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CRISPR in the Modern World and the Context of Cancer Immunology

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ABSTRACT: CRISPR-Cas systems are the primary concept utilized in gene editing today, as it is an adaptive immunity system that can be used to edit or change areas of life as we know it. There are a few primary steps in CRISPR: adaptation, biogenesis, and interference. CRISPR's first step is the CRISPR adaption phase, which provides genetic memory required for expressing phases that neutralize re-invading nucleic acids. After adaptation, biogenesis occurs, where the precursor cRNA is processed within the CRISPR repeats to generate mature crRNAs. In the last stage of CRISPR (Interference), the mature crRNAs that were formed in biogenesis are used as guides to interfere with invading nucleic acids. CRISPR is used in many fields worldwide, such as the selective breeding of animals and plants. However, its most practical use has been in cancer immunology. CRISPR is being used to edit genes in the body's immune system to make the immune system more defensible and more well-adapted to treat cancer, exposing flaws and helping fix these flaws very well. In this review, we explore the history of CRISPR, how CRISPR works, its useful applications, and its usefulness in the cancer immunology sector.

KEYWORDS: Biology, Gene Editing, CRISPR, Cancer Immunology.

Introduction

CRISPR (Clustered regularly interspaced short palindromic repeat)-Cas systems were first discovered in 1987 during an attempt to identify the protein responsible for the isozyme conversion of alkaline phosphate in E. cells that sequenced an *E. coli* DNA fragment which contained the *iap* (isozyme of alkaline phosphatase) gene by Y. Ishino.[11] For sequencing to take place, the cloned DNA fragment was produced by cloning the target DNA into an M13 vector (a filamentous bacteriophage specific to *E. coli*). During this sequencing in one of the fragments containing *iap*, one sequence repeated itself many times and was difficult to read due to secondary structure formation by the sequence. The sequence had dyad symmetry (base pair sequences are inverts of each other) sequences repeated along five or more times in the sequence, and so little was understood that it was not mentioned in the discussion of the paper. This was the first discovery of CRISPR, and it was barely possible even to read the sequences, let alone determine their function. However, the discovery of CRISPR in archaea a few years later was another changing point in the history of CRISPR. Francisco Mojica and his coworkers discovered similar repeated sequences in the archaeon (group of single-cell prokaryotic organisms which lack a defined nucleus) *Haloferax mediterranei*. The study's authors believed that the sequences could somehow be involved in regulating gene expression. Shortly after, yet another similar sequence was found in *Haloferax volcanii*. It was theorized that the sequences were involved in replicon portioning.[2] During the 90s, automated sequencing machines, and more efficient DNA sequencing procedures allowed scientists to access complete genome sequences. The unusual sequences continued to be sighted in bacterial and archaeal genomes, but not eukarya, as all three domains of life (Archaea, Bacteria, Eukarya) entered the genomics era. They went by many names, including short, regularly spaced repeats (SRSRs), spacer interspersed direct repeats (SPIDRs), and large clusters of tandem repeats (LCTRs).[2] Many and Francisco Mojica soon realized that these sequences' sudden growth and sighting were functionally related. Ruud Jansen and Francisco Mojica, along with others, proposed CRISPR in 2002. It began to become accepted by the community, preventing further confusion from the terms we discussed earlier.[2] Boundaries were created for common characteristics of CRISPR, such as (i) they are located in intergenic regions. (ii) they contain multiple short, direct repeats with minimal sequence variation, (iii) the repeats are interspersed with nonconserved sequences, and (iv) a common leader sequence of several hundred base pairs are located on one side of the repeat cluster.[9] Since CRISPR was present in too many inherently different domains of life; it pointed to them having a more general role. They were found in most archaeal genomes and about half of the bacterial genomes, making them the most widely distributed family of repeated sequences in prokaryotes. CRISPR sequences have not been found in any eukaryotic genome, including in humans, which are eukarya.[9] Furthermore, the growing pile of genomic sequences in the early 21st century led scientists to discover four conserved genes regularly adjacent to CRISPR regions and designed CRISPR-associated genes 1 to 4 (cas1 to cas4). For Cas1 and Cas2, no similarity to functional domains of any known protein was identified. However, Cas3 and Cas4 contained motifs characteristic of the superfamily (above a family but below an order biological classification) 2 helicases. As a result, Cas3 and Cas4 were hypothesized to be involved in DNA metabolism and repair, recombination, transcriptional regulation, and chromosome segregation.[12] The CRISPR function was discovered

