Narrative Review Article and Proposal

Harnessing A CRISPR-Cas9-Based Approach to Stimulate T Cells in Multiple Myeloma Patients by Targeting the PDCD1, HAVCR2 and LAG3 Genes

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ABSTRACT

Multiple myeloma (MM) is an incurable cancer that manifests within the bone marrow, affecting plasma cells. Although incidence and prevalence rates remain low, there has been a gradual increase worldwide in the incidence rates of MM over the past few decades. In the tumor microenvironment, plasma cells undergo mutations during development, leading to uncontrolled cell growth in the bone marrow and the production of abnormal monoclonal antibodies. These malignant plasma cells downregulate the T cell response via upregulated signaling pathways associated with the PDCD1, HAVCR2, and LAG3 genes in T cells. To reinvigorate deactivated T cells, this proposal puts forward an evidence-based approach that uses CRISPR-Cas9 to knock out the PDCD1, HAVCR2, and LAG3 genes in T cells to restore T cell cytotoxic functions against MM. Multiplex gene editing appears promising; however, additional research is required to fully encapsulate the complex nature of their interactions with MM. Since many cancers persist through the activation of redundant signaling pathways that are co-expressed, editing three genes simultaneously is essential to preventing MM from surviving or adapting through alternate pathways. This CRISPR-Cas9-based approach holds immense potential to advance patient outcomes through *ex vivo* T cell editing, but also raises considerations regarding the effectiveness of triple gene knockout. This proposal discusses *in vivo* and *in vitro* research that support such treatment, along with future considerations of coupling CRISPR-Cas9 with other known MM treatment mechanisms.

Keywords: Multiple Myeloma; CRISPR; T cells; PDCD1; HAVCR2; LAG3; PD-1; TIM-3

INTRODUCTION

The incidence of multiple myeloma (MM), the second most common hematologic cancer worldwide, has been gradually rising (1). Hematologic cancers, like MM, are blood cancers that originate in plasma cells, which are white blood cells primarily found in the bone marrow (2). Despite advances in medicine and technology, MM is still an incurable disease due to drug resistance and an immunosuppressive bone marrow microenvironment, resulting in its poor prognosis among patients (3, 4). A recent 2022 study indicated that there were nearly 188,000 MM cases and 121,000 deaths worldwide (5). The study predicted that, in the absence of changing rates, the incidence and mortality of MM will increase by 71% and 79% respectively, by 2045 in relation to 2022, underscoring the need for research on disease progression and viable treatment options to limit MM's

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mortality rate and unfavorable prognosis (5). MM can cause numerous symptoms that range in severity based on the stage of MM: from pain, tiredness, and depression, along with anemia, hypercalcemia, renal insufficiency, and skeletal fractures (6, 7). In the United States, the average risk of acquiring MM for men is 1 in 108 and 1 in 133 for women, but an individual's risk may vary based on other factors including race, age, and gender (8). In the United States, non-Hispanic black individuals have shown rising incidence rates: 12.02 in 1999 to 14.20 in 2020 per 100,000 people (9). MM is usually diagnosed among individuals who are 65 to 74 years old, with very few cases in individuals under the age of 40 (10). In the United States, the median age of diagnosis was around 69 years (10, 11). MM is more prevalent in men than women, where in 2022, 19,100 men compared to 15,370 women were diagnosed in the United States (12). Lifestyle choices can also influence MM progression. Practicing sports for half a year can play a protective role against developing MM; however, toxic environmental exposures and agents such as pesticides, asphalt, coal dust, organic vapors, metal dust, and exhaust fumes can increase likelihood of MM progression (13). Obesity is also an important risk factor, as prior studies have found a correlation between obesity and MM onset (14). However, more recent studies have found that although this relationship between obesity and MM exists, it may not be as statistically significant as once thought (15). In addition to obesity, family history contributes to MM pathogenesis as the risk is reported to be two to four times greater among first-degree relatives of MM and Monoclonal Gammopathy of Undetermined Significance (MGUS) patients; this relationship is much stronger in African Americans than Europeans and Americans (16). However, the relationship is less pronounced and is not as statistically significant for second-degree relatives (17). An individual's risk of developing MM increases when they have had prior immune infections such as Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)(18). HIV results in T cell depletion and dysfunction, along with B cell activation without specific antigen stimulation, producing weak or nonfunctional antibodies that create a weak immune response to cancers like MM (18). A meta-analysis by Grulich *et al*. found that the standardized incidence ratio for MM in HIV and AIDS patients was 2.71, suggesting that T cell depletion and immune deficiency, because of prior immune infections, significantly increase the risk of developing MM (19). Due to many barriers such as drug resistance and an immunosuppressive bone marrow environment, this manuscript aims to propose a novel treatment strategy for MM by incorporating a CRISPR-Cas9 mediated therapy. This approach seeks to restore T cell function within the immune system by knocking out three T cell inhibitory genes exploited by MM cells.

Pathogenesis of Multiple Myeloma

Characterized by the continuous growth and aggregation of plasma cells in the bone marrow, MM develops through a multistep progression. After developing in the bone marrow via hematopoietic stem cells, B cells migrate to the germinal center of a lymph node, or other secondary lymphoid organs; after encountering its target antigen, a B lymphocyte can develop genetic alterations during somatic hypermutation (SHM) and class-switch recombination (CSR) while the activation-induced deaminase (AID) enzyme is expressed (20, 21). During B cell maturation, SHM introduces mutations into the regions of the heavy and light chains within the complementarity-determining regions of B cells to enhance antigen-binding affinity (22). Meanwhile, CSR removes segments of the heavy chain locus, allowing B cells to express antibodies of different isotypes while having the same specificity (22). SHM and CSR occur frequently in B cells to provide antibodies with increased affinity for antigens and new effector functions to strengthen the immune response (23). SHM and CSR both rely on AID to create double-stranded breaks (DSBs) in the DNA at heavy and light chain loci of immunoglobulins, resulting in the diversification of immunoglobulin chains (24). However, when DSBs are improperly repaired or off-target effects occur, chromosomal translocations arise, which in turn may contribute to the onset of MM (24). Notably, AID has been linked to structural changes in certain chromosomes in monoclonal gammopathies like MM, such as the translocation of chromosomes 11 and 14[t(11;14)(q13;32)] (25). The post-germinal B cells can create plasmablasts, which have undergone SHM and CSR, that can terminally differentiate into mutated plasma cells in the bone marrow (26). The mutations within plasma cells vary, but many mutations are shared across patients in genes such as FAM46C. A 2018 study demonstrated that when overexpressed, the wild-type FAM46C gene results in considerable damage to MM cells (27). Mutations in this gene, specifically in MM patients, result in diminished cytotoxicity, suggesting that the FAM46C mutation in plasma cells promotes MM cell growth and survival (27). MM patients with

mutations in the NRAS, KRAS, and BRAF genes of plasma cells, components of the MAPK-pathway, demonstrate a reduced possibility of complete remission, a state with no signs or symptoms of cancer, after receiving a VRd therapy, which combines bortezomib (Velcade), lenalidomide (Revlimid), and dexamethasone to disrupt the MM cell cycle and stimulate the immune system (28). After migrating to the bone marrow, mutated plasma cells produce monoclonal(M) protein, representing MGUS. MGUS, a precursor to multiple myeloma, is an asymptomatic disorder defined by the presence of a serum monoclonal (M) protein and <10% malignant plasma cells in bone marrow without an MM diagnosis (29, 30). Arising after MGUS and before MM, smoldering multiple myeloma (SMM) is an asymptomatic condition and precursor to MM, but with more exaggerated conditions than MGUS, defined by the presence of serum M protein $\geq$ 3 g/dL and/or 10% to 60% malignant bone marrow plasma cells (31, 32). A 2020 study, utilizing next generation sequencing to study 214 SMM patients, found that SMM patients already harbor most of the genetic alterations necessary for the onset of MM (33). The researchers validated their findings in an external study to confirm that patients with the risk factors found in SMM had an increased risk of progression of MM, indicating that SMM is not an independent stage but rather a precursor of MM, similar to MGUS (33) (Figure 1).

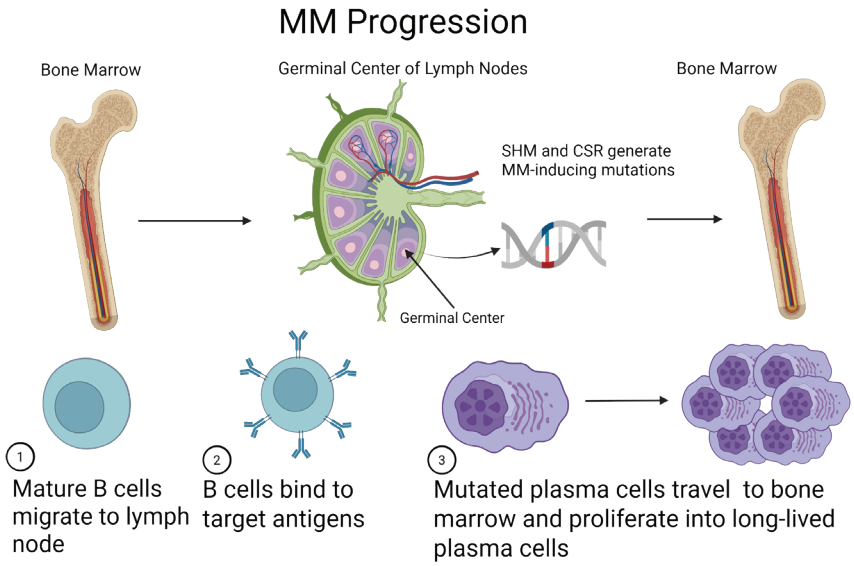

Figure 1. Overview of MM progression. Mature B cells migrate to secondary lymphoid organs and undergo SHM and CSR. Malignant plasma cells return to bone marrow and proliferate, driving disease progression and increasing the difficulty of treating MM due to its rapid proliferation. Understanding the stages of MM progression aids in highlighting areas where potential therapeutic interventions and therapies can be applied to prevent cell proliferation and bone marrow infiltration. Combining intervention strategies at multiple stages depicted above may improve therapeutic efficiency and success at improving patient outcomes. Created in BioRender. Patel, V. (2025) https://BioRender.com/bjekz0k

Two key laboratory techniques are used to diagnose MM by testing for M proteins: serum protein electrophoresis and immunofixation electrophoresis. Serum protein electrophoresis separates serum proteins by their electrical charge, allowing for the detection of abnormal M proteins, hallmarks of MM; whereas, immunofixation electrophoresis is a more sensitive technique that also separates proteins but instead uses antibodies to characterize specific immunoglobulin types (34). In a 2021 study, out of 105 MM cases, immunofixation electrophoresis detected MM in every case, but serum protein electrophoresis detected MM in 101 cases (96.19% success rate) (34). Although the number of correctly diagnosed cases varied by only

four, a seemingly marginal amount, this difference will become significant when the sample size increases. In addition, imaging techniques such as X-rays, computed tomography (CT), and magnetic resonance imaging (MRI) can be used to detect MM. Skeletal X-rays and CT scans detect lytic lesions and pathological fractures in bones, with CT scans offering higher sensitivity, while MRIs assess bone marrow involvement and infiltration before the bone is destroyed (35).

