles are also a common choice of delivery vehicles. They are more relevant in the context of CAR-T cells as they do not contain any viral components, and do not hold the potential to raise any immunogenicity concerns. However, this method lags in producing high-quality results in successful delivery as, firstly, it needs to escape the endosome, the cell can direct it into the lysosomal pathway which can degrade the lysosomal contents. Even if it is able to escape the endosome, it might face another barrier in translocating to the nucleus.

- ***Role of dendritic cells and their possible integration with the CAR-T cell therapy:***

T-cells, more specifically cytotoxic T-cells and T-helper cells are majorly used in CAR-T cell therapy. The usage of modified T-cells and using them is the baseline of CAR-T

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cell therapy because T-cells are one of the most effective immune cells but there is no need to be limited to using modified T-cells only. The immune cells often work in integration with each other in the immune system possessed by our body to produce a better immune response. One such dependency is that of T-cells on APCs. These APCs have a crucial role in T-cell activation. One such APC is a dendritic cell that has the ability to present exogenous antigens using MHC class II molecules to CD4+ helper T-cells, along with costimulatory molecules like CD86 and CD83.[82,83] DCs are a type of professional APC that is responsible for the three main signaling functions: antigen presentation, expression of costimulatory molecules and cytokine/chemokine secretion.

They are able to **train and activate** T-cells due to these qualities to fight pathogens and cancer cells.[83-85]

Dendritic cells are arguably the most effective antigen-presenting cells.[86] Improvement in their functionality can lead to better T-cell responses. A study was conducted by Raychaudhuri *et al.*[87] aimed at finding the pro-tumor lactate-induced reprogramming in intratumoral plasmacytoid cells. According to their findings, pDCs recruited by the tumor showed a lack of IFN-α generation and lactate-induced expansion of Tregs and tryptophan metabolism modulation in pDCs leading to the expansion of CD4+, FoxP3+, and Tregs in breast cancer.

- ***Genetic Targets in DCs to Improve their Function:***

GPR81 is a Gi protein-coupled receptor. Inhibition of its functioning can possibly involve a reduction in a cAMP generation driven by the Gαi subunit and cytosolic Ca^{2+} mobilization which is a function of the Gβγ subunit. To understand the functions of the Gβγ subunit gallein was used to inhibit the Gβγ subunit. It was found that 1μM completely nullified calcium influx. This Ca^{2+} mobilization regulates GPR81 downstreaming by either or both Ca^{2+}/calmodulin-dependent protein kinase (CAMKII), and calcineurin phosphate (CALN). Out of the two, when only CALN was inhibited a 'significant reversal of the inhibitory effect of lactate was registered'.[70]

In addition to the possibility of targeting GPR81, we can target other proteins like MCT-1 and MCT-2. Inhibition of MCT-1 and MCT-2 resulted in significant reversal of the lactate-mediated inhibition of IFN-α.[87] Another protein that was earlier discussed is TGF-β. Blocking TGF-β and TNF-β (Tumor Necrosis Factor) independently without blocking IL-10 resulted in a partial decline in the TUMSN (suppressants derived from tumors)-mediated inhibitory effect on IFN-α production of healthy pDC. Even better results involving complete stoppage of TUMSN-mediated inhibitory effect on IFN-α production were produced when TNF-α and TGF-β were simultaneously blocked. In fact, blocking IL-10 along with TNF-α or TGF-β does not show a distinct impact.[89] This brings to our attention the fact that although hypothetically, inhibition of all these proteins would serve anti-tumor effects only, however, inhibition of a particular set of proteins as in this case only TNF-α and TGF-β simultaneously resulted in optimum results in contrast to combining it with IL-10.

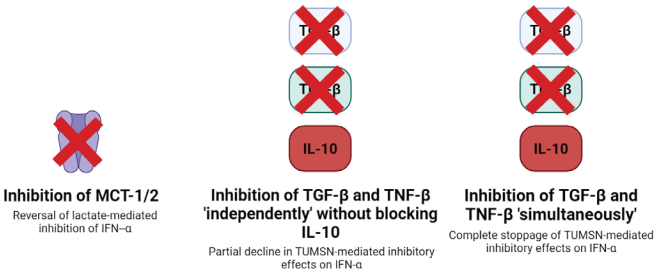

Figure 7: Varied responses generated by different combinations of inhibition.