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when Francisco Mojica and Christine Pourcel noticed that the spacer regions between the sequences were similar to bacteriophages, prophages, and plasmids. They proposed that CRISPR sequences function in a biological defense system similar to eukaryotic RNAi to protect cells from entering those foreign viruses. It was also suggested that CRISPR could cause the immune system to "photograph" these diseases and viruses to add to a memory of past afflicted viruses.[12] Alexander Bolotin later confirmed these results and noticed a correlation between spacers of phage origin and degree of resistance to phage infection, suggesting that CRISPR could be used to create antisense RNA (single-stranded RNA) that is complementary to a protein-coding mRNA).[8] The role of Cas proteins as effectors of prokaryotic immunity was highlighted in 2006 by Makarova and others, and this pinpointed the CRISPR-Cas system, with its memory component, it resembles the adaptive immune system of vertebrates (the crucial difference being the animal immune system is not inheritable).[7] CRISPR-Cas system's erratic distribution suggested high mobility, and their pervasiveness in archaea suggested it emerged in an ancient ancestor and spread horizontally and concluded that CRISPR-Cas systems could be manipulated to silence specific genes in organisms encoding Cas proteins.[4] The function of the CRISPR-Cas system was experimentally proven in 2007 when it was used as a prokaryotic acquired immune system in the lactic acid bacterium *Streptococcus thermophilus*.[7] Insertion of the phage sequence into the spacer region of the CRISPR of *S. thermophilus* made the strain resistant to the corresponding phage. Still, when the protospacer sequence was deleted, the bacterial resistance disappeared. Also, CRISPR-Cas systems were shown to restrict the transformation of plasmids that carried sequences similar to the CRISPR spacers. The group then reconstituted the immunity system using *E. coli* CRISPR, discovered in 1987.[3] They then proceeded to demonstrate that the processed RNA from the transcription of the CRISPR region function in harmony with the Cas proteins produced from the genes located next to the CRISPR. Also, the CRISPR-Cas system of *S. thermihilus* expressed in *E. coli* shows protection against phage infection and plasmid transformation by the CRISPR-Cas9 system. The specific reasoning behind using the cas9 protein was also demonstrated, which was that cas9 is the sole cas gene necessary for CRISPR-encoded interference, and CRISPR-Cas became widely known as the prokaryotic acquired immunity system. A review of CRISPR is necessary due to the quickening growth of the field and its sudden vitalization in the past few years. CRISPR is a relatively new technology, as opposed to household names such as chemotherapy, and a review of this topic would be vital to help those understand its benefits. There are many research articles about CRISPR. However, mine aims to target many different aspects, including the delivery of CRISPR and the treatments it can be used for. It also has a specific target on CRISPR's role in cancer immunology, a topic less widely covered than CRISPR's main area, where it targets genetically modified organisms and its ethical morality.

Discussion

Explanation of CRISPR:

