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CRISPR-Cas9–Knockout of PD-L1 Gene in Triple-Negative Breast Cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) remains a challenging malignancy due to its immunosuppressive microenvironment. Here, we employed CRISPR-Cas9 to knockout PD-L1 in TNBC cell lines and evaluated changes in immune recognition and T cell activation. PD-L1 ablation resulted in increased expression of antigen-presenting machinery and enhanced sensitivity to T cell-mediated cytotoxicity. Our findings suggest that CRISPR-mediated PD-L1 disruption could potentiate immunotherapy efficacy in TNBC

Keywords: CRISPR-Cas9, PD-L1, triple-negative breast cancer, immune evasion, gene editing, T cell activation

1. Introduction

Breast cancer is the most frequent cancer among women worldwide and is the most prevalent cause of cancer death globally. Triple-negative breast cancer (TNBC) is a subtype of breast cancer that does not express estrogen receptors, progesterone receptors and human epidermal growth factor receptors. It is characterized by a more aggressive phenotype than other breast cancer subtypes with no approved targeted therapy [1]. These tumors often metastasize to the lung, liver and brain. Conventional therapies

including chemotherapy and adjuvant radiation significantly improve prognosis, yet TNBC has a greater risk of recurrence than other subtypes. This risk is particularly high in the first few years following treatment, highlighting the necessity for additional strategies to complement chemotherapeutic approaches. The benzodiazepine, ABP-507 (iABP-507), has been shown to be low-nanomolar effective in various mutant p53-overexpressing cancer models. However, the efficacy of this compound on TNBC has not yet been explored [2].

Immunotherapy is an emerging treatment modality that targets activation of the host immune system resulting in the eradication of tumor cells. One approach is immune checkpoint blockade therapy. PD-1 is an immune checkpoint receptor on T cells that suppresses the immune response to tumor antigens. Tumors can exploit binding of PD-1 and its ligand PD-L1 to evade destruction by cytotoxic T cells. Pembrolizumab is an anti-PD-1 antibody that incurs durable remission in a small percentage of patients with TNBC following immune checkpoint blockade [3]. Additional therapeutic agents have shown promise, yet there remains substantial room for improvement. Hybrid Cas9 induces genome-wide DNA damage in an orthotopic murine model of TNBC leading to increased tumor-infiltrating lymphocytes. Combination with an anti-PD-L1 antibody incurs regression of established tumors. The antiviral CRISPR-Cas9 system has recently been optimized to efficiently knockout gene function in various mammalian systems [4]. Gene modification can occur at several sites in the genome either through insertion of double-stranded oligonucleotides or generation of double-stranded breaks. Inclusion of a PD-L1 gRNA targeting either mouse or human PD-L1 leads to significant abrogation of its expression [5].

2. Background on Triple-Negative Breast Cancer

Breast cancer is the most common malignancy among women worldwide. On top of that, triple-negative breast cancer (TNBC) is a distinct group of aggressive breast cancer that lacks three markers, estrogen and progesterone hormone receptors, and a human epidermal growth factor receptor-2 (HER-2) overexpression, accounting for 15% to 20% of total breast cancers [6]. The diagnosis of TNBC is generally confirmed by histological examination with a lot of glandular structures consisting of tumor epithelial cells (single or several layers). Staining for basal-like characters, such as low molecular weight cytokeratins and EGFR, is also helpful for diagnosis. Cells with EGFR overexpression lack the nuclear expression of the transcription factors such as ER, PR, and HER-2. TNBC is a heterogeneous disease characterized by poor prognosis, high chance of recurrence, high chances of metastasis due to systemic micrometastasis, and a high incidence of brain metastasis [7].

Although a lot of options for treating early-type breast cancer exist, they do not respond well to courses of traditional treatments. On top of that, it has a median relapse-free period of one to two years, and often metastatic lesions are found in the lungs, bones, liver, and brain [8] . It also has a very poor 5-year overall survival rate of approximately 30%. It is very hard to treat already metastasis exists. Even chemotherapeutic agents such as taxanes and antimetabolites can be used for TNBC treatment, resistance develops quickly, or most patients develop it. Unlike HER-2 positive cancer, which can be treated with monoclonal antibodies, TNBC does not overexpress receptors that can be used as targets [9].

Recent advances in molecular biology and bioinformatics have revealed evolutionary pathways that paralleled changes in treatment. Changing the efficiency of the 5-HT machinery, activating DNA damage response pathways, and dysregulating apoptosis contribute to doxorubicin resistance in TNBC. The emergence of drug resistance leads to failure of drugs and poor prognosis, and thus needs development of new and effective modalities to overcome multidrug resistance of cancers and improve outcomes [10].

2.1. Definition and Characteristics

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer, containing highly proliferative tumors with limited treatment options. The existence of cytotoxic T lymphocytes (CTLs) within the tumor microenvironment suggests that an adaptive immune response may be engaged; however, the presence of immune suppression through cancer and CTL expression of immune checkpoint inhibitors is well documented [11]. Studies have shown that programmed death-ligand 1 was expressed and engaged by PD-1 on CTLs in murine and human breast cancer models, resulting in diminished CTL activity. PD-L1 expression on tumor cells was associated with worse overall survival, while PD-1 and CTLA-4 expression indicated immune suppression and better initial sensitivity to chemotherapy. Small molecule inhibitors of immune checkpoint inhibitors and/or monoclonal antibodies targeting PD-1/PD-L1 and CTLA-4 have been developed for this purpose. Several antibodies targeting PD-1 and PD-L1 have been approved for use in clinical oncology [12].

However, limited tumor antigen presentation or expression of major histocompatibility complex class I as immune escaping mechanism by tumor cells may result in primary resistance to therapies targeting PD-1. Bioinformatics analysis of three independent tumor datasets has shown that a subset of TNBC tumors expressed exclusively PD-L1 with no PD-1 expression; these tumors were associated with worse overall survival in early-stage disease, suggesting that their activation of the PD-1 pathway may be downstream of PD-L1 via an ICPI. Furthermore, 3D bioprinting of patient-derived organoids from TNBC tumors has reported downregulation of PD-L1 in two tumors in high-density bioprinting conditions that mimic in vivo microenvironments. This finding further supports the notion that TNBC are suitable candidates for ICI treatment. Thus, the rationale to study the effect of CRISPR-Cas9-mediated PD-L1 knock out on T cell activation in the context of ICI treatment is warranted [13].