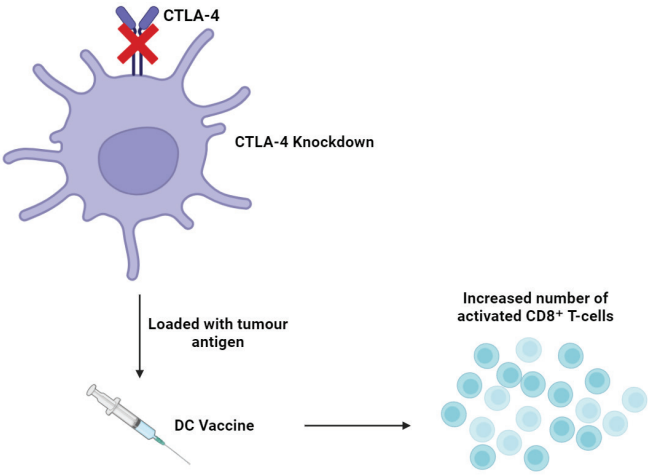

Figure 8: CTLA-4 knockdown in DCs can induce a greater volume of activated $CD8^+$ cells producing a better anti-tumor response.

Usage of siRNA-mediated knockdown of CTLA-4 in a DC vaccine proved to be effective producing a significantly increased number of activated CD8+ cells improving anti-tumor action marking its importance as a possible target gene.[90]

IL-10 is an important target in DCs. It is known to interfere in the interaction between DCs and CTLs for antigen-presentation which is a primary function of the DCs and a primary reason for their extension to the CAR-T cell therapy.[12,91-93] It is believed that IL-10 can regulate MHC-II and many costimulatory molecules and potentially reduce inflammatory response due to a reduction in the inflammatory cytokines secreted. Results from an experiment by Steinbrink *et al.*,[92] demonstrate that when IL-10-treated DC were used APCs for CD8+ T cells derived from peripheral blood and cord blood showed a decreased proliferation in all T-cell: DC ratio.

There is a wide array of potential targets including adenosine receptors, nitric oxide, and many more. Targeting them to produce optimum results might be a challenge as mentioned earlier, so further experimentation is required to decide the most suitable targets.

- ***Extracting and Culturing DCs:***

pDCs can be extracted from the blood through apheresis. Apheresis means the removal of a component of blood which can be anything like WBCs etc. and the remaining components returning back to the patient.[94] However, extracting DCs directly from the blood is challenging and less rewarding as they make up a very small amount (~0.2%) of the human blood mononuclear cells.[95] A review by Lozano *et al.*[96] talks about

the similar efficiencies of the modern apheresis systems, but the Spectra Optia system has shown some additional benefits in the collection of CD14+ cells which are mainly monocytes and macrophages required for DC production and also shows high efficiency in successfully separating it from other possible components which has often been seen as one of the key areas for improvement in the newer generations of systems.[97] After extracting these cells there is still a need for removing non-monocyte cells which can be possibly removed by methods like:

- Plastic Adherence
- Using magnetic beads coupled to CD14 antibodies
- Cell depletion using beads coupled to antibodies against CD2+ T cells
- Using CD19+ B cells
- Counterflow centrifugal elutriation

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is known to regulate DC homeostasis and DC development. GM-CSF supports the development and cell expansion *in vitro* [98] and *in vivo* respectively. It has been used to stimulate the differentiation of monocytes into DCs.[99] In fact an increased number of DCs have been found in the spleen and thymus of a transgenic mouse which were overexpressing GM-CSF indicating its effects *in vivo*.[100, 101] The maturation of monocytes into immature DC thus commonly involves culture with GM-CSF and IL-4 or 13.

It has been shown by Ohteki *et al*. that DCs can produce a significant amount of IFN-γ when infected with *Listeria monocytogenes* 96, 102 or when IL-12 is administered. This finding can provide scope for the development of more potent DCs. Alongside, other cytokines like IL-1β, TNF-α, IL-6, and Prostaglandin E2 (PGE2) bear the potential of producing 'mature' DCs with high motility.[96, 103] IL-6 and PGE2 are generally recognized as pro-tumor cytokine and lipid respectively. Armitage *et al*. mentioned it as a 'pan-immunosuppressive mediator' as it shows detrimental effects on cells of the immune system which function against the tumor-like cytotoxic T lymphocytes and Natural Killer cells, and type-I inflammation and supporting cells like Treg and MDSC, and type-II inflammation which are considered immunosuppressive and known to dim the effects of immunomodulatory cells.[12, 104] It is also associated with other ligands like PD-L1 which is known for its immunosuppressive nature.