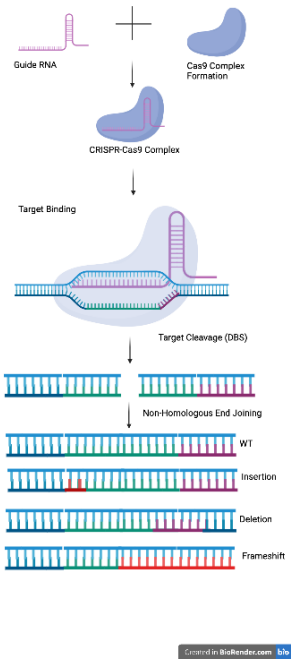

Figure 1: Simple Explanation of CRISPR, Created in Biorender.com

CRISPR is a naturally occurring RNA-guided endonuclease (an enzyme that breaks down a nucleotide chain into two or more shorter chains). CRISPR uses a short RNA sequence to drive the formation of a double-stranded break (DBS) at the targeted site, simplifying and greatly reducing the time needed for gene editing design and implementation. The CRISPR nuclei primarily use Type II due to the simple architecture of the effector, which has made it beneficial for genome editing technologies. CRISPR's first step is the CRISPR adaption phase, which provides genetic memory required for expressing phases that neutralize re-invading nucleic acids. In adaption, prespacers (a stretch of DNA) must select a suitable space to be put into, be generated properly, and be incorporated into arrays. The selection of the target sequence where the prespacer is put is not random; rather, a short sequence known as the adjacent protospacer motif (PAM) must be located directly next to the protospacer.[10] A Pam is recognized as any sequence within the genetic code that follows the nucleotide pattern of NGG, with N representing any possible nucleotide and G representing guanine. Then in the generation and incorporation phases, the prespacer is captured by Cas proteins. Then the bound prespacer and its Cas proteins are directed to the CRISPR nuclei, wherein a repeat sequence is duplicated, and the prespacer is put in between the two repeats as a novel spacer. Both the cas1 and cas2 genes are essential to his phase. The cas1 protein is a metal-dependent endonuclease that cleaves double-stranded DNA (dsDNA) at the CRISPR site. Cas2 is also a metal-dependent endonuclease, and together, cas1 and cas2 form a complex. In this complex, however, only cas1's nuclease activity is essential for adaptation, but in the absence of Cas2, cas1 is incapable of binding to the CRISPR locus, ma-

king both necessary in adaptation.[21] Also, a CRISPR repeat and a leader sequence (polynucleotide regions involved in gene expression) are necessary to integrate a spacer. The AT-rich leader sequence (typically upstream of the CRISPR locus) often contains the promoter that directs transcription of the locus into pre-crRNA. The leader sequence and its role in transcription are also involved in harboring recognition signals and directing the Cas1-Cas2 complex to this position. Adaptation allows the host organism to memorize the genetic material should it reencounter it and display its adaptive nature. After adaptation, biogenesis occurs, and transcription is the first part. In transcription, the precursor cRNA mentioned above is subsequently processed within the CRISPR repeats to generate mature crRNAs, usually composed of a repeat sequence recognized by Cas proteins in a structure. There are two classes of crRNA maturation, both of which yield mature guide cRNAs. In Class 1 and 3 systems. this occurs when trimming off the pre-crRNA by a nuclease occurs, and the mature cRNA is formed, which displays a hairpin structure. In class 2 systems, a tracrRNA is required to form mature cRNA, and the anti-repeat of the RNA enables the formation of an RNA duplex with each of the repeats of pre-crRNA, which Cas9 stabilizes. This duplex is then recognized and processed by RNase III, which yields an intermediate form of CRNA that undergoes further maturation to lead to the mature guide crRNA. In the last stage of CRISPR, the mature crRNAs that were formed in biogenesis are used as guides to interfere with invading nucleic acids. Each mature crRNA guides a Cas protein to complementary targets, and Cas nucleases degrade the target sequences. There are many possible ways that target degradation occurs. In Class 1 systems, Cascade (CIRPSR-associated complex for antiviral defense)-like complexes are used for target degradation. Class 2 systems utilize a single effector protein for target interference. Type I, II, and V systems can recognize the protospacer's Pam sequence upstream (downstream in type II) to avoid self-targeting. And type III systems do this via the tag of the mature crRNA, which cannot base pair with the target to enable degradation by the complex.[4] There are several ways of delivering CRISPR to their target cells, and three major categories: Viral-Mediated Delivery, Non-viral vector delivery, and Physical Delivery. Viral-Mediated Delivery is accomplished through two mechanisms, infection, and replication. During infection, a virus can recognize and enter a specific cell, and the viral genome can be released into the nucleus for replication. After replication, the infection cycle repeats, the virus containing the delivery material is transported to the target cells, and gene therapy is achieved by genome editing. Viral-Mediated Delivery includes subclasses, including Adeno-Associated Viral Vector-Mediated Delivery and Lentiviral Vector-Mediated Delivery. Then, there are non-viral vectors, of which the major delivery methods are polymeric materials, liposomes, cell-penetrating peptides, and cationic nanocarriers. Their major advantages are the capability to accommodate large components for delivery, reduced hazardous nature, and easy generation. Cationic vectors have a high probability of uptake, making them suitable for delivering gRna or Cas9 to the cells. At the same time, Cell-Penetrating Peptides are useful for having low off-target activity while also successfully tested in genome alternation of various cell types in the body (dermal fibroblasts, HeLa cells, human embryonic stem cells). Physical delivery methods include microinjection and electroporation. Due to its target specificity, simplicity, and high reproducibility, this method is the most promising without limitations. A needle with a diameter ranging from 0.5 to 20 μm is inserted into embryos, specifically in zebrafish and mosquitoes, for direct delivery to a target cell. Electroporation generates pores on cells and enables plasmids to enter the cells. The electroporator produced electric pulses that created pores.[5]