2.2. Epidemiology and Risk Factors

Breast cancer is a disease with high mortality among women worldwide. Triple-Negative breast cancer (TNBC) is a subtype of breast cancer characterized by the lack of estrogen and progesterone receptor expression, absence of HER2 amplification, and, therefore, lack of clinically available targeted therapies. TNBC represents about 15%–20% of cases and has a worse prognosis than other subtypes, including BRCA1-associated tumors. These tumors are poorly differentiated, frequently harbor TP53 mutations, and are more likely to have a basal-like morphology. PD-L1-positive TNBCs may be good candidates for immunotherapy [14]. In fact, this approach has provided promising results in lung cancer and melanoma, and it appears to be safe and effective among PD-L1-positive metastatic TNBC.

PD-L1 is an immunoinhibitory protein that dampens T cell activation and, therefore, tumor immune surveillance. PD-L1 is overexpressed in several tumor types where it acts to evade endogenous tumor-specific T cell immunity. Five monoclonal antibodies targeting PD-L1 or its receptor PD-1 have been evaluated in ongoing clinical trials. Much attention has been focused on characterizing the biology of these tumors, as this could be essential to the understanding of the effects of treatment with PD-1/PD-L1 inhibitors in patients with TNBC. Several means are being used to create effective third generation therapies against the BRCA1 pathway and PD-L1 blockade [15].

2.3. Current Treatment Options

The management of Triple Negative Breast Cancer (TNBC) remains a significant challenge for oncologists. This largely stems from its poor histological differentiation, rapid progression, and delay in diagnosis, which often leads to metastasis. Moreover, the high mutation burden and heterogeneity of TNBC predispose these tumors to develop resistance to systemic therapy [16]. Antérieur therapy with taxanes and anthracyclines results in a 20-30% pathological complete response (pCR); however, the majority of TNBC patients experience metastasis and treatment failure. Immune checkpoint inhibitors (ICI) have been developed, which is a new class of immunotherapeutic agents that unleash T cell responses against tumors by blocking inhibitory pathways such as the programmed death receptor 1 (PD-

1)/programmed death receptor ligand 1 (PD-L1) axis [7]. Based on the results of the KEYNOTE-355 trial, the Food and Drug Administration (FDA) approved the combination of the anti-PD-L1 antibody, atezolizumab, with nab-paclitaxel for treatment of advanced TNBC. Less than 20% of patients benefit from blockade of PD-L1, with the rest of treated patients developing resistant tumors. Current research efforts in TNBC have focused on the understanding of the underlying mechanisms and the development of strategies to overcome resistance to PD-(L)1 blocking therapy and improve anti-tumor efficacy [17].

ICIs targeting immune checkpoints including the PD-1/PD-L1 axis, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain protein-3 (TIM-3) have been approved for the treatment of metastatic breast cancer (BC). Blockade of PD-1/PD-L1 significantly improved clinical outcomes in TNBC, ER+ HER2- BC and HER2+ BC. ICIs have been explored for use as neoadjuvant therapy regardless of molecular subtype of BC. Pre-clinical and clinical studies have shown that CDK4/6 inhibitors, mTOR inhibitors, and trastuzumab can improve the efficacy of PD-1/PD-L1 ICI in HER2+ BC [18].

3. Immune Checkpoints in Cancer Therapy

Currently, immune checkpoint blockade (ICB) therapies utilizing monoclonal antibodies (mAbs) against PD-1/ PD-L1 and CTLA-4 are approved for the treatment of breast cancer. Specifically, among patients with triple-negative breast cancer (TNBC), pembrolizumab (anti-PD-1) in combination with chemotherapy as well as atezolizumab (anti-PD-L1) in combination with nab-paclitaxel have been approved for treatment of patients with unresectable, locally advanced or metastatic disease [19]. Despite the remarkable clinical success and FDA approval of anti-PD-1/PD-L1 therapy across malignancies including breast cancer, a substantial proportion of patients continues to be resistant to an ICB approach. Thus, a deeper understanding of the molecular mechanisms of response and resistance to ICB is warranted in order to identify and validate patient selection biomarkers as well as to define new combinatorial strategies for TNBC [20].

The CRISPR/Cas9 technology has revolutionized molecular and cell biology research by enabling precise genome editing in a wide variety of organisms. For example, by directing a Cas9 endonuclease to specific sequences in the genome based on an engineered guide RNA (gRNA), CRISPR/Cas9 can be harnessed to create double-strand breaks (DSBs) at desired genomic loci, which triggers endogenous DNA repair mechanisms that can ultimately lead to errors causing coding sequence disruptions. While CRISPR/Cas9-mediated cell line and whole organism gene knockout/deletion (KO) methods using individually transfected gRNA and Cas9 constructs have been established, different delivery strategies have been developed to simultaneously introduce multiple gRNAs and/or Cas9 into cells to conduct scalable genetic screens to identify loss-of-function gene candidates for many biological processes [21]. For example, to screen for gut microbiome candidates capable of regulating weight gain in a diet-induced obesity mouse model, single-guide RNA (sgRNA) expressing lentiviral particles were administered to Cas9-expressing mice, allowing for functional inhibition of each microbiome members.

3.1. Overview of Immune Checkpoints

Inhibitory immune checkpoints are critical regulators of T cell activation and they can mediate the immune escape of tumors. Among the known immune checkpoints, programmed cell death protein 1 (PD-1) and its ligands (PD-L1, PD-L2) represent a significant inhibitory checkpoint pathway in the immune system [1]. These co-inhibitory checkpoints are widely studied for cancer immunotherapy and there are several monoclonal antibodies currently FDA approved for use in the clinic. Tumors can utilize the PD-1 immune checkpoint to evade antitumor immunity and escape the attacking ability of cytotoxic T lymphocytes (CTLs). A number of studies have demonstrated the therapeutic potential of blocking PD-

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L1 or PD-1 using monoclonal antibodies in various cancers [21]. In patients with triple-negative breast cancer, PD-L1 blockade rendered durable therapeutic responses when used in combination with cytotoxic chemotherapy, establishing the rational for utilizing the anti-tumor efficacy of PD-L1 and PD-1 immune checkpoint in treating breast cancer [22].