The review by Lozano *et al*. also talks about a protocol developed in 2003 that involved incubation of monocytes with GM-CSF and IL-4 in the first 24 hours and treatment with proinflammatory cytokines for their activation in the next 24 hours. Overall, producing DCs in just 48 hours served many benefits and is even now used in clinical practice.[96]

The next step involves loading the dendritic cells with antigens so that they can present these to the T-cells. This step provides us with an option to load antigens specific to the target cells which in this case is tumor. However, tumor antigens are highly varied making it difficult to eradicate the neoplasm. Three instances included in a review by Sterner and Sterner [22] also bring to attention the major challenge in targeting any antigen. First, relapsed patients with acute lymphoblastic leukemia (ALL) when treated with CD19-targeted CAR-T cell therapy show considerable improvements. However, the tumor formulates a mechanism to evade these CAR-T cells involving downregulation/loss of CD19 antigen in about half the patients.[105, 106] Second, patients with multiple myeloma when treated with CAR-T cell therapy targeting BCM showed downregulation/loss of BCMA expression.[107-109] Third, for glioblastoma treated with CAR-T cell therapy targeting IL-13Ra, the recurrent tumor showed a decrease in IL13Ra2 expression.[110]

To evade these problems a common approach is to target multiple antigens. However, this approach is not as effective and can be possibly rejected as the antigen-presentation of DCs through incubation has a short half-life of antigen expression on DCs.[96, 111]

AGS-003 is another immunotherapy using DCs to present tumor-specific antigens to prime T-cells. Its approach is slightly different from directly incubating DCs with antigens and involves transfecting DCs with the tumor RNA. This way enables DCs to present an array of antigens. The approach for AGS-003 production adopted by Amin *et al*.,[112] involved 'tumor total RNA' isolation from metastasectomy sample, amplification of mRNA using RT/PCR, and *in vitro* transcription. Mature DCs are then transfected with the tumor RNA through electroporation (in this case).

The addition of genetically modified dendritic cells and possibly other cells like natural killer cells and B-cells to the current CAR-T cell therapy can turn the current therapy into a rather superior subset of the immune system specifically modified to increase the effectiveness in targeting tumor site(s). During T-cell activation in CAR-T cell production purification of autologous APCs is required, this can provide a juncture for genetically superior DCs to be introduced in the CAR-T cell therapy, however, this process requires multiple additional steps, making it more arduous to obtain. Another way to activate these T-cells is from beads coated with anti-CD3/anti-CD28 monoclonal antibodies or cell-based artificial APC (aAPCs).[113] Using aAPCs instead of natural DCs can help us evade the steps required in extracting these from blood and the further steps required in purification.

- ***CRISPR/Cas-9 mediated Multiplexing:***

While CRISPR is widely popular for its usage in gene editing, sometimes there is a need to add, or delete, etc. various genes. To KO multiple genes from the CAR-T cells and even other cells like DCs, we utilize multiplexing. In an attempt to generate more enhanced and potent CAR-T cells, Xiaojuan Liu *et al*.,[114] targeted TRAC, B2M, and PD-1. Here four sgRNAs were used to target TRAC, four to target B2M, and two sgRNA to target PD-1. This highlights the importance of using multiple sgRNAs to improve the disruption probability of the gene. The mixture of Cas9 and *in vitro* transcribed sgRNA was electroporated into the umbilical cord blood-isolated CD3+ T-cells. Two interesting takeaways from their experiment are that when T cells were transduced first and then electroporated instead of the other way round they produced CAR-T cells with 'better gene-editing efficiency and viabil-

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ity' and gene-editing proved to have a proliferative downside on the CAR-T cells with standard CAR-T cells proliferating 300-folds 15 days after activation whereas multiplexed CAR-T cells undergoing only 100-fold proliferation.