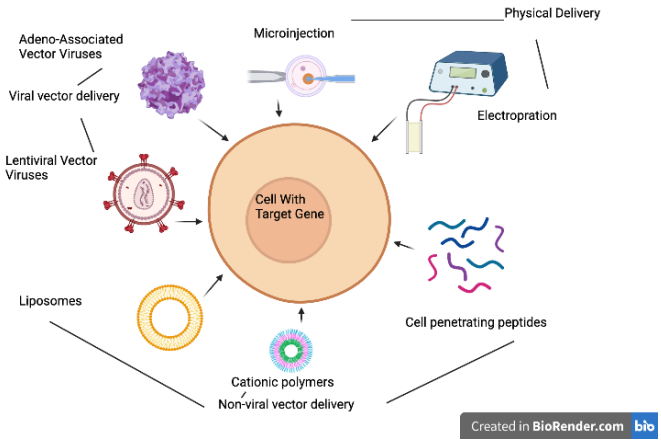

Figure 2: Methods of Inserting CRISPR into cells, Created with biorender.com

The Use and Popularity of CRISPR:

Due to its usefulness, CRISPR is currently being used in a large number of areas to improve both commercial & medical interests. CRISPR is used to improve the yield of crops and their adaptation to the environment in many fruits, which staple foods and provide essential nutrients to communities worldwide.[28] CRISPR can also be used in animal breeding to insert traits desired by breeders into animals such as dogs & cats. Moral dilemmas have been raised about inserting these traits into larger wildlife animals, including humans.[3] One of CRISPR's most promising areas is cancer immunotherapy, which is used to edit genes, potentially inhibiting the immune system and its processes.

Cancer immunotherapy is a type of cancer treatment that helps your immune system. It can help the immune system in a variety of ways. Within the immune system are different lineages of cells: Lymphoid and Myeloid. The Lymphoid cells include B and T cells, both of which specify even further to have more unique functions. The T lymphocyte differentiates into three main types: B cell progenitors, T cell progenitors, and Killer T cells. T cell progenitors then differentiate into three sub-classes of T cells: Helper T cells, Cytotoxic T cells, and Memory T cells.1 Helper T cells stimulate B cells so that they can make antibodies while also helping killer T cells develop. Memory T cells are antigen-expressed T cells that mediate a faster immune response upon repeat encounters, created after the body has dealt with a pathogen in case it reencounters it. Cytotoxic T cells (CD8 T cells) recognize foreign antigens combined with major histocompatibility complex class

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1 (MHC I) on the surface of cells and complete their killing function by releasing two types of cytotoxic protein: granzymes (which induce apoptosis in target cells) and perforin (which create holes in the target cell membrane).[22] Natural killer cells also recognize foreign antigens. They use similar processes to cytotoxic T cells to kill pathogen cells, the significant difference being that cytotoxic T cells have receptors specific for a particular microbe and can only kill target body cells with one microbe. In contrast, NK cells can destroy various microbe-infected body cells.

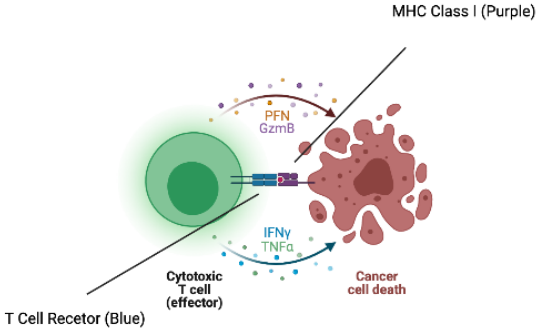

Figure 3: A diagram explaining how Cytotoxic T cells induce cell death in Cancer cells, created with Biorender.com

B cell progenitors differentiate into two subclasses of B cells: Plasma cells and Memory B cells. Plasma cells are large cells with an abundance of endoplasmic reticulum, which allows them to produce large amounts of antibodies, respond to signals from T cells during infection and continue to produce antibodies until the disease is controlled. Memory B cells are long-lived cells that remain within the body and allow faster reactions to future conditions, similar to Memory T cells.[15] Despite these immune responses, the immune system can sometimes fail to prevent pathogens or cancerous cells that have never been detected. Compared to other forms of cancer immunotherapy, such as radiotherapy or chemotherapy, CRISPR has a low-risk ratio compared to the other two, and as a result, less toxic possible side effects, including loss of hair, nausea, and many more. Chemotherapy is usually used to shrink your tumor so that invasive surgery can be performed to remove it from your body.[23] CRISPR involves no surgery, which mitigates the risk ratio and leads it to a better option. Additionally, chemotherapy is repetitive and allows cancerous cells to mutate and develop ways to block chemotherapy's effectiveness within the tumor. Chemotherapy can also cause cancer recurrence due to how much of the body it destroys, including weakening the immune system so that the body will be much more susceptible to other diseases.