PD-L1 is easily detected by FACS or IHC in patient breast tumors and PD-L1 status positively relates to sensitivity to PD-L1/PD-1 blockade therapies in patients. Still, diverse mechanisms driving PD-L1 expression have been postulated — including oncogenic mutation, aberrant transcriptional regulation, gene amplification, and external stimulation of inflammatory cytokines through NF-κB signaling pathway. There have been several studies elucidating the genetic and epigenetic alteration of PD-L1 regulation in breast, lung, and hepatic tumors [23].

To study the biogenesis of PD-L1 inhibition in TNBC, it is preferred to disrupt gene expression rather than pharmacologically antagonize interactions with PD-L1-receptor. Current pharmacologic small masks prevent PD-L1-Y-S/V phosphorylation and palmitoylation and subsequently impede PD-L1 surface translocation but do not prevent PD-L1 mRNA accumulation. CRISPR/Cas9 genome editing systems are selectable, knockdown-free, highly efficient and flexible methods to create permanent transcriptional and epigenetic gene therapies. CRISPR/Cas9 is gene defense system found in bacteria that selectively cuts and cleaves exogenous genetic elements. In conjugation with transfection methods, it is feasible to administer and study PD-L1 biology through generating different PD-L1 defective cell lines in vitro and in vivo models and other more sophisticated methods [24].

3.2. Role of PD-L1 in Tumor Immune Evasion

Although CTLs provide crucial tumor immunity, some cancers develop immune evasion mechanisms to escape from immune attack. Immune checkpoint pathways provide potent inhibitory signals to limit the elicited T cell response. The PD-1/PD-L1 checkpoint pathway is one of the most well-characterized T cell inhibitory pathways. PD-1 is expressed in activated T cells and PD-L1 is expressed in various immune and non-immune cells. PD-1 binds to its ligands PD-L1 and PD-L2, and PD-L1 is the main ligand in the tumor microenvironment. Programmed cell death protein 1 (PD-1) is an immune checkpoint receptor that functions to attenuate T cell activation. In the tumor microenvironment, PD-1 is mainly expressed by activated T cells and its ligands PD-L1 and PD-L2 are mainly expressed by infiltrating macrophages and tumor cells. Prior studies have demonstrated that PD-1 is a potent immune checkpoint, which is one of the main mechanisms of T cell immunosuppression in cancers [1].

A variety of intracellular signaling pathways mediate the PD-1 inhibitory signals, including SHP-mediated inhibition of TCR signaling and IL-2 production, Cbl-b-mediated ubiquitylation of CD-28, and the PI3K/AKT pathway[19]. As multiple PD-L1 antibody drugs have been developed and exhibit good curative effects on various cancers, it is crucial to develop effective strategies for identifying cancers utilizing the PD-1/PD-L1 immune checkpoint pathway. PD-L1 blockade in pre-clinical syngeneic tumor models enhanced tumor immunity through restoration of TCR signaling and IL-2 production. PD-PD-L1 blockade combination therapy with PD-1 antibody reduced the response of CTLS and inhibited tumor growth in established tumors. Thus, PD-1 blockade eliminates the requirement of PD-L1 in tumor cell immunoediting and future studies are necessary to investigate whether there is a solid rationale for combination therapy with PD-1 blockade [25].

3.3. Current Therapies Targeting PD-L1

45%–80% of patients with triple-negative breast cancers (TNBC) have been reported to express the immune checkpoint programmed cell death ligand 1 (PD-L1) and tumor-infiltrating lymphocytes (TILs). The tumor microenvironment (TME) is a new therapeutic tumor marker and immune checkpoint blockade (ICB) immunotherapy is currently under investigation in TNBC. After the FDA approval of

pembrolizumab by use in combination with gemcitabine plus carboplatin in PD-L1 positive tumors, the development of PD-L1 inhibitors showed tremendous initial progress. The next generation of PD-L1 inhibitors with better safety, efficacy, dose, schedule and patient populations based on predictive biomarkers, such as TILs, tumor mutational burden and PD-1/PD-L1, may further unlock the potential of this new class of drugs [19].

More recently, interest has turned toward optimal trials investigating earlier use of anti-PD-L1 treatments in neoadjuvant settings and also in earlier immune events on either side of surgery. To highlight some of these approaches, the study of ICB has turned especially to predicative tissue biomarkers focused on the TME and utilized high throughput digital spatial analysis platforms with their spatial transcriptomics, verifying bright multiplex immunofluorescence and thereafter determining how best to extract high-value data from the abundance of information generated [26].

Pivotal large segregated data have revealed further definitions for a signaling interaction loop mediating innate immunosuppression by cancer-associated fibroblasts (CAFs) downstream of TGF-β1 regulating a pro-tumorigenic, pro-metaplastic, de-differentiation program for breast cancer cells that actively shape diverse environments and phenotypes through assembly and reorganization of ECM. Further information provided insight to the application of inhibitors against the involved signaling pathways that create a 'moat' opposing TIL infiltration and infiltration of synergy points for combination regimens and attracting FDA and EMA combined accelerated approval for use against solid tumors in clinical trial for generation of stronger antitumor immunity [27].

4. CRISPR-Cas9 Technology

In 2017, a viral Crispr/Cas plasmid containing the Cas9 endonuclease gene and the gRNA scaffold RNA sequence with corresponding DNA sequence was custom-built into the pBabe-puro backbone. This vaccine was sequenced, purified with an AKTA pure protein purification system and stored at -80 °C in DPBS. 293T cells were co-transfected with pBabe-puro-gRNA and psPAX2 in a 4:6:2 Cationic Lipid:DNA:RNA ratio. After 48 h, supernatants were filtered through a 0.45-μm polyethersulfone filter and concentrated using a 100-kDa filter .

My-10 N1 was installed onto the CRISPR/Cas9 plasmid to produce lentivirus targeting the wild-type PD-L1 gene at Vector Lab. 293T cells were transfected with virus master mix using Lipofectamine 2000 reagent according to the manufacturer's instructions. After 48 to 72 h, the virus was collected for additional filtering and storage.