Current Treatment Strategies and Limitations

Treatment options for MM have evolved in recent years due to advancements in drug therapies and stem cell transplants. Among current drug therapies, proteasome inhibitors are a key aspect of treatment plans for MM and are used alongside other drugs. The proteasome is a multi-subunit protein complex, consisting of proteases that degrade and process intracellular proteins by breaking peptide bonds (36). Since MM cells produce large amounts of M protein, proteases are used to degrade them and prevent aggregation (36). Proteasome inhibitors hinder protease function, resulting in the aggregation of misfolded proteins that would otherwise be degraded, leading to antiproliferative signals, disrupted cell cycle regulation, and activation of apoptotic pathways (36). However, proteasome inhibitors have been linked to off-target effects in the cardiovascular system due to the buildup of misfolded proteins in cardiomyocytes, giving rise to cardiovascular complications like hypertension, pulmonary hypertension, heart failure, arrhythmias, ischemic heart disease, and thromboembolism (37). Therefore, further experiments on proteasome inhibitors and their off-target effects are crucial to understanding their safety, along with the mechanisms that cause cardiovascular complications. Immunomodulatory drugs (IMiDs) are also a promising avenue for treatment. When introduced to MM cells, IMiDs limit growth and induce apoptosis (38). They promote the immune response and limit the effects of the immunosuppressive environment of the bone marrow by eliminating the attraction between MM cells and the bone marrow, inhibiting angiogenesis, and upregulating killer T cells (38). However, the effectiveness of IMiDs can decline with time. Lenalidomide (LEN), a common IMiD, has strong anti-MM activity; despite this, patients eventually relapse and become LEN resistant(39). As the number of patients receiving LEN increases, LEN resistance decreases the effectiveness of IMiDs in treating MM (39). Both proteasome inhibitors and immunomodulatory drugs have led to considerable improvements in MM prophylaxis, but off-target effects and drug resistance still hinder their effectiveness. Thus, newer treatment options should also be considered, notably monoclonal antibodies (mAbs). mAbs can result in varying immune responses by targeting and binding to a receptor on cancer cells and inhibiting downstream activity (40). mAbs can initiate apoptosis via the delivery of a drug, toxin, or radioisotope into the cancerous cell (40). The large amount of surface antigens on MM cells makes these cells effective targets for mAbs therapy, but these surface antigens are also found on normal cells, leading to non-specific killing (40). Therefore, it is important to consider the off-target effects of mAbs therapy and look to additional therapeutic options. Hematopoietic stem cell transplants (HSCTs), especially autologous HSCTs, are a promising approach within the field of regenerative medicine to treat cancers like MM. Autologous HSCTs involve collecting hematopoietic stem cells (HSCs) and progenitor cells from the bone marrow through a bone marrow harvest or from the peripheral blood, followed by chemotherapy to eliminate malignant plasma cells, and finally transferring the cells back into patients (41). Transferred stem cells travel to the bone marrow and initiate the production of new plasma cells (41). However, the effectiveness of autologous HSCTs is limited by the quantity and quality of HSCs, as studies indicate a positive correlation between the number of HSCs collected and the engraftment time of neutrophil and platelet counts, indicating that patients recover blood counts at a faster rate (42). Novel therapeutic agents intended to promote survival among MM patients can potentially delay HSC mobilization and collection, delaying hematopoietic recovery and reducing the effectiveness of autologous HSCTs (42). Some of the adverse side effects of HSCTs in the early post-transplantation period include deep myelotoxic agranulocytosis, disruption of mucous membranes of gastrointestinal tract and a high risk of infectious complications (43).

While MM originates in malignant B cells, it also profoundly impairs T cell function and response. Treatment options to address T cells include bispecific antibodies and chimeric antigen receptor (CAR) T cell therapy. Bispecific antibodies simultaneously engage and link endogenous T cells with malignant plasma cells by binding to CD3 on T cells and an extracellular antigen on malignant cells, such as B cell maturation antigen (BCMA) and G-protein coupled receptors. This promotes T cell activation and release of cytolytic molecules like perforin and granzyme B into

malignant cells (44). Bispecific antibodies have shown unprecedented response rates in patients with refractory MM (44). CAR T cell therapy, an emerging treatment option, involves extracting and engineering a patient's T cells with a chimeric receptor that recognizes a specific MM antigen, such as BCMA, which is normally found on mature B cells and upregulated during MM (45). The extracellular portion, which is linked to T cells through a transmembrane binding domain, comprises a single-chain fragment that binds to tumor-specific antigens on MM cells (45). CARs independently recognize tumor-specific antigens without the need for major histocompatibility complex (MHC), which normally aids in antigen recognition, and subsequently activate the T cell response (46). Upon activation, T cells release cytolytic molecules such as granzymes, perforin, and pro-inflammatory cytokines, into malignant cells (45). However, bispecific antibodies and CAR-T therapy can lead to exaggerated T cell and cytokine levels, resulting in life-threatening cytokine release syndrome (CRS) (47). Similar to mAbs, bispecific antibodies, and CAR-T cell therapy, must target an antigen that is unique to MM cells and minimally expressed on healthy cells; otherwise, they pose a risk to healthy cells (48). Due to antigenic escape, where MM cells develop genetic variation, which leads to tumor subpopulations that lack the target antigen, recipients of CAR-T cell therapy can relapse (49).

CRISPR-Cas9 Gene Editing as a Therapeutic Strategy

As seen above, MM can be treated using a wide variety of therapies, each with its advantages and disadvantages. A novel and effective approach involves gene editing techniques to correct mutations in T cells in MM patients, notably through clustered regularly interspaced short palindromic repeats and associated protein 9 (CRISPR-Cas9). Discovered in 1987 by Yoshizumi Ishino in *E. coli*, CRISPR sequences were a unique form of a bacterial adaptive immune response that identified and destroyed foreign DNA (50). Repurposed for gene editing, CRISPR, with the Cas9 nuclease, is used to make DNA fragment deletions and insertions across eukaryotic genomes (51). CRISPR-Cas9 gene editing can be divided into 3 stages: *recognition, cleavage, and repair* (52). Synthesized *in vitro*, a single guide RNA (sgRNA) detects and *recognizes* the target nucleotide sequence on the specific gene (53). The sgRNA contains a spacer region that defines the target DNA sequence and guides Cas9 to the precise spot in the genome to cut, where the spacer will bind to the target DNA via base-pairing, while the trans-activating CRISPR RNA(tracrRNA) ensures proper binding and activation of Cas9 (54). When the target sequence is found, Cas9 will *cleave* the DNA, generating DSBs at a site about 3 base pairs upstream of the protospacer adjacent motif (PAM), which aids in recognition of the target sequence (53). After DNA cleavage, DNA can be repaired via two pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (55). NHEJ repairs DSBs by combining the remaining DNA fragments on each side of the removed sequence; this is commonly used but can result in mutations via random insertions and deletions (53). On the other hand, HDR requires a homologous DNA template or extra DNA templates, which increases the precision of DNA insertion and deletion (56). To harness the potential of CRISPR-Cas9 for precise gene editing, researchers are using CRISPR to combat complex diseases and cancers like MM (Figure 2).

Immune Checkpoints in T Cell Dysfunction

Given the precision of CRISPR-Cas9 as a gene editing technique, there are many possibilities for targeted genetic interventions for MM that focus on addressing T-cell-specific genes of interest. These genes include Programmed Cell Death 1 (PDCD1), Hepatitis A virus cellular receptor 2 (HAVCR2), and Lymphocyte-Activation Gene 3 (LAG3). B-cell genes, such as FAM46C, NRAS, KRAS. BRAF, were not considered in this proposal in part due to the complexity and variability of their immunoglobulin locus (27, 28, 57).

PDCD1, found on chromosome 2 at position 2q37.3, is a gene coding for an immune-inhibitory receptor, PD-1, that is expressed on T cells (58,59). The transmembrane receptor PD-1 has two ligands: programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2). For this review, we will be focusing mainly on PD-L1, as the function of PD-L2 is not yet fully understood (59). After binding to the physiological ligand (PD-L1), PD-1 inactivates T cell receptor-mediated signaling by dephosphorylating intermediates such as Zap-70 through an intracellular signaling cascade (60). PD-1 ultimately suppresses T cell proliferation, leading to a decrease in cytotoxic activity, serving as an "off" switch for T cells (60). Although the PD-1 signaling pathway normally functions to limit excessive T cell activity, it can also be manipulated by cancer cells. MM cells express significantly higher levels of PD-L1

compared to healthy cells, as the expression of PD-L1 is increasingly induced by the MM microenvironment (61). Upregulation of PD-L1 results in increased binding to the PD-1 receptors on T cells and rising T-cell deactivation, suppressing T cell proliferation and cytotoxic activity and ultimately allowing MM cells to persist in the bone marrow (61).

Located on chromosome 5 and position 5q33.2, HAVCR2 encodes T cell immunoglobulin and mucin domain-3 (TIM-3), a transmembrane receptor on T cells (specifically CD4+ helper cells, $CD8^+$ cytotoxic cells, and regulatory T cells) (58, 62). TIM-3 binds with four ligands, Galectin-9 (Gal-9), phosphatidylserine (PtdSer), HMGB1, and CEACAM1, which bind to distinct regions in the TIM-3 Immunoglobulin variable region (IgV) domain (62, 63). Gal-9, a tandem-repeat β-Galactoside-binding lectin encoded by LGALS9, binds to the glycosylated IgV domain of TIM-3, causing a conformational shift that triggers inhibitory signaling in T cells (64). The binding between Gal-9 and TIM-3 receptor initiates apoptosis of $TIM3^+$ expressing T cells and suppression of helper T cell-mediated immunity (65). Data has shown that the TIM-3 and Gal-9 pathway has evolved to ensure effective termination of effector T cells (66). Cells that express TIM-3 have also been shown to simultaneously express PDCD1, and PDCD1 blockage led to TIM-3 upregulation, suggesting that PD-1 and TIM-3 may work in tandem and that T cells expressing both are most dysfunctional (67, 68). In MM patients, TIM-3 is overexpressed in $CD4^+$ T cell subsets, while Gal-9 levels are increased in peripheral blood (69). This overactivation of the TIM-3 and Gal-9 pathway results in suppression of T cells and diminished secretion of key pro-inflammatory cytokines like IFNγ, resulting in immune dysfunction and tumor escape (69).