CRISPR usage in cancer immunotherapy is essential for two significant reasons: the risk factor and how it can progress and grow to match the cancer. As spoken about before, CRISPR is much less invasive than current processes available and would allow for a much higher survival rate, meaning that people will be able to recover from their cancer much better after being inflicted with it. This also means that the treatment will harm their body less because it is less invasive and has less toxic and detrimental side effects on the body, leading to better protection against future cancerous cells & illnesses. Also, chemotherapy is highly stagnant; its processes do not continue to develop as the cancer does, and as a result, it is ineffective among a large majority of its patients.[19] CRISPR can be specifically designed for every patient so that it can be used to treat different problems that may arise in findings and create solutions to these problems effectively and continuously throughout the future.[19] These reasons play a significant role in why CRISPR has recently become popular. The ability to treat conditions on a case-by-case basis is an ability that, if doctors have it, will be extremely useful, especially in the field of cancer. Using CRISPR to specifically identify what is wrong with the patient, the most effective way to treat it is less invasive, less grueling, and less risky for the patient involved.

CRISPR in Cancer Immunotherapy:

Recently, one method used in CRISPR screening is the knockout (or silencing) or activation of genes. CAR T cell therapy has always faced the challenge of CRS (cytokine release syndrome), along with its significant contributors, IL6 and IL1.[32] One of CRS's leading contributors is GM-CSF, which has remained abundant as one of the inflammatory cytokines released by CAR T cells. However, CRISPR-Cas9 was used to knock out GM-CSF production in CAR T-cells.[32] This was achieved through CRISPR-edited GM-CSF knockout in CAR T cell secreting anti-IL6 scFv and IL1RA. This knocking out of genes in CAR T cells revealed that all three subjects received a response, of which two did not feel the effects of CRS and one received grade 2 CRS, none of which were affected by neurotoxicity. Another use of knocking out genes is in PD-L1 on tumor cells, where monoclonal antibody therapy emerged as an effective treatment.

However, only a minority of patients were able to present immune responses. Thus a CRISPR-Cas9 editing system delivered by cationic copolymer aPBAE was developed to downregulate PD-L1 expression via knocking out the Cdk5 (Cyclin-dependent kinase 5) gene *in vivo*.[6] This knockout of Cdk5 led to tumor growth inhibition in murine melanomas and lung metastasis suppression in triple-negative breast cancer while also demonstrating to aPBAE/Cas9-Cdk5 treatments created T cell responses in tumor microenvironments in that the population of CD8+ T cells was increased significantly while Tregs decreased. Silencing genes is also effective, specifically in the tumor microenvironment where silencing of Hif1a via histone H3 methylation (promoted by CRISPR/dCas9 system) was achieved.[8] The *Hif1a* silenced macrophage substituted as an inheritable tumor suppressing phenotype, and intertumoral injection of *Hif1a* Epigenetically Repressed Macrophages (HERMs) reprogrammed the immune suppressive microenvironment to the active microenvironment. This, in turn, reduced tumor burden, prolonged overall survival, and inhibited tumor angiogenesis. Activating genes through CRISPR can also be helpful in certain situations, such as NF-κB inhibitors. With these inhibitors, over-activation usually occurs in tumor microenvironments, leading to undesirable side effects. By activating NF-κB's gene expression (Nage), utilizing NK-FB's over-activation would lead to induced cell death in cancer cells but had no impact on regular cells.[33] Nage consisted of an NF-κB specific promoter formed by fusing an NF-κB

decoy sequence with a minimal promoter. This, in turn, could be bound together by the intracellular over-activated NF-κB and activate the expression of downstream effector genes in an NF-κB-manner. This demonstrates that CRISPR/Cas9 can be used to overexpress Nage, which leads to induced cell death of cancer cells.