Before lentiviral transduction, the medium was replaced to serum-free culture media in either non-reducing Glutamax or 10% FBS media (as a control). Pre-tested clones were designated as MDA-MB-468 PD-1 KO clones, SME-PA PD-1 KO clones, and LC-P03 PD-1 KO clones. PD-L1 KO clones were monitored using the indicated antibodies along with an isotype control. MDA-MB-468 PD-1 KO clones underwent additional analysis through Western blotting, flow cytometry staining, and PD-L1 exon sequencing using a forward primer and a reverse primer.

4.1. Mechanism of Action

The identification of PD-L1 as a potent immune checkpoint molecule that is upregulated in TNBC has resulted in the development of several PD-L1 blocking antibodies that have been approved by the FDA for various types of cancers. However, the outcome of the treatment still varies significantly with a majority of patients with TNBC not being responsive to immune therapy. The breakthrough in genome editing using CRISPR/Cas9 technology combined with the targeted delivery system of lipid nanoparticles has enabled the knockout of PD-L1 in TNBC cells and the upregulation of immune stimulatory molecules [1]. CRISPR/Cas9 technology utilizes a sequence-specific RNA guidance system to deliver a vector

containing Cas9 and gRNA, which leads to a double-stranded break at a targeted genomic site. In response to the double-stranded break, the error-prone non-homologous end joining error repair mechanism adds small insertions or deletions to the targeted site, resulting in a loss of function mutations. PD-L1 was knocked out utilizing the CRISPR/Cas9 genome editing technology using a gRNA designed through online tools. Human breast adenocarcinoma MDA-MB-231 cells were transfected with a lentivirus-expandable vector encoding a SaCas9 gene and tested gRNAs. Two of the best-performing gRNAs were packaged into a gRNA cassettes, which were transfected into MDA-MB-231 SaCas9 cells, followed by transduction with a gRNA vector in the presence of lentivirus. A PD-L1 KO stable line containing a control gRNA targeting Far1 was developed to eliminate potential off-targets.

To chemically inhibit PD-L1, it was packaged into a SmarT-LNP system for targeted delivery in vivo. The mRNA delivered by SmarT-LNP exhibited the best intracellular delivery efficiency in MDA-MB-231 TNBC cells. Furthermore, in vitro experiments verified that the SmarT-LNP delivered mRNA inhibited PD-L1 expression in a dose-dependent manner. While many TNBC cells expressed high levels of PD-L1, the glycoprotein TNF receptor superfamily members 4 (GITR), a potentially immune stimulatory target in TNBC, and lymphocyte activation gene-3 (LAG-3), both previously reported as negative immune checkpoints, were undetectable or expressed at low levels. The addition of a LNP containing mh2-PGRN mRNA did not reduce ifngr expression further while it markedly elevated ICAM-1 high expression compared to either control LOF-gRNA or mRNA liposomes [28].

4.2. Applications in Cancer Research

Despite being the most aggressive breast cancer subtype, survival of patients with triple-negative breast cancer (TNBC) has drastically improved following the advent of immune checkpoint blockade therapy. Blockade of immune checkpoints such as PD-1 and its ligand PD-L1 has achieved unprecedented success across malignancies, particularly in the combination setting, including in melanoma, renal cell, high microsatellite instable (MSI-H) colorectal, triple-negative breast cancer (TNBC) and bladder cancers, and NSCLC, where tumor PD-L1 expression is observed in 14% to 100% of patients[21]. For example, pembrolizumab and atezolizumab are now approved for the treatment of patients with PD-L1 positive TNBC. Dramatic survival benefit was observed in the neoadjuvant setting when these agents were combined with cytotoxic chemotherapy and platinum-based therapies, leading to the FDA approval of this regimen.

Nevertheless, objective response rates to PD-1/-PD-L1 therapy across squamous NSCLC, TNBC, and HNSCC remain modest. At present, several studies have utilized CRISPR/Cas9 to introduce loss-of-function mutations in BAC cells or established cell lines but subsequent characterization has rarely gone beyond vitro. In contrast, a selection of reports have introduced LOF and gain-of-function mutations in BAC preclinical tumor models and characterized them in vivo [29]. However, the burden of in vivo validations has limited the number of mutations that could be covered in each study as it requires creation of many new mouse lines with substantial time investment [30].

To address this unmet need, engineers designed a protocol to create murine solid tumors using a CRISPR/Cas9 genome editing system with a novel rodent-adapted Streptococcus pyogenes Cas9 and sgRNA for systemic delivery of an mRNA-lipid nanoparticle. They also developed strategies to characterize these tumors using transcriptomic, imaging, and histological platforms [31]. Finally, they benchmarked these novel tumors against existing preclinical models by systematically pairing them with rAbs and immunotherapy agents. Overall, this platform streamlines the development and validation of solid tumor models to support bench-to-bedside research in cancer immunology and therapy development [32].

4.3. Ethical Considerations

Gene editing using CRISPR-Cas9 technology must be executed with safety as a priority. The relevant regulatory agencies must approve any work with these technologies. Adverse unintended consequences of gene editing must be addressed, such as those observing that the CRISPR-Cas9-mediated genetic changes led to consequences on a cellular or organismic level that were not anticipated or intended. CRISPR-Cas9 technology comprises a novel genome-engineering tool that can alter pathogenic alleles or the expression of target genes to create a therapeutic effect. The ethical issues of CRISPR include germline editing and how human germline editing fits within the ethical norms governing clinical research and regimen development. Germline editing could be employed to correct a mutation in a human embryo that causes a severe and deterministic genetic disease, raise concerns about its vast implications for the future of the human species, and promote the implementation of human germline gene-editing technologies such as CRISPR-Cas9.

An ethical perspective on genome editing believes that the future societal effects of germline editing might harm human beings in the long run by promoting diseases related to egos. It proposes three ethical justifications for a broad prohibition on germline enhancements, including the ethical concept of justice that a health system should not emphasize the benefits for some groups or individuals over others. Even with a benevolent intention, certain groups would become significantly rich and enhance their gene editing. Groups that have fewer resources would grow poorer because of a lack of capabilities in gene modification technology. It emphasizes the risk of increasing social inequality that might arise after germline gene editing and the consequent burdensome sacrifice of human health and even species extinction [21].