Found on chromosome 12 at position 12p13.31, LAG3 is a gene coding for the inhibitory receptor LAG-3 found on the surface of activated $CD4^+$ and $CD8^+$ T cells (58, 70). It is a transmembrane glycoprotein consisting of a transmembrane region, a cytoplasmic region, and an extracellular region with four IgSF domains (71). The primary ligands that bind to LAG-3 are MHC-II, which downregulate T cell proliferation, and Gal-3, which inhibits T cell responses to tumors (71). In MM patients, studies have found increased LAG3 expression on proliferating $CD3^+$ T cells in the peripheral blood and bone marrow, along with increased expression of the ligand Gal-3 in MM cells, indicating that LAG3 hinders T cell proliferation when overexpressed (72).

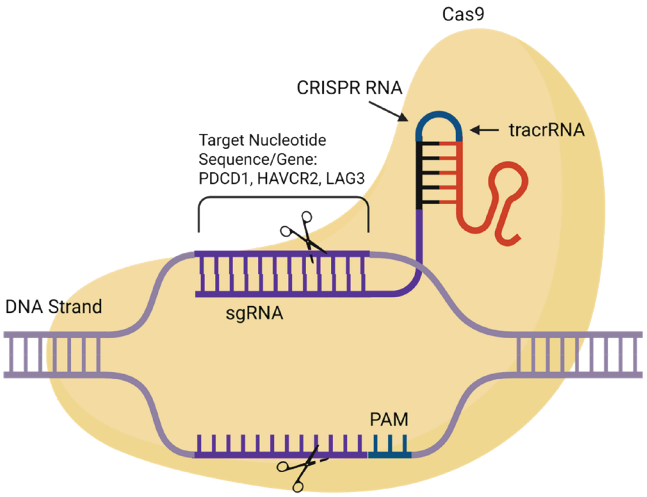

Figure 2. Overview of the mechanism of CRISPR-Cas9 gene-editing. The Cas9 protein, directed by a single guide RNA (sgRNA), binds to a specific target sequence on the DNA strand adjacent to the PAM and introduces a DSB, enabling genetic modifications. This gene editing technique can be harnessed within MM patients to disrupt inhibitory genes in T cells to restore immune function. Gene editing can be applied in preclinical models to evaluate the therapy's effectiveness in overcoming immune suppression. Created in BioRender. Patel, V. (2025) https://BioRender.com/h4ie3ep

Although MM manifests within plasma cells, there are some limitations associated with using gene editing techniques such as CRISPR-Cas9 to edit B cells. This is due to the complexity and variability of each B cell's immunoglobulin variable locus, which requires tightly regulated expression for the B cell receptor and its secreted antibody isoforms to mimic natural occurring antibody production (57). An alternative treatment for MM involves gene-editing T cells to improve their targeting of MM cells and bolster the weakened immune response, focusing on the PDCD1, HAVCR2, and LAG3 genes. By knocking out the PDCD1, HAVCR2, and LAG3 genes, T cell-specific gene therapies can restore T cell response and function, improving the cytotoxicity of the immune response against MM cells and improving patient outcomes worldwide.

GENES OF INTEREST

The following sections will explore the genes targeted in this proposal, as well as the existing evidence and literature on CRISPR-based gene editing

approaches for altering these T cell genes in MM patients.

CRISPR Targeting of PDCD1

PDCD1, coding for the PD-1 immune-inhibitory receptor, binds to the PD-L1 ligand to inhibit T cell receptor-mediated signaling through an intracellular signaling cascade, resulting in decreased helper and killer T cell proliferation and cytotoxic activity (59). In comparison to healthy cells, MM cells express a higher level of the PD-1 receptor, resulting in increased T cell suppression and a weakened immune response against MM cells (59).

Since PD-L1 binds to the receptor PD-1, one way to enhance the T cell response in MM patients can involve deleting the PDCD1 gene to terminate PD-1 production and prevent PD-L1 from binding to the receptor, thereby stopping T cell exhaustion and promoting proliferation. A 2020 study tested the feasibility and safety of CRISPR-Cas9 gene editing to engineer and infuse T cells into three patients with refractory cancer, with two diagnosed with advanced multiple myeloma and another diagnosed with liposarcoma (73). CRISPR-Cas9 was delivered *ex vivo* to T cells via electroporation. The researchers edited two genes encoding the endogenous T cell receptor (TCR) chains, TCRA and TCRB, as well as the PDCD1 gene (coding for PD-1), to enhance the anti-tumor response (73). They then delivered an infusion of 1×10^8 engineered T cells. In MM patients, approximately 25% of T cells had mutations in the PDCD1 gene. But four months after the infusion, only about 5% of the transgenic T cells lacked the gene. At the same time, the remaining T cells showed upregulation of genes associated with central memory, such as IL7R and TCF7, over time, possibly indicating that the T cells were transitioning into a longer-lasting, central memory phenotype (73). Although the number of T cells with the PDCD1 edit decreased after follow-up, the expression of central memory genes highlights how targeting PDCD1 may have initially triggered a signaling pathway that promotes memory-associated genes and supports early T cell expansion. Compared to the two TCR sgRNAs, the sgRNA for PDCD1 showed the most specificity, as very few off-target mutations were identified in over 7000 cleavage sites, depicting the limited off-target effects associated with editing PDCD1 (73). Of the three individuals involved in the study, neither developed humoral responses to the Cas9 nuclease, meaning their immune systems did not react against Cas9. Since the patients did not develop antibodies to Cas9, this study is a good indicator that the transfused T cells were able to persist within the body without being targeted by the immune system, suggesting that CRISPR-Cas9 therapies aimed at removing PDCD1 may be both safe and feasible for immunocompromised MM patients without triggering adverse side effects. However, the authors also suggest two possible explanations: the amount of Cas9 in the infused cells was too little to trigger an immune response, or the patients already had weakened immune responses due to MM, making them unable to initiate an immune response (73). Nonetheless, this study suggests that gene editing techniques like CRISPR-Cas9 can be utilized to remove the PDCD1 gene from MM patients safely. Ultimately, the modified T cells remained effective for about 9 months and demonstrated minimal immunogenicity (73). Bone marrow biopsies in the two MM patients confirmed the presence of engineered T cells in the bone marrow. Even though the biopsies revealed a residual tumor, there was a reduction in the target antigens NY-ESO-1 and LAGE-1, with this being temporary in one MM patient and permanent in another (73). These findings suggest that the engineered T cells were actively recognizing and killing malignant plasma cells expressing the antigens, serving as an indicator of an improved immune response. The authors reported that all patients eventually experienced disease progression as of December 2019, with one dying from progressive MM (73). The researchers noted that the initial clinical results have shown acceptable safety, but the small cohort size may limit significant results from emerging, as more clinical trials with patients receiving infusions of CRISPR-Cas9 engineered T cells with the removal of the PDCD1 gene will be required (73). As the first-in-human trial using CRISPR-Cas9 to edit T cells in MM patients, this study demonstrates the technical feasibility and molecular impact of PDCD1 gene disruption. The T cells successfully engrafted and persisted *in vivo*, with evidence of antigen-specific activity. Many more *in vivo* trials will need to be conducted to further assess the safety and efficacy of this approach. Despite limitations such as small cohort size, this study provides proof-of-concept that PDCD1 gene deletion can be safe, does not create a humoral immune response, and results in an increase in the number of activated T cells, enhancing the immune response (73).

Studies using *ex vivo* methods have demonstrated the capabilities of CRISPR-Cas9-based T cell editing to contribute to the MM immune response. In 2017,

Zhao *et al*. isolated primary cytotoxic T lymphocytes (CTLs) – $CD3^+$ and $CD8^+$ cells – from peripheral blood mononuclear cells (PBMCs) from healthy donors, with the efficacy of isolation verified by flow cytometry and confirming a purity of 95% $CD3^+$ and $CD8^+$ cells *in vitro*(74). The CTLs were then transduced with a lentivirus carrying CRISPR-Cas9 and PD-1 guide RNAs, resulting in PD-1 knockout (KO) in the transduced CTLs. The researchers then tested whether PD-1 KO would improve the anti-tumor activity of CTLs against the MM.1S cancer cell line. After co-culturing MM cells with CTLs (either control or PD-1 KO) for 24 hours, flow cytometry indicated that the cell viability of MM cells found in the PD-1 KO culture decreased to 63.49 ± 2.71% compared to control cultures. There was also a decrease in tumor cells in the PD-1 KO culture, as MM.1S cells were only 21.9% of the PD-1 KO culture compared to 46.1% of the control culture(74). The decrease in MM cells indicates that PD-1 KO had a noticeable effect on T cells and thus contributed to increased cytotoxicity, which led to an improved and increased immune response against MM cells. After incubating the MM.1S cancer cell line with CTLs, the PD-1 KO culture exhibited 50% more MM cell death in comparison to the control CTL culture. 16.2% of MM cells in the PD-1 KO culture entered the late apoptotic stage, compared to 8.7% in the control culture (74). These findings suggest that PD-1 KO CTLs triggered increased apoptosis in tumor cells compared to the control CTLs and that PD-1 KO enhanced anti-tumor activity among T cells. Known to play a role in apoptosis, caspases are activated through protein cleavage and dimerization. Through immunoblotting, the PD-1 KO culture was found to have increased forms of caspase-3, caspase-7, caspase-8, and caspase-9 in regard to the control culture. After incubating MM cell lysates with caspase substrates, it was found that caspase-3, caspase-8, and caspase-9 were approximately 8.2, 6.58, and 4.92 times higher in the PD-1 KO culture than the control, suggesting increased cleavage of proteins involved in cell death (74). The researchers then tested if PD-1 KO, in addition to improving T cell activity, could increase cytokine secretion. The PD-1 KO CTLs were shown to secrete approximately 2.43 and 1.92 times more of cytokines TNF-α and IFN-γ, respectively, than the control culture, contributing to an increase in T cell function within the PD-1 KO culture (74).