The genes must be located within the genome first to determine genes that need to be knocked out, silenced, or activated. Firstly, we have an assay of predicted off-targets with two standard assays: mismatch-detection nuclease assay and next-generation sequencing. Off-targets are when sgRNA binding at a site does not lead to DNA mutation or cutting and may not have any functional consequence. This is caused by not every position in the gRNA needing to match the target DNA. In mismatch-detection nuclease assay, DNA from cells treated with Cas9 is PCR supplemented, transformed, and re-hybridized to form hetero-duplex DNA. This hetero-duplex DNA contains one wild-type strand and one strand with genetic variations in nucleotide chains. These mismatches can be recognized and cleaved using mismatch detection nucleases, primarily through Surveyor nucleases or T7 endonucleases.[30] On the other hand, the PCR can also be sequenced using NGS, which combines the advantages of sequencing chemistries, matrices, and bioinformatics technology to allow massive sequencing of DNA sequences, and NGS approaches can be more precise than mismatch-detection nuclease assays thanks to the DNA fragmentation, library preparation, and short time scale of the process.[25] In another process called systematic mutagenesis, mutagenic analysis of the target DNA was used to evaluate its importance using several factors such as position, identity, and the number of mismatches. It is still unclear whether the variation represents a specificity requirement or is confounded by unidentified changes in the target DNA. Mutations in the target DNA could change the abundance of the target DNA, which would alter target efficiency.[31] These mutations could also disrupt Cas9 binding sites for endogenous proteins. In a paper evaluating the systematic mutagenesis of the *Escherichia coli* genome,[14] the system is based on in vitro transposition of a modified Tn5 element, the Sce-poson, into linear fragments of each open reading frame. The transposon has both positive (kanamycin resistance) and negative (I-SceI recognition sites) selectable markers for the isolation of mutates and subsequent allele replacements.

The Sce-poson insertions can be replaced by marker-less mutations (using the I-SceI homing endonuclease) to select against retention, which allows a Sce-poson-containing gene to be targeted for designed or random modifications and permits the engineering of strains growth multiple mutations. Another way of determining genes is Whole genome sequencing (WGS). WGS works by selecting the order of bases in the genome of an organism and can detect small indels and Cas9-induced structural changes but can miss off-target sites if they are bound without cutting or perfectly repaired cuts. This is demonstrated in how Whole-genome sequencing revealed rare off-target mutations in grapevines edited by Cas9,[29] where WGS of grapevines were completed and off-target sites were identified. In contrast, only one off-target indel mutation was identified.

Lastly, DSB capture and sequencing can also identify target cells. Since Cas9 and other DNA endonucleases involve DSBs, many approaches to identifying off-target sites are applied to Cas9-treated cells to uncover genome-wide cutting sites. DSB capture approaches are physiologically more relevant but can be both more biased (due to the effect of local chromatin and sequence composition near the cutting site) and less efficient since DSBs exist fairly transiently.[17] For example, in a paper where 36 ZFN off-target sites were detected by an *in vitro* selection, one was identified by DSB capture.[26] DSB capture can also identify many false positives (since DSBs can be generated by cellular processes independent of Cas9 cutting); however, proper controls (cells treated with no Cas9 or sgRNA) can be used to filter these false positives.

CRISPR is primarily used to modify lymphocytes within the body and can treat various conditions and diseases with the immune system. Chimeric Antigen Receptor (CAR) T cells help treat malignant tumors, but the effectiveness towards solid cancers remains challenging. Nanobody-based CAR T cells designed by CRISPR-Cas9 for cancer immunotherapy are used to treat solid tumors in CAR T cells.[20] CD105, whose expression is upregulated on endothelial and cancer cells, is used as a drug target. Sequences are inserted for anti-CD105-nanobody-linked standard cassette genes into the AAVS1 site using the CRISPR-Cas9 system to show the generation of a CD105 anti-CD105 nanobody. A co-culture with CD105+ target cells led to the activation of the anti-CD105 CAR T cells. The activated anti-CD105 CAR T cells displayed the ability to multiply, have activated cytotoxic T cell characters, effectively kill CD105+ target cells *in vitro*, and produce pro-inflammatory cytokines. This treatment inhibited the growth of CD105+ tumors, reducing their weight and prolonging the survival time of the tumor-bearing mice. This means that nanobody-based CAR T cells have functionality as an antitumor agent in human tumor xenograft models and that anti-CD105 CAR T cells engineered by the CRISPR-Cas9 system are a threat to solid tumors by targeting the CD105 antigen. However, CAR T cells have significant limitations regarding their widespread use in cancer treatment. Development of cytokine release syndrome (CRS) and neurotoxicity (NT) can occur, as well as effector function, limited expansion, and anti-tumor activity in solid tumors. A solution for these limitations is to edit the genome of CAR T cells during the manufacturing process.[34] This editing is done using the CRISPR-Cas9 system in CAR T cells via transduction with a lentiviral construct (which contains gRNA to granulocyte-macrophage colony-stimulating factor (GM-CSF) and Cas9). The edited CAR T cells produce less GM-CSF while maintaining critical T cell functionality. This results in enhanced anti-tumor activity compared to wild-type CAR T cells in vivo. Another study focusing on the adoptive transfer of T cells to express a TCR shows potential in hematological and solid tumors.[16] A major issue of transducing T cells with a transgenic TCR is the preexisting expression of TCR in these recipient T cells. The endogenous TCRs compete with the transgenic TCR for surface expression and allow