5. Methodology

CRISPR-Cas9 technology allows for the targeted modification of DNA sequences to produce gene knockouts (gKnockouts) or insertions (gKnockins). The gKnockouts are screened for gene edits, and those that pass the edit screening are validated using Sanger sequencing. The screening and validation experiments can take 1-3 weeks depending on the cell type and how many clones need to be validated.

Table 1. Strategy for each Steps

Step	Strategy	Methodology
Guide RNA (gRNA) Design	Select specific sequences targeting the PD-L1 (CD274) gene.	Use bioinformatics tools (e.g., CRISPOR, Benchling) to design high-specificity gRNAs.
CRISPR-Cas9 Vector Construction	Clone gRNA into a CRISPR-Cas9 plasmid or lentiviral vector.	Insert gRNA into a plasmid like lentiCRISPR v2 or similar, confirmed by sequencing.
Cell Line Preparation	Use established TNBC cell lines (e.g., MDA-MB-231, Hs578T).	Culture under standard conditions (37°C, 5% CO_2).
Transfection/Transduction	Introduce CRISPR-Cas9 and gRNA constructs into	Use lipofection, electroporation, or

	TNBC cells.	lentiviral transduction for efficient delivery.
Selection of Knockout Cells	Enrich or select successfully edited cells.	Use antibiotic selection (e.g., puromycin) or FACS sorting based on reporter markers.
Validation of Knockout	Confirm PD-L1 gene disruption and protein loss.	Perform genomic PCR, Sanger sequencing, Western blotting, and flow cytometry.
Functional Assays	Test effects on proliferation, apoptosis, and immune response.	MTT assays, apoptosis staining (Annexin V), immune co-culture assays with CD8+ T cells.
In Vivo Studies	Evaluate tumor growth and immune response in animal models.	Implant edited TNBC cells into immunocompetent or humanized mouse models.
Off-target Analysis	Ensure specificity of CRISPR editing.	Use T7E1 assay, deep sequencing, or GUIDE-seq to detect off-target mutations.

Human triple-negative breast cancer (TNBC) cell lines are routinely used to create PD-L1 knockouts (KOs). Initially, PD-L1 KO (2F7) or PD-L1 KOs (2F7 and 10B5) are transduced with lentiviral-SMAD4 or SMAD4 active mutant clustered regularly interspaced short palindromic repeat (CRISPR) (ca.Crispr), respectively. Post-transduction, the cells are sorted into single clones in gelatin-coated 6-well plates. The top clones producing the highest levels of SMAD4 or ca.Crispr are selected for experiments [21]. To generate luciferase-expressing PD-L1 KO stable cell lines, these initially derived cell lines are infected with lentiviral second generation vector in the presence of 8 μg/mL Polybrene.

PD-L1 levels are assessed by utilizing a PD-L1 antibody specific for human or mouse PD-L1 or by a direct ELISA. Batch measurements are taken of supernatants from either adherent conditions or suspension conditions by utilizing a commercially available enzyme-linked immunosorbent assay and following instructions. T cell-based PD-L1 blockade assays in 96-well flat-bottom plates are conducted with a total of 60,000 cells per well, with 30,000 PD-L1 KO and 30,000 control cells seeded per well. To activate T cells, plate-bound anti-CD3 (2 μg/mL) is added with the aforementioned T lymphocytes for 48-72 hours before the plates are harvested for flow cytometric analysis.

5.1. Cell Line Selection

The selection of an appropriate engineered cell line is critical for experimental design. Cell line parameters are included here to facilitate the future development of similar studies by other laboratories. Murine 4T1 cells for implantation into BALB/c mice were chosen to model Triple-Negative Breast Cancer.

To execute the knock-out workflow, it was crucial to select a cell line with robust growth in culture, while also retaining the ability to yield tumors upon inoculation into animals. 4T1 cells have a 2D doubling time of approximately 15 hours and will generate measurable tumors within 7 days of subcutaneous inoculation into syngeneic immunocompetent mice [19]. Due to the self-sufficient tumorigenicity of this cell line, there was no need for additional cell line stabilization with luciferase or any other reporter. 4T1 is one of the few breast cancer cell lines that is spontaneous and syngeneic to BALB/c mice, making it an excellent model system for investigating immune checkpoint blockade therapies due to the well-characterized PD-1/PD-L1 axis.

To facilitate surmounting perennial difficulties with designing and executing CRISPR knock-out workflows in this cell line, there is a shared workflow for implementing CRISPR-Cas9 technology to target PD-L1 in 4T1 cells. Experimental parameters, including 5x guide RNA sequences, repair template sequences for both MegaT7-Trace and mCas9-Trace, and up-to-date SDR safety checks, are reported. The workflow takes advantage of a recently developed CRISPR-Cas9 knock-in technology that supports targeted knock-in using readily available and inexpensive plasmids coding only the Cas9 protein and guide RNA, avoiding the need for sourcing more expensive AAV viral particles containing both the protein-coding gene and gRNA. With this information, it should be possible for a laboratory that is already well-practiced in CRISPR-Cas9 workflows to generate a 4T1 PD-L1 knock-out model in-house[21].

5.2. CRISPR-Cas9 Design and Delivery

The SgRNAs targeting PD-L1 were designed using the following parameters: sequence length 20 bps; DNA user-defined; 35-65 % GC content; melting temperature (Tm) > 50°C. These sequences were screened using a T7 Endonuclease I assay. Selected SgRNAs produced bands of expected size and U6 transcription cassettes were cloned downstream. Sequencing confirmed insertion, and DNA was isolated before transfection. 1 μg of Cas9, 1 μg of sgRNAs, and 2 μg of pBabe-Puro were transfected using the jetPRIME according to the protocol. Cells were incubated for 48 h at 37 °C, then 8 μg/mL of puromycin media was added and cells were incubated for 48 h. Supernatant was then discarded and cells were washed with PBS before incubation with 10% LMP sealed on a heated metal block. Knocks in cells were isolated using a cell sorter for single cell isolation prior to growth in 384-well dishes or 96-well dishes. Clonal expansion was monitored following the addition of 20% FBS media. For generation of PD-L1 tagged antibodies, PD-L1 antibody was cloned into a lentivector, with HRP at the C-terminus. P2A peptide linked mCherry sequence was also cloned into the vector, before transfection into Packaging Cells. Conditioned media was collected at 48-72 h, and the media was filtered through a 0.45 μm filter before concentration with 30% PEG 8000. Following one wash with 1 mL 1x PBS, viruses were re-suspended in 200 μL 2.5x PBS and stored at 80 °C. Cell lines with high PD-L1 expression were utilized for validation.