The effectiveness of the PD-1 KO CTLs in suppressing MM tumor growth was tested *in vivo* with a human tumor xenograft mouse model. Two groups of mice were inoculated with 5×10^6 MM.1S cells and subsequently treated with either control CTLs or PD-1 KO CTLs (74). After 2.5 weeks, the tumor became detectable in the control culture; whereas, tumor growth in the PD-1 KO culture was significantly repressed. After 52 days, all mice within the control group died from progressive tumors; however, only 40% of the mice in the PD-1 KO culture died after the same amount of time, as the PD-1 KO treatment was shown to prolong the overall survival of the host mice (74). Although mice died in both studies, mice with PD-1 KO in CTLs showed significantly prolonged survival, suggesting that PD-1 KO effectively suppressed MM progression *in vivo.* These findings highlight the potential for a CRISPR-Cas9-based PD-1 KO to be used in human gene therapies that aim to boost T cell function among MM patients with an overexpressed PDCD1 gene. This approach highlights that human T cells can be safely edited to remove the PDCD1 gene, which has proven to be feasible in suppressing MM and improving survival both *in vitro* and *in vivo*, supporting its potential application to human gene therapies.

In a 2025 study by Zhao *et al*., engineered TCR T cells were edited to target a specific tumor antigen, express the PD-L1 ligand via lentiviral transduction of CD274, and lack the PD-1 receptor by knocking out the PDCD1 gene (75). The melanoma antigen gene (MAGE) family consists of subfamilies that are normally expressed in tumors and frequently used as cancer-specific antigen targets for TCR-T cells due to their cancer-specific characteristics and intracellular expression (76). Thus, the MAGE-C2 (MC2) receptor was chosen due to its presence in many different cancer types, including MM (75, 76). Human $CD8^+$ T cells were engineered with a TCR targeting the MC2 antigen. After transduction with the MC2-specific TCR, subsets of the cells were further modified: some were transduced to overexpress PD-L1, and others were edited via CRISPR-Cas9 to knock out the endogenous PDCD1 gene. PD-L1 was expressed to selectively activate the PD-1 pathway in tumor cells. These modifications resulted in four distinct TCR-T cell models: MC2-targeting TCR-T cells (MC2-TCR-T cells); MC2-targeting and PD-1 knockout TCR-T cells (MC2-TCR-T^{PD1-}cells); PD-L1-expressing and MC2-targeting TCR-T cells (PDL1-MC2-TCR-T cells); and PD-L1-expressing, MC2-targeting, and PD-1-knockout TCR-T cells (PDL1-MC2-TCR-T^{PD1-}cells) (75). Flow cytometry confirmed a highly efficient PD-1 disruption as the proportion of $PD\text{-}1^+$ T cells decreased from 35.5% to 9% after editing, indicating a robust PDCD1 knockout. The co-expression of PD-L1 in the

T cells was used to activate the PD-1 signaling pathway in malignant cells, triggering tumor suppressive effects inside malignant cells expressing the PD-1 receptor. The cytotoxicity, or killing ability, of the engineered T cells was measured through co-culture experiments with different $MC2^{+}$ tumor target cells (75). The PDL1-MC2-TCR-T^{PD1-} cells exhibited the highest cytotoxicity against lab-engineered $PD\text{-}1^{+}$ target cells, followed by MC2-TCR-T^{PD1-}cells, MC2-TCR-T cells, and PDL1-MC2-TCR-T cells, demonstrating that combined PDCD1 knockout along with PD-L1 co-expression yielded the strongest tumor cell killing, especially against PD-1 expressing targets (75).

The authors also examined cytotoxicity against PD-1-negative targets (75). Even here, the $PD\text{-}1^{-}$ T cells performed better than the controls: MC2-TCR-T^{PD1-}cells exhibit a killing percentage of 70% compared to 50% by the wild-type MC2-TCR-T cells that still express PD-1, demonstrating that PDCD1 deletion can still enhance T cell cytotoxicity regardless of whether the target cells express PD-1. Importantly, PD-1 knockout boosted T cell activation markers. Between MC2-TCR-T and MC2-TCR-T^{PD1-}, the latter secreted significantly more of the cytokine TNF-α and slightly more of the cytokine IFN-γ, suggesting that removing PDCD1 increases T cell cytokine production (75). These *in vitro* findings were also confirmed *in vivo*. The researchers developed a xenograft mouse model to compare the effects of the T cell lines on tumors, injecting immunodeficient mice with MC2-A02-$K562^{PD1+}$ cells that were genetically labeled with luciferase to serve as a tumor-bearing mouse model. MC2-TCR-$T^{PD1-}$and PDL1-MC2-TCR-T^{PD1-} cells at low (1×10^6) and high (5×10^6) doses, along with a high dose of control TCR-T cells, were subsequently injected into tumor-bearing mice. Tumor growth was monitored by bioluminescence imaging and measurements of tumor size (75). PDL1-MC2-TCR-T^{PD1-} was once again found to be more effective at suppressing tumor growth, and the high doses of MC2-TCR-$T^{PD1-}$and PDL1-MC2-TCR-T^{PD1} cells were more effective than low doses. Mice injected with the low and high MC2-TCR-T^{PD1-} cells had lower total flux (luciferase presence) and smaller tumor volume than the control T cell line that expressed PD-1. PD-1 knockout in T cells (with or without PD-L1) achieved significantly greater tumor control than the $PD\text{-}1^{+}$ controls, consistent with the *in vitro* data (75). Thus, multiple trials confirm that $PD\text{-}1^{-}$ T cells kill more tumor cells than do their $PD\text{-}1^{+}$ counterparts, as seen in both *in vitro* and *in vivo* trials. These findings provide strong evidence that removing PDCD1 in T cells can boost the anti-tumor T cell response. None of the mice in the treatment groups had exhibited any signs of off-target toxicity resulting from the TCR-T cell therapy (75). Although there were no off-target effects detected, the long-term effects of this therapy cannot be ruled out. The researchers did not address the long-term persistence and exhaustion of the T cells within the mouse models, which is important to understand their clinical durability. This study demonstrates that cancer-specific TCR-T cells can successfully deliver a ligand to cancer antigen-expressing malignant cells and induce activation of the target receptor. In the context of MM, this approach has not been well studied, but the results of this study detailing the cytotoxicity of T cells after removing PDCD1 strongly support the theory that PDCD1 removal can enhance T cell cytotoxicity within MM patients (75).

CRISPR Targeting of HAVCR2

In addition to PDCD1, HAVCR2 is a promising gene to target in T cells of MM patients. HAVCR2 encodes the TIM-3 receptor in $CD4^{+}$ and $CD8^{+}$ T cells, which binds with Galectin-9, phosphatidylserine, HMGB1, and CEACAM1. Gal-9 binds to the glycosylated IgV domain of TIM-3 in T cells (62). The binding of Gal-9 and TIM-3 triggers inhibitory signaling and initiates apoptosis of $TIM\text{-}3^{+}$ expressing T cells. Cells that express HAVCR2 have also been shown to overexpress PDCD1 (65). In MM cells, TIM-3 is overexpressed in CD4+ T cell subsets, while Gal-9 levels are increased in peripheral blood. This leads to T cell suppression and diminished secretion of pro-inflammatory cytokines like IFNγ (69).

Targeting the HAVCR2 gene using CRISPR can be coupled with TCR gene editing toward an antigen. A study conducted in 2024 demonstrates this concept of genetically engineering T cells to resist exhaustion (77). T cells were isolated from PBMCs of healthy donors, which were activated with anti-CD3/anti-CD28-coated magnetic beads. T cells were electroporated with ribonucleoprotein (RNP) complexes that contain Cas9 and sgRNAs. Subsequently, T cells were introduced to a lentiviral vector coding for the NY-ESO1-specific TCR that allows T cells to recognize and bind to the NY-ESO-1 antigen expressed on MM cells (77). Wild-type MM cells (U266 and MM1.s cell lines) were incubated with lentiviruses coding for knock-in of HLA-A2, NY-ESO-1, ΔLNGFR, luciferase, and green fluorescence protein (GFP), along with IFNγ, Gal-9, and HMGB1. CRISPR-Cas9 was used to target the endogenous T cell receptor alpha chain and HAVCR2, LAG3, or 2B4 genes (77). Within *in vitro* co-culture

assays, MM cells were cultured with two types of T cells: one group had the IR genes knocked out (TCR_{ED}-IR_{KO}) and another retained these genes (TCR_{ED}-IR_{COMP}). Results showed that in short-term co-culture assays, knockout of HAVCR2, LAG3, or 2B4 did not significantly affect killing efficiency or degranulation of T cells, but a slight improvement in killing efficiency was seen in TCR_{ED}-IR_{KO} cells at low effector-to-target ratios against the U266 and MM1.s cell lines. TCR_{ED}-IR_{KO} cells, particularly those with HAVCR2, LAG3, or 2B4 knockout, produced an increased amount of pro-inflammatory cytokines and effector molecules like IL-2, TNFα, sFasL, and perforin compared to control T cells. (77) Disruption of HAVCR2, which resulted in the knockout of the TIM-3 receptor, resulted in the preservation of T cell degranulation capacity, specifically under chronic stimulation conditions. This demonstrates that HAVCR2 knockout enhances T cells' ability to release cytotoxic granules, which are crucial for killing target cells. The *in vitro* study demonstrated efficient HAVCR2 disruption: quantification of NHEJ events at the HAVCR2 locus indicates a median efficiency of 91.6%, and 94.7% of T cells were $CD3^-$-$TIM\text{-}3^-$ (HAVCR2 knock out) following double gene disruption of TRAC, which encodes a region of the TCR alpha chain, and HAVCR2. The data demonstrate that creating large amounts of differentiated T cells, targeting a specific tumor antigen and lacking IRs, can be efficiently done (77). Moreover, disruption of IRs like TIM-3 did not affect the growth or memory phenotype of edited T cells *in vitro*(77).

An *in vivo* animal model trial was also performed, where CRISPR-Cas9 was performed *ex vivo* on human T cells and then transferred into mice (77). The human T cells underwent delivery of RNPs via electroporation, targeting the TRAC gene and one selected IR. These T cells were then introduced into immunocompromised mice, which were injected with luciferase-containing U266 cells for non-invasive monitoring of tumor growth. The *in vivo* results show that T cells with disrupted IRs had stronger anti-tumor activity compared to T cells lacking IR disruption (77). Moreover, in the tumor rechallenge model used to assess the long-term persistence of the efficiency of T cells, the TCR_{ED}-IR_{KO} performed better than TCR_{ED}-IR_{COMP} cells in terms of killing cancer cells. TCR_{ED}-$TIM\text{-}3_{KO}$ cells proved to be highly effective, ultimately eradicating the second tumor engraftment in the rechallenge model (77).