- ***Controlling CAR-T cell cytotoxicity:***

Kill or suicide switches are increasingly used either for the purpose of cancer cell lysis or for therapies which may include controlling and mitigating the immunogenic effects of CAR-T cells. Its basic mechanism involves converting a harmless pro-drug into a toxic product. The two prominent suicide genes integrated with the CAR-T cells are herpes simplex virus thymidine kinase (HSV-TK) and inducible caspase-9 (iCasp9).[115]

HSV-TK has been used to tackle the effects of GvHD (Graft versus Host Disease). The HSV-TK is made a part of the CAR. Then, treating it with ganciclovir (GCV) is known to control the CAR-T cells by apoptosis.[115-117] A research by Beltinger *et al.*[118] describes the current understanding of the mechanism of the HSV-TK/GCV combination which involves:

• Phosphorylation of GCV by TK

• Phosphorylation of the previously produced phosphorylated form of GCV to GCV-triphosphate

• Its incorporation results in the inhibition of DNA synthesis and also causes breaks in the strand.[100]

• This ultimately results in caspase activation which results in cell necrosis.

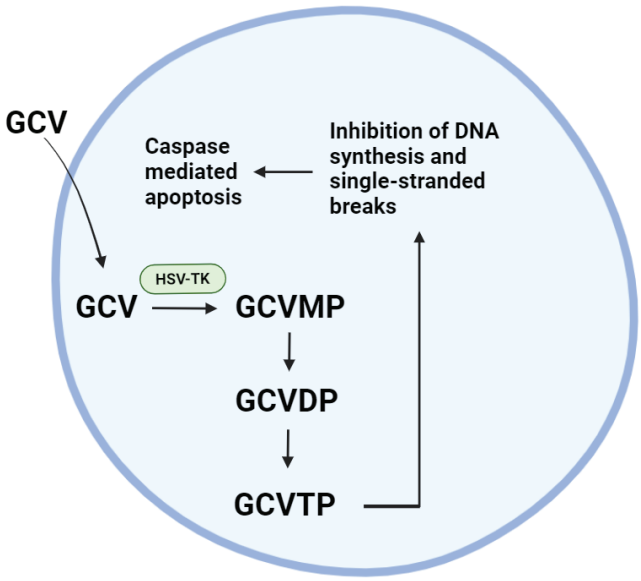

Figure 9: Mechanism of HSV-TK/GCV combination.

According to their research, the TK/GCV promotes the build-up of p53 which causes the movement of CD95 such that it causes cell apoptosis with the help of Fas-associated death domain protein (FADD). However, slow response and dependence on the cell's proliferation and growth cycle do not make it the most viable method.[115, 119]

A mechanism of iCasp9 functioning is proposed by Straathof *et al.* According to them, dimerization of the inactive caspase monomer results in its activation. Interactions between the CARD domains of Apaf-1 and caspase 9 are important for successful dimerization. However, they noticed that when caspase 9 was joined with 2 FKBPs it might result in spontaneous dimerization and observed toxicity, whereas, when 1 FKBP was used better expression of gene and relatively lower pace of dimerization and decreased toxicity in comparison to the former. Overall, it is a feasible OFF switch which is currently most practically suitable for CAR-T cells. Hoyos *et al.*[120] demonstrated successful apoptosis of iCasp9-IL15-CD19 CAR-T cells by treating it with AP1903.[115] In another research by Stasi *et al.* the dimerizing drug used for the iCasp9 to function successfully neutralized >90% CAR-T cells.[115, 121]