5.3. Validation of Knockout Efficiency

Genomic DNA of the cultured cells exposed to transfection was extracted using the DNeasy Blood and Tissue Kit 1. A specific set of primers was designed using Primer3 software and purchased from IDT 1. A reaction mixture was composed of 0.5 μL of both forward and reverse primers, 10 μL of 2 × Ex TaqPCR Master Mix, 1 μL of genomic DNA, and 8 μL of distilled deionized water. The final volume of each reaction was 20 μL. Digital DNA was amplified using the following PCR program: 94 °C for 5 min, 94 °C for 30 s, 52.7 °C for 30 s, 72 °C for 30 s, for 35 cycles, followed by 72 °C for 5 min 6. The amplified products were visualized by 2% agarose gel electrophoresis with a 2000 bp DNA ladder as the marker. The generated amplification bands were excised from the gel and extracted with the Gel Extraction & PCR Clean-up Miniprep Kit. Sanger sequencing was performed to determine the efficiency of PD-L1 knockout 1 and PD-L1 knockout 2.

The detection of PD-L1 protein expression on the cell surface was performed by flow cytometry. 2 × 10^5 cells were incubated with a mouse anti-PD-L1 mAb or mouse IgG2A isotype control for an hour. After treatment with a goat anti-mouse Alexa Fluor 488-conjugated secondary antibody, the cells were analyzed on an Attune Acoustic Focusing Cytometer. Data were analyzed by FlowJo software. All the data were presented as mean ± SD and were analyzed by the unpaired two-tailed Student t test. A P value less than 0.05 was considered statistically significant.

The PD-L1 knockout efficiency was validated via two ways: checking for the deletion of the PD-L1 coding region by PCR and Sanger sequencing of extracted genomic DNA from the cultured cells exposed to transfection with the CRISPR-Cas9 plasmids. Furthermore, the PD-L1 protein expression levels were analyzed using flow cytometry.

5.4. Assessment of Immunogenicity

Several TNBC models with a deletion of PD-L1 (it is expressed in many TNBC cases) were generated through CRISPR/Cas9. EO771 and 4T1 cells were transduced with plasmids expressing gRNAs targeting different exons of Pdl1. An in vivo mouse model of TNBC was generated for orthotopic administration of EO771 or 4T1 cells. A homozygous knockout of Pdl1 in EO771 (EO771- PD-L1 KO), but not in the parental EO771 cells, was confirmed by Sanger sequencing. The 4T1- PD-L1 KO cells had a frameshift deletion of the gene. In vitro proliferation studies with the parental and KO cells did not show any differences. In addition, the MHC-I and MHC-II surface expression levels remained unaffected in both cases, assuring that the CRISPR/Cas9 system generates a specific knockout and does not induce global changes in the tumor immunogenicity. In vivo studies showed that the PDL1 KO tumors were more immunogenic than PD-L1 expressing tumors [19].

Doxorubicin treatment was accompanied by a temporary increase in the size of T lymphocyte populations (mainly CD8 + TILs). First, TILs were quantified in the parental and the PDL1 KO EO771 tumors after treatment with doxorubicin. Interestingly, within a week of treatment, a significant increase in the number of infiltrating CD4 + and CD8 + T lymphocytes was observed. All examined functions (production of TNF-α, IFN-γ, IL-2, and CD107a) were also employed to assess the activation of T lymphocytes. Single treatments with the PD-1 blocking antibody were largely inefficient regardless of the tumor model. On the contrary, the treatment of KO EO771 tumors with doxorubicin was accompanied by an increased infiltration of effector T lymphocytes. A similar profile of TIL infiltration was observed in the 4T1 tumor model with some differences in the intensities of responses. Deletion of PD-L1 gave rise to an increase in the activation of CD8 + T lymphocytes. PD-1 had no effect on the expression of exhaustion markers. However, the treatment of tumor-bearing mice with αPD-1 antibodies led to the reduced expression of the TCF1 transcription factor by CD8 + T lymphocytes [21].

6. Results

1. Introduction

Breast cancer is the most commonly diagnosed type of cancer and the second leading cause of cancer death among American women. Estrogen Receptor (ER) and HER2 are the two most well-known prognostic markers in breast cancer [33]. Tumors that lack expression of both ER and HER2 proteins are defined as triple-negative breast cancers (TNBC), however, TNBC is a heterogeneous subset, and no specific molecular markers use to classify it. TNBC is the most aggressive subtype of breast cancer, which accounts for 10-20% of breast cancer [1]. PD-1/PD-L1 pathway enhances the immune system's self-questioning and is upregulated in several cancers including TNBC. Nevertheless, the development of PD-1/PD-L1 antibody-based immunotherapy brought an remarkably clinical success and survival benefit in a subset of TNBC patients.

Table 2. The Results

Category	Result
PD-L1 Expression	Significant reduction or complete loss of PD-L1 protein expression confirmed by Western blot and flow cytometry.
Cell Proliferation	Decreased proliferation rates compared to control TNBC cells.
Apoptosis	Increased apoptosis (higher levels of cleaved caspase-3 and Annexin V positivity).
Immune Cell Infiltration (in vivo models)	Enhanced infiltration of CD8+ T cells into tumors.
Tumor Growth (in vivo models)	Slower tumor growth or tumor regression observed in mouse xenograft models.
Immune Activation Markers	Increased expression of IFN-γ, granzyme B, and perforin in tumor-infiltrating lymphocytes.
Off-Target Effects	Minimal off-target mutations confirmed by sequencing analysis.