The high rate of T cells with disrupted TIM-3 expression (94.6%) after CRISPR was introduced depicts the efficiency of CRISPR in modifying its target sequence (77). The ability of TCR_{ED}-$TIM\text{-}3_{KO}$ cells to eradicate rechallenged tumors suggests that CRISPR-Cas9 knockout of HAVCR2 results in a more persistent and long-lasting response in treating MM, addressing the challenge of limited T cell persistence in chronic tumor environments. However, further trials will be necessary to assess the long-term effectiveness of the methodologies employed in this study. These findings reveal that disrupting IRs like TIM-3 can improve anti-tumor responses as seen with increased cytokine production in TCR_{ED}-IR_{KO} cells (77). Limitations of this study include the lack of reliable surface markers to quantify IR-disrupted cells and that mice were immunodeficient and thus could not model the entire tumor microenvironment in humans. Nonetheless, this study demonstrates that T cells with HAVCR2 disruptions lead to increased T cell activity and tumor decline. Given the high rate of TIM-3 disruption and T cell persistence, the methods of this study provide a proof-of-concept that HAVCR2 disruption may be a favorable therapy for MM patients, especially those with weakened immune systems (77).

CRISPR Targeting of LAG3

Like HAVCR2, LAG3 is another promising target for CRISPR-Cas9 therapies. The LAG3 gene codes for the LAG-3 transmembrane glycoprotein, which is known to bind to MHC-II and Gal-3, which downregulate T cell proliferation and inhibit the T cell response to tumors, respectively (70, 71). MM patients are shown to have increased LAG-3 expression on $CD3^+$ T cells, along with increased expression of Gal-3, resulting in decreased T cell proliferation and an inhibited immune response (72).

In addition to targeting HAVCR2, Cianciotti *et al*. edited the LAG3 gene along with TCR (77). To understand the research methods and samples used, reference the section above on HAVCR2. After T cells were introduced to a lentiviral vector for the NY-ESO1-specific TCR, CRISPR-Cas9 was used to disrupt the LAG3 gene *in vitro*. After double genetic disruption of the TCR and LAG3 gene, a median of 92.7% of T cells were $CD3^-$-$LAG\text{-}3^-$, confirming the high efficiency of LAG3 knockout, which was also indicated by a 76.8% median efficiency of genetic disruption quantified by NHEJ events at the LAG3 locus (77). Since LAG3 knockout efficiency measured via NHEJ is less than the percentage of $CD3^-$-$LAG\text{-}3^-$ cells, this may indicate that even partial knockout of the LAG3 gene can lead to the

absence of LAG-3 protein. Nonetheless, these findings illustrate the effectiveness of CRISPR in editing the LAG3 gene within T cells *in vitro*. LAG-3 disruption was found to prevent the upregulation of additional inhibitory receptors on T cells, compared to HAVCR2 and 2B4, which preserved T cell granulation, suggesting that many genes contribute to the tumor environment of MM, and no single gene solely contributes to MM (77). Gene expression analysis revealed that LAG3 disruption resulted in the enrichment of genes associated with the cell cycle, DNA replication, and nucleotide metabolism. 757 genes were uniquely expressed on TCR_{ED}-$LAG\text{-}3_{KO}$ cells, such as genes involved with T cell activation and effector functions, including FASLG, TNFSF15, CD72, and PGK1 (77). Gene ontology analysis of TCR_{ED}-$LAG\text{-}3_{KO}$ cells also found strengthened glycolytic processes and ADP, NAD, and nucleotide metabolism, indicating that LAG-3 disruption in antigen-specific T cells resulted in increased metabolic activity (77).

Similar results were observed *in vivo* using xenograft mouse models, where high doses of TCR_{ED}-$LAG\text{-}3_{KO}$ elicited a significantly stronger anti-tumor response compared to TCR_{ED}-IR_{COMP} cells. Only TCR_{ED}-IR_{KO} cells could completely eradicate the second tumor engraftment, highlighting their ability to resist exhaustion (77). Transcriptional findings revealed that TCR_{ED}-$LAG\text{-}3_{KO}$ exhibited the highest and most consistent expansion profile after tumor rechallenge, as the TCR_{ED}-$LAG\text{-}3_{KO}$ group had three of its six mice containing human CD3 total cells/mL higher than 100, compared to TCR_{ED}-IR_{COMP} and TCR_{ED}-$TIM\text{-}3_{KO}$, which had zero and one mice with more than 100 human CD3 T cells per mL, respectively. The data suggest that TCR_{ED}-$LAG\text{-}3_{KO}$ proliferated much more extensively than the other T cells tested and can bolster the anti-tumor response against MM (77). This ability to proliferate rapidly and persist after tumor rechallenge is crucial for long-lasting immunity and persistence. The study found that in a high tumor burden setting, the CR_{ED}-$LAG\text{-}3_{KO}$ cells that infiltrated the bone marrow show reduced numbers of inhibitory molecules and receptors such as CTLA-4, PD-1, and TIM-3 under conditions of antigen persistence compared to TCR_{ED}-IR_{COMP} cells (77).

In this study, LAG3 gene editing via CRISPR-Cas9 proved to be highly efficient, as seen by the percentage of cells that had the $CD3^{-}$-$LAG\text{-}3^{-}$ phenotype (77). *In vitro,* the LAG3 gene was also found to affect different aspects of the immune response as it prevented upregulation of additional inhibitory molecules on T cells (77). TIM-3 and 2B4 preserved T cell granulation, shedding light on the variety of genes that may contribute to the MM tumor environment. LAG3 disruption did not affect cell growth or the memory phenotype of T cells. Rather, genes that were enriched were involved in proliferation and the cell cycle. TCR_{ED}-$LAG\text{-}3_{KO}$ cells also expressed smaller amounts of inhibitory molecules, such as CTLA-4, PD-1, and TIM-3, which leads to the avoidance of exhaustion pathways in T cells that limit the immune response, allowing TCR_{ED}-$LAG\text{-}3_{KO}$ cells to exhibit increased cytotoxicity (77).

The studies discussed previously utilized CRISPR to edit T cell inhibitory receptors (TIR) by modifying the PDCD1, HAVCR2, and LAG-3 genes (73–75, 77). However, since each of these genes controls different aspects of the immune response in MM patients, a more comprehensive approach involves triple gene editing of these checkpoint molecules in T cells to enhance the anti-tumor response and possibly avoid the side effects of single immune checkpoint blockade(60,65,71)COCM 1.032BitNameN.

In a 2022 study, CRISPR-Cas9 was used to simultaneously create permanent disruptions in PD-1, TIM-3, and LAG-3 in $CD8^{+}$ T cells (78). The study designed CRISPR RNA sequences that induce Cas9 DSBs to bind to specific exons on the target genes in the T cells. To first validate the effectiveness of the crRNAs, the murine T lymphoblast cell line EL4 was transfected via nucleofection with RNPs containing Cas9. EL4 cells were isolated and diluted. Then, the cells were lysed, and expression of PD-1, TIM-3, and LAG-3 was analyzed by western blot (78). EL4 clones with a reduction in protein expression of more than 30% were analyzed via DNA sequencing, which confirmed the presence of DNA modifications at the Cas9 binding sites with over 50% reduction in expression of PD-1, TIM-3, and LAG-3. Similarly, CRISPR was tested on $CD8^{+}$ T cells from the MataHari BLITC (MH) and OT-1 transgenic mouse models (78). $CD8^{+}$ T cells were isolated from the spleen in mice, cultured *in vitro* for one week, and transfected with Cas9 RNPs and crRNAs. T cells were subjected to long-term activation to induce expression of TIR. MH $CD8^{+}$ T cells were stimulated via splenocytes derived from $Rag^{-/-}$ immunocompromised mice (78). After 72 hours of incubation, cells with no gene knockout (MH $CD8^{+}$) had increased LAG-3 expression but no upregulation of PD-1 and TIM-3. However, the triple gene knockout MH cells (3KO MH $CD8^{+}$) had no upregulation of LAG-3, demonstrating the inhibitory effects of the therapy on LAG-3 expression. Through anti-CD3/CD28-

mediated activation, it was found that PD-1 and TIM-3 expression was induced to a lesser extent than in the control, demonstrating the comparable improvements in 3KO MH $CD8^+$ cells compared to control cells (78). Suppression of PD-1, TIM-3, and LAG-3 was similarly observed in the 3KO OT-1 $CD8^+$ T cells, which were stimulated with OVA peptide pulsed with $RAG^{-/-}$ splenocytes. These results convey the efficacy of CRISPR-Cas9 gene editing in simultaneously inhibiting the expression of PD-1, TIM-3, and LAG-3 in different T cell lines (MH and OT-1), suggesting this therapy could be applied to different types of T cells (78). To test immune functionality, 3KO MH CD8+ T cells' cytokine production, specifically IFNγ, was compared to that of the control $CD8^+$ T cells. Findings demonstrated that 3KO MH $CD8^+$ T cells and transection control T cells (TC) produce similar levels of IFNγ as non-transfected control T cells (NT) when stimulated with UTY peptide and anti-CD/3CD28 antibodies (78). Thus, this study confirms that simultaneously editing three genes in $CD8^+$ T cells maintains T cells' ability to produce inflammatory cytokines, which is a crucial part of the anti-tumor response. To assess cytotoxicity, MH $CD8^+$ T cells were cultured for 72 hours with anti-CD3/CD28 antibodies, and subsequently incubated with the MB49 cell line, as cytotoxicity was quantified through MTT cell viability assay. Across all three groups (3KO, TC, NC), MH $CD8^+$ cells had similar cytotoxicity against MB49, with killing rates of more than 80% for a 10:1 effector-to-target ratio, suggesting 3KO T cells did not lose their cytotoxic functions. Moreover, tumor-specific activation didn't result in the expression of PD-1, TIM-3 and LAG-3 in 3KO T cells (78).