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mixed dimer formation (which may harbor autoreactive specialties). To get around this issue, the study describes a system in which the endogenous TCRβ is knocked out using a CRISPR-Cas9 system and transduction with a cancer-receptive receptor. The TCR replacement strategy resulted in increased surface expression of transgenic αβ and γδ TCRs, which in turn led to stronger and more engineered response T cells to their target cancer cell lines. Moreover, the TCR plus modified T cells were up to a thousand times more sensitive to antigen than standard TCR-transduced T cells, and transduction with a pan-cancer–reactive γδ TCR used alongside a CRISPR/Cas9 system knockout of the endogenous αβ TCR led to more efficient redirection of $CD4^+$ and $CD8^+$ T cells compared with standard TCR transfer. Multiplex CRISPR-Cas9 editing is also extremely popular for editing T cells since it can edit several genes simultaneously with one system, making it more effective for editing several T cells. This is shown in multiplex CRISPR engineering of T cells in patients with refractory cancer.[27] In this study, two genes encoding the T Cell Receptor (TCR) chains, and TCRα (TRAC) and TCRβ (TRBC), were deleted in T cells to reduce T cell mispairing and to enhance the expression of a synthetic cancer-specific TCR transgene. Additionally, the removal of a third gene that encoded PDCD1 (Programmed Cell Death protein 1) was completed to improve antitumor immunity. The transfer of the CRISPR-Cas9-engineered T cells into patients resulted in durable engraftment at all three genomic loci. While chromosomal translocations were detected, their frequency decreased over time. These modified T cells persisted for up to 9 months and were effective at fighting the cancer cells, showing immunogenicity hopefulness. Another study utilizing multiple CRISPR-Cas9 editing is transfection-based multiplex delivery of the CRISPR-Cas9 system, which allowed the editing of multiple gene sets in individual cells.[18] This method induced pancreatic cancer in mouse models and exploited CRISPR-Cas9 mutational signatures for phylogenetic tracking of metastatic disease. CRISPR-Cas9 system multiplexing can enable key applications, according to the study. These include chromosome engineering, *in vivo* synthetic lethality screening, and chromosome engineering. Negative selection screening in the pancreas using the multiplexed CRISPR-Cas 9 system displayed the vulnerability of pancreatic cells to Brca2-inactivation in a Kras-mutant context. This is because the only target gene that was not mutated was Brca2, even though its sgRNAs are functional, which indicates negative selection of Brca2. Homozygous Brca2 inactivation also inhibited Kras-dependent PDAC formation but promoted tumorigenesis in a Trp53 mutant background (when Trp53-dependent cell cycle checkpoints are altered in the mouse). This data shows that negative screening is possible in mouse pancreas and could be used to explore therapeutic vulnerabilities and synthetic lethality *in vivo*. The CRISPR-Cas9 system can genetically modify macrophages to enhance their effectiveness. This is described in a study where arginine nanoparticles (ArgNPs) are used to deliver CRISPR-Cas9 systems into cells to generate SIRP-α knockout macrophages.[35] The ArgNP codelivers the sgRNA and Cas9 protein required for editing these macrophages. The purpose of these macrophages and editing them is so that they can continue to perform phagocytosis on cancer cells. Cancer cells exhibit a signal that disables macrophages from performing phagocytosis on them, and by disabling this signal, the macrophages can be more effective, even by 4-fold. This improved attack on cancer cells by macrophages makes this strategy for weaponizing macrophages extremely effective. Another way that genetically edited macrophages can be delivered is through lentivirus-driven engineering, which allows macrophages to express therapeutic payloads (including interleukin (IL-12)). The effect of genetically edited macrophages (GEMs) was assessed using *in vitro* coculture studies. GEMs secreted IL-12 on T cells and on the GEMs themselves, which allowed them to secrete cytokines and full-length cytokines checkpoint antibodies, as well as proteins that can overcome these barriers. GEMs can express their therapeutic payloads in xenograft mouse models and a truncated non-signaling CD19 surface protein for elimination. IL-12 secreting GEMs activated T cells, reduced tumor growth (resulting in extended survival in vivo), and induced interferon-gamma (IFNγ) *in vitro*. IFNγ signaling cascades were also observed in mice treated with mouse bone marrow-derived GEMs secreting rodent IL-12. When these findings were replicated in an *ex vivo* tumor compromised on intact MEs, the GEMs induced tumor cell death, IFNγ-stimulated genes and proteins, and chemokines. CRISPR-Cas9 systems can also be used to edit B cells, another major component of the immune system. One such process in this study is where B cells are engineered using the CRISPR-Cas9 system with a targeted nuclease.[13] B cells are effective in CRISPR-Cas9 gene editing due to their large amount of protein secreted (as antibodies) and persist for the organism's life as plasma cells. In the study, CD19+ B cells from PBCMs (peripheral blood mononuclear cells) are enriched, followed by the activation, expansion, and electroporation of CRISPR-Cas9 reagents. As a result, the B cells in the culture increased 10x and outgrew the IgD+ IgM+ CD27− naïve subset from 35% to over 80% of the total culture. The B cells in the culture are receptive to nucleic acid delivery via electroporation three days after stimulation at peak on Day 7. Using chemically modified sgRNAs and Alt-R-gRNAs targeting CD19 with Cas9 mRNA or protein, genetic and protein knockout of CD19 at rates over 70% was achieved. Furthermore, testing sgRNAs targeting the AAVS1 safe harbor site using Cas9 and AAV6 to deliver donor template encoding a splice acceptor-EGFP cassette led to up to 25% site-specific integration frequencies. B cells are also used as synthetic antigen receptors in this promising study which describes their molecular optimization, design, and genomic integration.[24] These B cells are called Chimeric B cell receptors (CBCRs). Their expression and detection were optimized by modifying the extracellular surface tag, the transmembrane regions, and intracellular signaling domains. As a result, they steadily integrated a series of CBCR variants into immortalized B-cell hybridomas using CRISPR-Cas9. They also developed a method of reliably delivering cassettes of several kb sizes into the genome of murine primary B cells using CRISPR-Cas9 induced HDR (Homol-