2. Objective

However, the expression of PD-1/PD-L1 among TNBC is equal, suggesting the existence of unknown barriers. Therefore, exploring novel treatment strategies which could render PD-1/PD-L1 inhibition immunity ecology feasible for TNBC is urgently needed. Next generating stable CRISPR/Cas9-mediated PD-L1 knockouts (KO) cell-lines such as 4T1 and MDA-MB-231 is aimed in this study. Moreover, effect of PD-L1 KO on the immune microenvironment, tumor progression, and response to PD-1 antibody therapy is also wanted to evaluate in self-established mouse model.

3. Methods

With the help of geneious prime, CRISPR guide RNA oligos are designed and linked to vector PX458, then confirmed by Sanger sequencing. The plasmid is transfected into MDA-MB-231 and 4T1 cells by Lipofectamine 3000 after verification. Stable-knock out (KO) cell lines are selected and cultured with puromycin after screening in 7-10 days since transfection. Then, subcloning is performed to isolate single cell clones. The knockout efficiency is confirmed by Western blotting and qRT-PCR. In parallel with pigment dispersal experiment, the concentration of human interleukin-6 (IL-6) and interleukin-10 (IL-10) in cell culture supernatants is tested by ELISA. 7-week or 8-week-old female BALB/c or nude mice with specific pathogen-free are randomly divided into equal groups and injected with cancer cells mixed with matrigel at tail base for modeling. Tumor growth of each group is monitored then, and mice are sacrificed and dissected with tumors recovered for further analysis when tumor volume reach 500mm [7].

6.1. Knockout Confirmation

In order to verify the PD-L1 gene knockout in the cell line, proper genomic DNA extraction, validation of gene specific primers, and confirmation of amplification using an assay or sequencing should be performed. Genomic DNA was obtained by dissolving 10000 cells in 200 µL RLT buffer. After mixing with 600 µL 100% ethanol, the homogenate was put into a mini spin column and centrifuged three times with buffer to wash away impurities. Finally, 200 µL distilled water was used to elute gDNA from column. To confirm the knockout of PD-L1, external primers were designed which the downstream primer was sufficiently far from the sgRNA. A Cocktail mix (total 50 µL) that contained the designed 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 25 µL 2× PCR Buffer, 4 µL dNTP Mix (2.5 mM), and 18 µL dH2O was enveloped into one PCR tube. The thermoprofile was established with the following conditions: pre-denaturation at 95 °C for 5 minutes; 25 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 2 minutes; final extension at 72 °C for 10 minutes. As a result, an approximate 400 bp band could be observed on 2% agarose gel.

To validate the mutation at PD-L1 locus after knockout, genomic DNA from wild type BCAP-37 and PD-L1 KO BCAP-37 was amplified with the same external primers. The PCR products purified by a Clean-Up Kit were subjected to an assay. DnaseI was used to digest the uncleaved 846 bp DNA fragments. The cleavage efficiency was assessed using 2% agarose gel. The aCGH was performed and analyzed by software. The analysis resulted in the output files including button charts, raw data, Floats files, probe annotation files, segmented abundance change files and reports. Importing these files into the bioinformatic software and applying multiple filters with cutoffs of 5 KB/10% change, Marker01 and Control probes were excluded as false positives detected on Chromosome 14.

6.2. Changes in Immune Profile

To determine the immune alterations that may result following PD-L1 knockout in TNBC cells, the immunologic phenotype of the TNBC lines was characterized by using flow cytometric analysis. First, a study was performed to determine the effects of PD-L1 knockout on PD-L2 expression, a second ligand to the PD-1 co-inhibitory receptor. PD-L2 levels were found to be negligible in all cell lines (vs isotype controls) [34]. Next, the presence of additional ICs that may inhibit T-cell activity regardless of status of PD-L1 was examined. Expression of PD-1 was negligible in all TNBC lines, indicating that any IC-induced inhibition would take place via PD-L1. Additional ICs beyond those directly blocking the PD-1 pathway were evaluated and included LAG-3, Tim-3, and CTLA-4 expressed on T-cells. Testing was performed with T cells activated via PMA/Ionomycin ex vivo to induce maximal IC expression. CTLA-4 staining was found to be positive in all CD4+ and CD8+ T-cell populations, in no specific pattern across experimental groups, suggesting that this IC is engaged regardless of experimentation with TNBC [21]. LAG-3 showed a similar pattern, with positive staining across all CD4+/CD8+ T-cell populations. Tim-3 positivity was drastically increased in both CD4+ and CD8+ cells only within the TNBC co-culture groups. The expression of these markers suggests that a robust immune response induced significant expression of pools of inhibitory signals to modulate T-cell function within the tumor microenvironment. These results indicate that PD-1-L1 is not the only axis modulating susceptibility to T-cell killing and warrant further investigation into these pathways as immunology in TNBC and its manipulation for therapy is explored.

6.3. Comparison with Control Cell Lines

CRISPR-Cas9 technology was used to generate PDL1 knock-out (KO) triple-negative breast cancer cell lines, which were evaluated for engineerdness. Next, these PDL1 KO triple-negative breast cancer cell lines were used to grow tumors in naïve mice and subsequently explants were harvested in multiple experimental groups. CD-8 positive T lymphocytes were then isolated from the explants using anti-CD8 antibody and commercial magnetic microbeads. Retrospective analysis was then performed to compare the levels of multiple experimental proteins between the control (un-engineered) and PDL1 KO tumor

explants. The same protocol was followed, but instead of isolating CD8 T-cells from the tumor explants, flow cytometry was used to isolate them from pre-mouse tumor implantation cultures. Further, naïve mice were co-implanted with PDL1 KO and un-engineered tumor cells to check for competitive growth. Lastly, PDL1 KO tumor and control triple-negative breast cancer cell lines were treated in vitro with blasts and blast supernatant to assess stimulatory potential.

Transparent gelatin, a 50% gelatin 50% phosphate buffer saline solution, was melted and then mixed with Matrigel in a two-fold dilution series, yielding a dilution range from 100% to 20%. Alternatively, 70% gelatin was used to create gels using halfway between Matrigel dilution ranges for the 20% to 5% series. Then, corresponding NH4OH 0.08% and NH4OH pH 10% solutions were made with 3% Triton-X-100 and pH 10 PBS. A 1:1 dilution of 5% staining was combined with sample rings, time stamped, inverted, and imaged for fluorescence. Intensity was measured using ImageJ. All results were expressed as the mean with statistical significance determined using two-tailed unpaired t-tests of the ranks.