The researchers, in addition to *in vitro* trials, conducted an *in vivo* trial to test the ability of 3KO $CD8^+$ T cells to engraft, persist, and cause anti-tumor activity (78). 3KO, TC, and NT $CD8^+$ cells were introduced into immunocompetent female mice with B-16-OVA tumors. On day 16, engrafted mice with 3KO OT-1 $CD8^+$ T cells had significantly higher survival rates (75%) compared to TC (~ 40%) and NT (~ 30%) cells (78). To analyze if higher tumor targeting by 3KO OT-1 $CD8^+$ cells was related to the observed tumor growth suppression and higher survival times, tumors were searched for OVA-peptide-specific T cells (Vβ5). Flow cytometry revealed a slight, but insignificant, increase in 3KO OT-1 cells in the tumor compared to TC and NT cells (78). Testing the ability of T cells to enter and remain in the tumor environment, researchers altered $CD8^+$ T cells to express renilla luciferase. Eight days later, mice were injected with coelenterazine, the substrate for renilla luciferase, and the resulting luminescence indicated that 3KO resulted in a stronger T cell signaling at the peak of the tumor, depicting a high concentration of 3KO T cells invading and persisting in the tumor. 3KO cells lasted in the tumor microenvironment for at least eight days without becoming exhausted (78). Nonetheless, these cells need to survive for much longer to be truly effective, and more studies are needed to assess long-term viability. Mice with B-16-OVA tumors were evaluated to assess routine health for signs of toxicity from the therapy. Ultimately, all mice (3KO, TC, and NT) maintained adequate health conditions during the experiment, suggesting that a triple gene CRISPR-based therapy could be a suitable and safe approach for addressing tumors (78).

Although the mice were reported not to have any drastic health outcomes during the experiment, their long-term health of the mice was not explicitly stated (78). These mice were also immunocompetent, a stark contrast from prior studies discussed, where mice used were all immunodeficient (75, 77). MM patients tend to already have weakened immune systems due to an inactivated immune response resulting from factors such as T cell inhibition, a state that is not well represented in immunocompetent mice (18). Although the study found that cytokine levels and cytotoxicity did not significantly decrease due to triple knockout, they also did not result in a heightened immune response to counter the tumor. Other studies that targeted each gene (PDCD1, HAVCR2, and LAG-3) separately reported seeing higher cytokine production and increased cytotoxicity, but this study sheds light on the idea that triple knockout may keep the cytokine levels and cytotoxicity stable (73–75, 77). Examining the survival rates reveals that triple knockout significantly enhances survival rates, as 3KO OT-1 $CD8^+$ T cells exhibit approximately 35% and 45% higher survival rates compared to TC and NT cells. Considering that inhibiting IRs only results in a 15-25% response rate in monotherapies, this study demonstrates that triple gene knockout using CRISPR is feasible and results in promising outcomes that can increase survival rates (78–85). Although the tumor microenvironment of MM may differ from that of this study, these findings demonstrate the feasibility of triple gene knockout using CRISPR and the promising outcomes of preserving cytokine levels and cytotoxicity while increasing survival rates *in vivo* (78). Triple gene editing of all three genes proved to be an effective

and successful method of reducing tumor growth, a technique that could be replicated within MM patients.

These studies provide compelling evidence for a CRISPR-based knockout technique to eliminate PDCD1, HAVCR2, and TIM3 genes from MM T cells, but it is important to consider the mechanisms by which CRISPR will be delivered into T cells (Table 1).

DELIVERY MECHANISMS

For CRISPR to serve as a reliable treatment for MM, it requires an effective delivery mechanism. An effective mechanism for delivering CRISPR-Cas9 into T cells can be done *ex vivo*, and T cells can be subsequently injected into MM patients. After T cells

Table 1. Summary of key points and findings from selected studies about CRISPR-Cas9 applications

Reference	CRISPR Delivery and Editing of Genes	Study Model	Main Findings	Limitations	Survival Benefits	Therapeutic Implications
73	*Ex vivo* T cells electroporated via RNP complexes and injected back to patients after CRISPR-Cas9 edits on PDCD1, T cells transduced with lentiviral vector to express NY-ESO-1 cancer-specific TCRα, TCRβ	3 refractory cancer patients (2 MM patients), autologous T cells	CRISPR-Cas9-edited T cells trafficked to tumor sites in all 3 patients, reduction in target antigens NY-ESO-1 and/or LAGE-1, minimal immunogenicity	Low fraction of cells with all edits, small sample size, minimal off-target effects, one patient death due to progressive MM	Edited T cells persisted for 9 months in the human body	Feasibility and safety of multiplex CRISPR editing in human T cells, edited T cells are durable in the human body, demonstrate need for improved efficiency, limiting off-target effects and long-term monitoring
74	CRISPR-Cas9 KO of PDCD1 in CTLs, delivered via lentiviral transduction	*In vitro* CTLs co-cultured with MM.1S cell line, *In vivo* mouse models	*In vitro:* PD-1 KO CTLs result in 36% more MM cell death, increased apoptosis, caspase activation, increased cytokine production, *in vivo:* mice survival time and cytokine secretion increased	Only PDCD1 was targeted, safety, long-term durability of edited CTLs not fully assessed	~60% of xenografted mice treated with PD-1 KO CTLs, compared to 0% of CTL treated mice, survived until day 60	Feasibility of editing the PD-1 checkpoint (PDCD1 KO) via CRISPR-Cas9; PD-1 KO enhanced CTL cytotoxicity and survival time *in vivo*
75	PDCD1 knockout via CRISPR-Cas9 (lipid nanoparticles), PD-L1 overexpressed via lentiviral transduction, MAGE-C2 specific TCR inserted into T cells	*In vitro* co-culture with MAGE-C2^{+} target cells *In vivo* mice models with MC2-A02-K562^{PD1+}	*In vitro* PDL1-MC2-TCR-T^{PD1-} show highest cytotoxicity and cytokine production, *in vivo* these cells suppressed tumor growth more effectively than PD-1^{-} cells while showing no off-target toxicity	Focus on MC2 antigen, long-term durability and persistence not fully assessed	All treated tumor-bearing mice survived for 25 days (end of observation period), PDL1-MC2-TCR-T^{PD1-} acquired enhanced tumor-suppressing abilities	Support for a nuanced therapy combining PD-1 knockout with PD-L1 delivery to tumors

Continued Table 1. Summary of key points and findings from selected studies about CRISPR-Cas9 applications

Reference	CRISPR Delivery and Editing of Genes	Study Model	Main Findings	Limitations	Survival Benefits	Therapeutic Implications
77	Multiplex CRISPR-Cas9 editing of HAVCR2, LAG3, or 2B4 and TCR in human T cells, delivered via electroporation of Cas9 RNPs	*In vitro* human T cells (TCR_{ED}-IR_{KO}) co-cultured with U266 and MM1.s cells expressing target antigen, *In vivo* mouse models with U266 and tumor rechallenge	*In vitro* TCR_{ED}-IR_{KO} show slight improvement in killing efficiency at low effector-target ratios, increased pro-inflammatory cytokines, HAVCR2 & LAG3 expression promotes degranulation and prevents upregulation of other inhibitory receptors, respectively, *In vivo,* TCR_{ED}-$TIM3_{KO}$ were highly effective in rechallenge tumor, TCR_{ED}-$LAG3_{KO}$ had most expansion	Single IRs knocked out per T cells, although tumor models did include rechallenge, long-term safety and persistence are not addressed, focus was on antigens like NY-ESO-1	Mice with IR-disrupted T cells were found to have a slight increase in survival rates	Editing IRs can improve function of transferred T cells in cases of chronic antigen exposure and high burden tumor settings, suggests IRs display varying functions
78	CRISPR-Cas9 gene editing of PDCD1, HAVCR2 and LAG3 in $CD8^+$ simultaneously	*In vitro* $CD8^+$ cells activated via CD3/CD28 or OVA peptide from mouse models, *in vivo* 3KO OT-1 $CD8^+$ T into mice with B-16 OVA tumors	*In vitro* 3KO in $CD8^+$ T cells showed reduction in PDCD1, HAVCR2 and LAG3 expression, cytokine levels relatively similar between 3KO and control T cells, *in vivo* 3KO OT-1 $CD8^+$ mice had slower tumor growth and improved survival, no drastic health outcomes reported	Preclinical and may not translate fully to humans, long-term health effects not fully assessed	At day 16, overall survival rate of 3 KO mice (75%) was significantly higher than that of N.T. and T.C. mice (~30% and 40%, respectively)	Simultaneous knockout of PDCD1, HAVCR2 and TIM3 improves T cell therapy; suggests 3KO could possibly be more effective than single or double KO, support multiplex gene editing

are isolated from an individual's PBMCs, cells can be stimulated *in vitro* to promote proliferation and growth to generate an adequate number of T cells. However, prolonged culture times can lead to phenotypic changes in T cells and T cell exhaustion (86). Thus, to prevent *ex vivo* T cell exhaustion, T cells should proliferate for less than two weeks, as they typically lose cytotoxicity and function after more than two weeks (87). By mixing Cas9 protein and sgRNA targeting a specific gene, an RNP complex can be formed. In an RNP complex, Cas9-sgRNA is already pre-assembled and active, so it begins editing the genome immediately upon entry, with no need for transcription and translation (88). RNPs naturally degrade after editing is complete and are not continuously produced, unlike in plasmid delivery of CRISPR, which lowers the risk of unintended edits and off-target mutations (89). After T cells are cultured, a suitable delivery approach would be to use electroporation to deliver short, quick pulses of high voltage current into cells to create pores in the cell membrane through which CRISPR-Cas9 can enter (90). This approach was used in a first-in-human phase 1 clinical trial by Stadtmauer *et al*., where autologous T cells were first taken out of MM and liposarcoma patients and expanded *ex vivo* for 8 to 12 days (73). Researchers electroporated RNP complexes consisting of Cas9 and sgRNA targeting PDCD1 into T cells, finding that approximately 20% of the cells had PDCD1 edited prior to being infused into MM patients (73). Additionally, they noted that the sgRNA for PDCD1 was extremely specific, resulting in very few off-target mutations in over 7000 sites of cleavage, indicating high precision. MM patients were then infused with 1×10^8 cells per kilogram (73). Although this study targeted PDCD1, TRAC, and TRBC, similar methods could target HAVCR2 and LAG3. Ultimately, the modified T cells lasted for nine months, highlighting the durability of these CRISPR-edited *ex vivo* T cells (73). In addition to the Stadtmauer *et al*. study, which conducted multiplex editing on PDCD1, TRAC, and TRBC, a study by Ciraolo *et al*. also edited three genes simultaneously, similar to what is being put forth in this proposal (78). Researchers utilized RNP complexes consisting of Cas9 and multiple sgRNAs targeting PDCD1, HAVCR2, and LAG3. The complexes were delivered into CD8+ cells via nucleofection, a form of electroporation. T cells were activated and proliferated both before and after the introduction of CRISPR-Cas9. PDCD1, HAVCR2, and LAG3 exhibited high cutting frequency determination (CFD) scores of 87, 98, and 80, respectively, demonstrating the high specificity of each sgRNA to the respective target sequence (78). Both studies demonstrate the efficacy of *ex vivo* CRISPR editing (73, 78). Another possible delivery method is *in vivo* T-cell engineering, which involves direct delivery of CRISPR components into the body, bypassing *ex vivo* manipulation and reinfusion of T cells; however, problems may arise since *in vivo* gene editing requires the ability to deliver the CRISPR components to the relevant organs and tissues (91). Thus, for this proposal, a reliable yet effective method for delivering CRISPR to target the PDCD1, HAVCR2, and TIM3 genes would be to culture T cells *ex vivo*, deliver RNP complexes containing Cas9 and sgRNAs for the three genes, and inject the modified T cells back into the body.