ON-switches are another way of manually regulating the CAR-T cells. The advantage presented by ON-switches is the precise controllability they offer through which CAR-T cell reactivity can be adjusted to achieve optimum effects. An interesting molecular ON-switch is proposed by Zajc *et al.*[122] The mechanism of this switch is based on human retinol binding protein 4 (hRBP4) and an orally available drug A1120. They engineered two protein scaffolds, FN3 and rcSso7d which help to identify the changes induced by A1120 and induce conformation of hRBP4. The hRBP4 does not have any free glycosylation sites or free cysteines. This is responsible for its stability and better expression. In addition to this, the specificity of A1120 was also proved. Several molecules that are possibly capable of binding to hRBP4 were tested, resulting in only A1120 being able to show significant hRBP4 binding and almost negligible results with retinoic acid and other molecules. In an attempt to create an ON switch CAR, chain I is created such that it comprises: RS3 binder (on a second generation CAR backbone) and extracellular IgG1-Fc spacer domain and chain II contains: hRBP4, scFv targeting CD19 and an IgG1-Fc spacer domain. A1120 is integral for the CAR-T cell activation as it is only in its presence that chain I and chain II can combine. The efficacy of its mechanism was also substantiated when tested, with the negative control CAR-T cells showing efficacies similar to T-cells with only chain I or no CAR which showed cytotoxicity levels in parallel with natural T-cells Efficient lysis was observed in the NALM6 cell line being used for the test when A1120 was present.

Another switch called Chemically Regulated -SH2-delivered Inhibitory tail developed by Sahillioglu *et al.*[123] highlights the problems posed by the irreversible nature of the other currently available switches. In the context of currently present switches like HSV-TK, there is not much scope for titration of T-cell functions. The switch aims to provide better controllability or titration of the T-cells. The basis of the research is around the presence of an activating signaling complex. It was hypothesized that if an inhibitory signal is brought to this complex '**in a titratable manner**' then it can lay the foundation of the switch's mechanism. In the search for elements that would fit this description, it was found that signaling through CARs and TCRs involves phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMS) and the fact that Zap70 kinase binds to these ITAMS with the help of SH2 subunits. For SH2 domain-assisted inhibitory tail delivery, the PD1 intracellular domain was chosen primarily due to its reversible effect. This led to the formation of Zap70 2xSH2 domain-PD1 tail fusion protein (Zap90-PD1). Assessment of

Zap70-PD1 efficacy was made, by testing the different possible combinations like with free PD1 tail and with Zap70 2xSH2 domains without PD1. It was noted that it was only when Zap70-PD1 was used that extremely effective suppression of cytokine production and T-cell degranulation could be seen. Not only this but Zap70-PD1 can be controlled by other drugs like the fusion proteins created in this case which consist of HCV NS3/4A and a degron which can result in protein degradation. It works by an interesting mechanism that requires a hepatitis C virus (HCV) protease inhibitor. The HCV protease cleaves the linker between the protein and the degron. Since, now the linker is cleaved, degron which is responsible for the degradation of the protein can no longer cause this. But in the presence of the HCV inhibitor the fusion protein is broken down by the proteasome. Not only in theory but it was also tested, showing downregulation of fusion protein level in CD4+ and CD8+ cells.

Conclusion

CAR-T cell therapy is a unique method of treating cancer in comparison to the other alternatives present. This can be attributed to the exciting results of its current application in treating blood cancer. Not only this but it also possesses the potential to be used in treating solid tumors which can become possible if we can identify the additional immunosuppressive factors presented by the solid neoplasms and then modify the next generation of CAR-T cells to be able to easily evade these challenges. In this review, I have tried to describe some of the immunosuppressant elements posed by the tumor which in combination with some T-cell mechanisms can induce an immunoregulatory response decreasing the cytotoxic potential of T-cells. One way of dealing with this is by making genetic deletions in T-cells which removes the T-cell counterpart responsible for the immunoregulatory effect.

The immune system is not just limited to T-cells but is recognized as a system of many cells working together to achieve the same overall result by serving different aspects. An example of this is illustrated by dendritic cells which, although, might not be as efficient in directly combating tumor cells as T-cells are, however, serve an integral role in their growth and activation. If similar genetic deletions can be made in dendritic cells which can improve their persistence at tumor sites, then it will ultimately result in better activation as well as tumor-antigen presentation, one of the major challenges with using CAR-T cell therapy.

While all these modifications bear great potential for the future success of CAR-T cell therapy, the uncontrollable nature of the induced cells can have life-threatening consequences. Through the years, we have developed numerous measures to better control the outcomes of these adoptive therapies. I have tried to include currently widely accepted methods for managing cytotoxicity like HSV-TK and iCasp9 and also a suicide switch CRASH-IT which provides better control of the T-cell functions.

A combination of all these methods along with currently viable methods like combination therapy can help overcome the current challenges to a great extent and can pave the road for its future approval for treating solid cancers.

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