ogy Directed Repair). Also, they illustrated the antigen reception and surface expression of a synthetic CBCR in primary B cells.

Conclusion

CRISPR is constantly growing and changing in the context of cancer immunotherapy. CRISPR can be used to either activate, knock out, or silence genes, all of which have different purposes. The knockout process can be used to knock out genes such as GM-CSF, which is a significant contributor to Cytokine Release Syndrome (CRS) and would be used to improve CAR T cell therapy. Silencing genes can be used in Hif1a silenced macrophages, reprogramming the immune suppressive microenvironment, reducing tumor burden, and prolonging overall survival. Activating genes were functional in Nage, where utilizing NF-kB's over-activation could be used to induce cell death. Genes can be located within the body by multiple processes, including assay of predicted off-targets, systematic mutagenesis, and whole genome sequencing. CRISPR can be used to modify lymphocytes within the body in a variety of ways. It modifies immune cells such as macrophages, T & B cells, and multiple cells simultaneously in Multiplex CRISPR-Cas9 engineering. These cells can be modified to prevent tumors from growing larger and even help the body fight back naturally against cancer. CRISPR is a highly complicated field that has continued to progress and grow. With it, the field of cancer immunology will continue to progress and become safer for people. I believe that clinical trials will soon begin on people. Eventually, CRISPR will reach a stage where it can be implemented as an option instead of chemotherapy or radiotherapy. With it, CRISPR will most likely become more streamlined, and as more studies continue to gain insight into CRISPR, it will become a quicker and much easier process. This will lead to worldwide availability and possibly even embryo CRISPR implementation (placing CRISPR/Cas9 technology within embryos so future generations may be born with specific traits) to fight off diseases such as malaria or AIDS before they even are given chances to progress. This could also lead to animal editing for future purposes and even human editing in traits. This could be adverse as societies and countries could use the CRISPR-Cas9 system for personal gain, which would be detrimental to society and possibly hurt science's cause. CRISPR has a bright future ahead of it and is growing at a much faster rate than ever before.

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