DISCUSSION

Due to complexities associated with MM, many therapies have been put forth as possible treatment mechanisms. The mentioned studies, trials, and *in vitro* and *in vivo* experiments reinforce a proposal aimed at editing the PDCD1, HAVCR2, and LAG3 genes in T cells. Through gene editing technologies like CRISPR-Cas9, these three genes of interest can be modified to restore the T cell response and contribute to anti-tumor activity against MM cells, corroborating an effective and feasible treatment for MM (Figure 3).

This proposal aims to simultaneously knock out the PDCD1, HAVCR2, and TIM3 genes via a single RNP complex containing Cas9 and multiple sgRNAs. A majority of the literature on the topic addresses the knockout of one of these genes. However, a triple knockout may result in different responses and effects within the immune system against MM compared to the individual knockout of a specific gene. Past studies measuring the efficiency and effectiveness of LAG3 blockage have found that inhibiting LAG3 as a monotherapy is insufficient to reverse tumor-induced immune suppression and activate the anti-tumor response, demonstrating that LAG3 inhibition should be paired with other anti-tumor treatments to elicit a stronger immune response (78, 92–97). To better understand the unique effects of triple gene knockout, it is essential to consult the literature that measures anti-tumor responses within cells with the knockout of these three genes. Ciraolo *et al*. tested this triple gene knockout *in vitro* and *in vivo*, finding that cytokine levels, as well as the cytotoxicity of T cells, remained stable and did not increase significantly

(78). However, studies mentioned above that edited a singular gene reported an increase in cytokine production and cytotoxicity (74, 75, 77). Ciraolo *et al*.'s study encapsulates the idea that triple gene knockout is a more nuanced approach to treating MM that has yet to be intensely researched, and the effects of three gene knockout may not be what is observed when each of the target genes is edited individually, as seen with cytokine production and cytotoxicity levels. Thus, conclusions about triple gene knockout should not be made based on the results from editing each gene individually. Within the MM tumor environment, editing PDCD1, HAVCR2, and LAG3 simultaneously can result in varying anti-tumor responses that differ greatly from what is observed in monotherapies.

Limitations

The unique location of MM, originating in the bone marrow, presents challenges in effectively treating due to the immunosuppressive qualities of the bone marrow. Within the bone marrow microenvironment, osteoclasts have been shown to shield MM cells against the anti-tumor response, specifically against T cell-mediated cytotoxicity by inhibiting the proliferative abilities of $CD4^+$ and $CD8^+$ T cells (98). PD-L1, Gal-9, along with other immune checkpoint molecules, which induce deactivation or apoptosis of T cells, are significantly upregulated during osteoclastogenesis (98). These results show that among MM cells, other cells, like osteoclasts, contribute to a weakened immune response against MM cells, even after T cell activation. Additionally, myeloid-derived suppressor cells, tumor-associated M2-like macrophages, $CD38^+$ regulatory B and T cells, have been shown to inhibit both memory and cytotoxic effector T cells (99). Through the secretion of cytokines like IL-6, VEGF, and stromal cell-derived factor 1 (SDF-1), the bone marrow microenvironment influences the progression of MM by promoting an immunosuppressive environment that appears within the early stages of MM and strengthens as the disease progresses, increasing the difficulty of treating MM (100, 101). This suppression of the immune response leads to resistance to many immunotherapies, resulting in relapses and ultimately death in MM patients (101). Thus, the unique inhibitory signals and cellular composition of the bone marrow microenvironment create a significant translational challenge for immune therapies: therapies that are known to be effective in preclinical studies or other cancer types, such as CRISPR-Cas9 induced gene knockouts, may have limited efficiency and success in MM patients due to the immunosuppressive nature of the bone marrow. Understanding the relationship between MM cells and the immune microenvironment is crucial to developing effective immunotherapies that achieve long-term reduction of MM. Regarding osteoclastic inhibition, anti-PD-L1 mAb and IDO inhibitors can partly overcome osteoclast inhibition and improve T cell responses against MM cells (98). IMiDs, protease inhibitors, mAb, adoptive T cell therapy, and inhibitors of anti-apoptotic signaling pathways are effective therapeutic strategies that can be leveraged to design an immunomodulatory approach that combats the immunosuppressive bone marrow microenvironment; regardless, the bone marrow microenvironment still poses significant challenges that can reduce the effectiveness of many MM therapies (99).

In addition to the immunosuppressive nature of the MM tumor microenvironment, the narrow sample size of studies done on this topic is a limiting factor. A 2020 study referenced previously infused T cells that had their PDCD1 gene knocked out via CRISPR-Cas9 into human patients with MM, the first-in-human phase 1 clinical trial to test CRISPR in three patients with refractory cancer, two of which were MM (73). After the introduction of *ex vivo* edited T cells, the MM patients exhibited an improved T cell response as seen by long-term presence of the edited T cells and possible activation of a signaling pathway that supports T cell expansion. Of the two MM patients, one died, but both MM patients demonstrated survival following PDCD1 knockout (73). Nevertheless, an outcome seen in two patients cannot support conclusions that could be applied to an entire population, as more human clinical trials will need to be conducted to verify these results. This study also did not test simultaneous knockout of the three genes of interest referenced in this paper; rather, it focused on editing PDCD1 along with two genes encoding T cell receptor chains. The human clinical trials in this study establish a foundation for future research centered around MM and CRISPR-based T cell editing specific to the PDCD1, HAVCR2, and LAG3 genes, yet the lack of MM clinical studies utilizing CRISPR-Cas9 to edit the three genes of interest is still a limiting factor that needs to be addressed to understand the effects of this proposed therapy in MM patients.

A previously referenced study from 2022 that harnessed CRISPR-Cas9 to edit all three genes of interest simultaneously, PDCD1, HAVCR2, and LAG-

3, showed promising results regarding the stability of the anti-tumor response while also highlighting differences that arose compared to monotherapies that address the genes individually (78). When tested *in vivo*, the triple gene knock-out T cells were transfused into immunocompetent mice, meaning that the xenografted mice initially displayed a normal immune response (78). However, this may not model the true immune response found in MM patients, as individuals with MM may have already acquired prior immune infections such as HIV and AIDS or have a suppressed immune system (18). This results in an initially weakened immune response that contributes to the onset of MM in humans. Thus, a typical MM patient would have a somewhat, if not severely, weakened immune system, but the xenografted mice did not exhibit this, suggesting that the results obtained from the experiment may overestimate the anti-tumor effects of triple gene knockout of PDCD1, HAVCR2, and LAG3. Nevertheless, the study still provides evidence in support of a multiplex CRISPR-Cas9 knockout therapy. Further research that uses immunodeficient mice in addition to human clinical trials is essential to model some of the complexities associated with the weakened immune system in MM patients. Additionally, the variability of patient immune status can hinder the effectiveness of CRISPR-Cas9 therapies for MM patients as preexisting differences in systemic and regional immunity can be a major determinant of the efficacy of a CRISPR-based immune therapy for MM patients (102). The inherently immunosuppressive bone marrow environment, along with heterogeneity across different lesions, creates a challenge for CRISPR-Cas9 based therapies. Specifically, alterations arising from MM, such as differences in immune suppression, T cell exhaustion and the local microenvironment across different regions, ultimately limit the uniform persistence, safety, and activity of engineered T cells. Together, these factors highlight the importance and challenge of tailoring strategies that can optimize the safety and efficiency of CRISPR-Cas9 therapies in MM patients with varying immune status (102).

Co-expression of PDCD1, HAVCR2 AND LAG3

The strength of MM may rely on the many pathways through individual genes and proteins that contribute to its overall onset and strong persistence within the body. In MM samples, T cells from the $CD8^+$ effector and IFN-responsive subsets showed increased expression of PDCD1, HAVCR2, and TIM3 in tumors with weak immune responses, compared to tumors with stronger immune responses (103). These genes were significantly overexpressed in MM patients, compared with healthy patients. Moreover, upon relapse, patients have increased expression of these immune checkpoint receptors (104). Thus, concurrently editing these receptors can prove to be a synergistic method for mitigating tumor growth and reducing the risk of relapse.

Because these immune checkpoints are all highly expressed in MM patients and samples, they all likely work together to support MM cell survival and proliferation by inhibiting the T cell response through receptor-ligand interactions (105). When multiple receptors are co-expressed, they often result in overlapping signal transduction pathways, as seen with the PDCD1, HAVCR2, and LAG3 that reinforce inhibitory effects on T cells. A CRISPR-based approach that focuses on knocking out the genes encoding these receptors is an effective strategy that aims to reduce and downregulate individual pathways that drive MM cell growth. Tumor cells in MM are dependent on multiple pathways that protect them from T cells through redundant pathways. Simultaneously editing the PDCD1, HAVCR2, and LAG3 genes would cut off multiple pathways that promote MM cell persistence within the bone marrow and prevent the cancer from surviving through alternate pathways, which occurs when only one of the receptors is knocked out. With there being various genes that are overexpressed in early-stage and relapsed MM patients, it becomes imperative to focus on eliminating multiple genes to limit MM progression, aiming to strengthen an already diminished T cell response.

A critical challenge seen in MM patients is that patients initially respond well to a therapy, but soon relapse due to adaptive resistance mechanisms (106). This evolving resistance within MM cells may arise due to the expression of multiple receptors and ligands that trigger similar functions in MM cells. When a singular receptor is targeted, another receptor, which is co-expressed, compensates for the loss of the previous receptor and becomes upregulated, hindering the effectiveness of the treatment and activating a similar transduction pathway that hinders the T cell attack. Using electroporation to deliver multiple sgRNAs, a treatment can be developed that reduces the risk of MM cells switching to an alternate pathway, increasing the likelihood of reactivating T cells and decreasing the risk of relapse to ultimately result in the long-term decline of tumor cells. MM is an aggressive yet convoluted

cancer that relies on multiple signal-transmitting pathways. Thus, our understanding of how to treat it must build off our knowledge of these pathways and their relationships with each other, creating a treatment that targets redundant pathways to eliminate the risk of relapse.

Future Directions

The evidence put forward in this review provides strong criteria for a CRISPR-Cas9 treatment for MM patients. Considering some limitations of this approach, future trials and treatments could focus on coupling a CRISPR treatment with other tested or gold standard therapies. Among the other therapies discussed in this proposal, most have limitations that have yet to be properly addressed. This proposal intends to edit T cells to reawaken the immune response. To that end, suitable therapies – specific to T cells – that could be coupled with CRISPR include bispecific antibodies and CAR-T cell therapy. Bispecific antibodies work by binding to CD3 on T cells and an extracellular antigen on malignant cells to ultimately direct T cells towards specific antigen-expressing cells (44). After transmitting CRISPR-infused T cells, which were edited *ex vivo*, back into human patients, bispecific antibodies could be subsequently injected to direct the newly edited T cells toward malignant plasma cells. Considering the mechanisms in which bispecific antibodies direct T cells toward tumor cells, a combined CRISPR and bispecific antibody could be useful in directing newly infused CRISPR T cells to MM cells to accelerate effector and tumor cell interactions, augmenting overall efficiency in destroying tumor cells in MM patients. Nonetheless, bispecific antibodies must target an antigen solely expressed on tumor cells and not on healthy cells to prevent non-specific killing (48). If antigens that are solely expressed in MM cells and minimally expressed in healthy immune cells are found, bispecific antibody therapy could be a promising approach that can be coupled with CRISPR to effectively link the cytotoxic T cells with MM cells. Due to the limited research on this topic, it is unclear how the integration of these two therapies will manifest in a clinical setting, considering MM's complex interactions with the tumor microenvironment. Additionally, the adverse side effects of bispecific antibodies, such as cytokine release syndrome, must be carefully evaluated and considered before coupling this therapy with CRISPR (47). These considerations underscore the need for more *in vivo* clinical studies that can fully elucidate the safety, effectiveness, and long-term durability of this combinatorial approach.

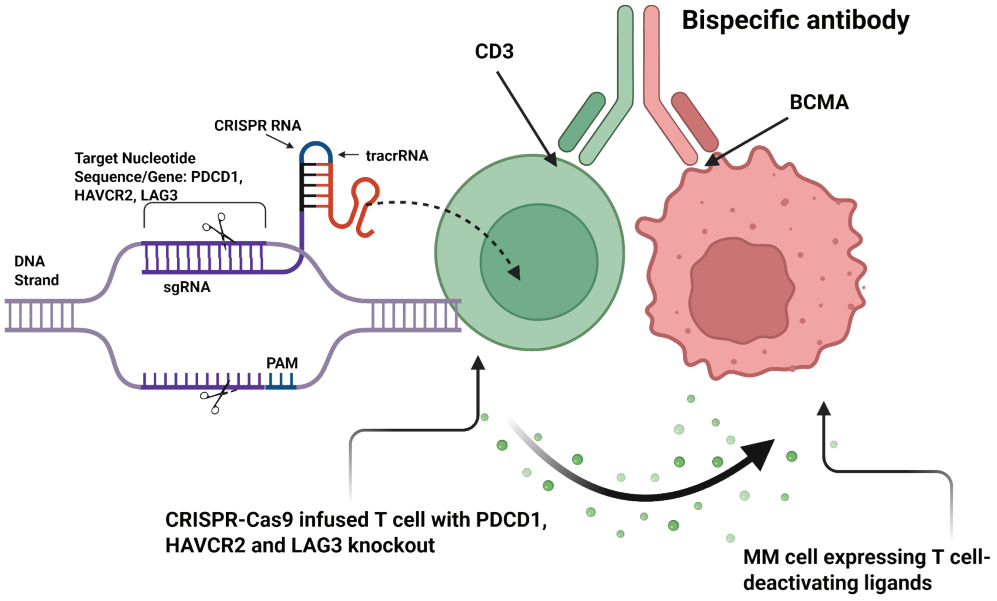

Figure 3. Schematic representation of CRISPR-Cas9 Therapy coupled with Bispecific Antibodies. Bispecific Antibodies bind to CD3 on T cells and BCMA on MM cells, ultimately directing the T cell response toward MM cells through a combined therapy that can be applied after CRISPR-Cas9 is delivered to T cells. This approach synergistically enhances CRISPR-Cas9-edited-T cell toxicity toward MM cells through the use of bispecific antibodies. Created in BioRender. Patel, V. (2025) https://BioRender.com/bjekz0k

Similarly, CAR-T cell therapy holds potential to be an effective treatment that can be paired with CRISPR-Cas9, considering its potential to engineer T cells with a chimeric receptor that recognizes MM antigens and release cytolytic molecules into malignant cells. Despite promising data, CAR-T cell therapy is still hindered by safety, logistical, and biological challenges as it poses similar risks as do bispecific antibodies in terms of non-specific killing due to antigen presence on healthy and tumor cells, along with antigen escape (48). With this approach, T cells are subjected heavy editing of antigen receptors along with immune checkpoint receptors. This cumulative burden could compromise the persistence and efficacy of engineered T cells in their killing efficiency of MM cells (107). Moreover, the increased frequency of gene editing events raises the risk of off-target mutations arising due to high editing rates, which can potentially lead to unintended genomic alterations that could alter T cell functionality and safety. While simultaneous editing of PDCD1, HAVCR2, and LAG3 through CRISPR and antigenic receptors offers the potential to enhance T cell cytotoxicity toward MM cells, it also introduces significant challenges related to T cell exhaustion, persistence, and genomic safety, with some treatments being developed to mitigate these effects (108). Nonetheless, these effects must be carefully studied and understood before applying CAR-T cell therapy in clinical trials for MM patients. It is also important to question how these therapies would be delivered alongside CRISPR. This proposal argues for a delivery mechanism that cultures T cells *ex vivo*, delivers RNP complexes that contain Cas9 and sgRNAs associated with PDCD1, HAVCR2, and LAG3, and injects modified T cells back into the body. Future work on MM treatment could focus on combining CRISPR-Cas9 with either bispecific antibodies or CAR-T cell therapy, but the long-term effects of both approaches should be comprehensively studied before being applied in human clinical trials.

Bioethical Considerations and Translational Barriers

The use of CRISPR-Cas9 in T cell therapies raises important bioethical considerations. Editing *ex vivo* results in permanent modifications to human cells, which can create unintended genomic consequences and long-term risks, including off-target mutations and impacts on immune function. Some ethical responsibilities include informing patients, obtaining consent, and monitoring patient outcomes in the long-term to assess the safety of using CRISPR on human cells (109). Regulatory approaches and oversight are essential to prevent misuse of this proposed therapy and to ensure that potential therapeutic benefits for patients are not valued more than the possibility of significantly harming patients. Additionally, as treatments using CRISPR are being developed, it becomes imperative that there is equitable access to CRISPR therapies to prevent widening disparities in patient care across different socioeconomic backgrounds (110). CRISPR's ability to introduce heritable germline mutations also raises concerns regarding unintended societal outcomes, such as non-therapeutic genetic enhancement and the creation of designer babies (110). Future applications of CRISPR as an immunotherapy for MM must address these ethical challenges that come with using gene editing techniques (111).

Many translational barriers may limit CRISPR usage among human patients. Patients who have undergone CRISPR-Cas9 therapies will require long-term follow up to assess safety and potential adverse side effects of the therapy as the effects on patient health may not be seen immediately after the therapy. Continuous monitoring is essential to ensuring therapeutic efficacy and mitigating unforeseen risks with gene editing in human patients (112). Many regulatory approvals are required for CRISPR-based therapies to be approved by the FDA as even minor changes in manufacturing require comprehensive comparability data to ensure that there are no drastic changes to the safety and efficacy of the therapy (113, 114). Regulatory guidelines also highlight the need for patient follow-ups to understand long-terms risks associated with the therapy (113, 114). Despite the high of upfront costs of CRISPR gene therapies, with Casgevy, the world's first approved CRISPR cell therapy being priced at $2.2 million per patient, studies have shown that cell and gene therapies like CRISPR have evidence demonstrating their cost-effectiveness, indicating they have value in the long term; however, there is much uncertainty about their long-term value as much of the modeling results are found through extrapolations (115, 116).

CONCLUSIONS

This proposal highlights various factors that support the use of CRISPR-Cas9 to edit T cells in MM patients, specifically focusing on the PDCD1, HAVCR2, and LAG3 genes, while also pointing out limitations and future directions. Editing of these immune checkpoint genes offers a promising avenue for overcoming T cell

exhaustion and improving cytotoxic function in MM patients. Moving forward, the integration of CRISPR with other approaches, such as bispecific antibodies and CAR-T cells, could be an emerging possibility. Rigorous preclinical studies that carefully optimize CRISPR-based gene editing techniques and assess the long-term safety of this treatment will be essential in translating this proposed treatment into an effective clinical intervention that can hinder MM prevalence and contribute to the decline of the disease. While obstacles persist, CRISPR-Cas9-mediated editing is a compelling and evidence-backed approach that could revolutionize the future of T cell-based therapies used to treat multiple myeloma with the goal of improving patient outcomes worldwide. This manuscript advances the field of CRISPR-based T cell immunotherapy centered around MM by elucidating the rationale for simultaneous CRISPR-Cas9 editing of PDCD1, HAVCR2 and LAG3 in T cells from MM patients, rather than targeting individual genes. By targeting multiple MM pathways, this manuscript offers a method to enhance T cell persistence and bolster the immune system while also addressing gaps in research on CRISPR monotherapies, with the aim of providing a foundation for multiplex gene editing within MM patients. This proposal addresses critical gaps in adoptive T cell therapy for MM and establishes a framework for CRISPR editing of PDCD1, HAVCR2 and LAG3 for future clinical applications in MM patients.

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CONFLICT OF INTERESTS

The author declares that there are no conflicts of interest related to this work.

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