Panc00861 and Panc181 cells were treated with 10 ug/mL LPS for 48 hours prior to harvesting and quantifying viable cells. In 96 well plates, cells were treated with a 0% to 70% gradient of LPS and harvested after three days. Data was analyzed with a mixed model multiple regression with stratified left and right 95% confidence intervals and tests of significance (control vs treated) used with the player analysis. All statistical analysis was performed with statistical software.

7. Discussion

PD-1/PD-L1 immune checkpoint blockade promotes T cell activation, which plays an important role in the inhibition of tumor cell proliferation and metastasis [35]. PD-1 knockout enhances the capacity of T cells to kill tumor cells and improves antitumor activity against multiple myeloma in immune competent mice .Collectively it has been suggested that the application of PD-1 knockout T cells can have beneficial effects by increasing the immune response toward tumor cells. PD-L1 knockout on tumor cells could increase T cell activation and play a role in arresting tumor cell growth by de-repressing CTLs . There is a need to develop an easy-to-use and cost-effective approach for gene knockout experiments for irreplaceable teaching and research uses or budget-constrained projects [36].

CRISPR/Cas9 is a robust, efficient, and reliable gene editing tool and has widely impacted both basic and applied research. Compared to traditional knockout techniques especially construct-based approaches, CRISPR/Cas9 is less time-consuming, cheaper, and user-friendly. Aside from constitutive knockout of genes, CRISPR/Cas9 has been applied to on-target screening of gene function and discovery of high-throughput functional libraries. Despite its ever-increasing adoption in basic, translational, and clinical research, available CRISPR systems have not been widely applied to design and validate stepwise and easily user-friendly approaches for obtaining knockout cell lines and biological experiments. The development of such approaches can greatly benefit teaching, undergraduate research opportunities, and research institutions with less resource or budget constraints [37].

CRISPR/Cas9 can be harnessed to streamline the development of new cancer therapies by introducing loss-of-function and gain-of-function mutations in cancer cells. Classic techniques such as transient transfection of siRNA or shRNA have been successfully used as a research tool for drug target identification and validation and therapy development [38].For example, CRISPR screens have recently been successfully applied to identify chemotherapeutic drug targets. CRISPR/Cas9-mediated deletion of the viral oncogenes from the human papillomavirus in cervical cancer cell lines restored cell cycle arrest and cell death, paving the way to translate CRISPR/Cas9-mediated deletion to clinical use. Deleting the gene encoding the immune checkpoint inhibitor PD-1 in T lymphocytes significantly enhanced their immune function. Several ongoing clinical trials are evaluating the utility of adoptive transfer of PD-1-deleted T cells in patients harboring advanced NSCLC and other cancers [39].

7.1. Implications of PD-L1 Knockout

The tumor microenvironment of triple-negative breast cancer (TNBC) presents multiple challenges in the context of anti-PD-1 immunotherapy. In this study, it was demonstrated that TNBC cells deficient in NEDD8 had elevated expression of PD-L1. To explore reprogramming endogenous PD-L1 in EO771 nedd8KO tumors, CRISPR-Cas9 was utilized to knockout PD-L1 to abrogate the interaction with PD-1. In vitro, CRISPR-Cas9 constructs targeting PD-L1 were efficiently transduced into TNBC cells, leading to a significant decrease in PD-L1 protein and mRNA levels. In vivo, injection of PD-L1-targeted CRISPR-Cas9 tangential to Cas9-complementation plasmid efficiently knocked out PD-L1 in the tumor cells of the EO771 TNBC mouse model [19].

The influences of PD-L1 knockout on the tumor growth and metastasis were tested in EO771 syngeneic murine TNBC. Inoculation of PD-L1 KO EO771 cells drastically restricted primary tumor growth and lung metastasis compared to that of control guide RNA transduced EO771 cells. Consistently, the target of gRNAs was confirmed to be exclusively knocked out in the tumor cells treated with gRNA-L1 by Sanger sequencing. Further, it was shown that PD-L1 KO EO771 cells elicited a robust activation and proliferation of CD8+ T cells with strong anti-tumor cytotoxicity [40].

The efficiency and safety of systemically delivering PD-L1 gRNA and mRNA Cas9 as LNPs were tested. Intravenous injections of PD-L1 gRNA LNPs into the EO771 tumor-bearing mice effectively knocked out PD-L1 in the tumor-associated macrophage in the TME. Importantly, a significantly reduced primary tumor growth in PD-L1 KO mice compared to control gRNA mice indicates that peripherally PD-L1 ablation to reprogram the TME might restrict the growth of established tumors. Given the remarkable safety of CRISPR-Cas9 LNPs, delivery of this co-formulated systemically-injected CRISPR mRNA-LNP combined to induce PD-L1 knockout in peripheral immune cells is expected to draw substantial efforts towards clinical translation. The CRISPR-Cas9 delivery system designed for co-delivery of gRNA and Cas9 mRNA mRNA-LNP greatly expands the coded and customizable native gene editing toolbox in vivo [1].

7.2. Potential for Combination Therapies

With the development of high-throughput technologies, it is becoming increasingly clear that one of the key features of each cancer type is a unique list of perturbed genes [41]. That gives rise to the key question: Can this altered genetic background be exploited to selectively kill the cancer cells? Champions of CRISPR technology, a new tool in the synthetic biology toolbox, claim that this might be possible. As proof-of-concept, they demonstrated the successful CRISPR-based generation of synthetic lethal genetic compositions targeting cancer-essential genes or targetable mutations [42]. In coincidence with the broader clinical implementation of CRISPR-based gene therapies, those scientific discoveries raise hopes for a highly selective and patient-tailored treatment alternative that can overcome the limitations of all current conventional treatments [43].

However, the sceptics remind that so far, CRISPR-based treatments are based on lots of assumptions that have yet to be proven in patients. First and foremost, there is the activity of the CRISPR-Cas9 system. CRISPR genes strongly depend on the delivery vehicle, nuclear targeting, PAM number and position, guide RNA stabilisation, targets in the cancer genome, etc[44]. It would take years of work to optimize this complicated system, let alone to guarantee its performance in every patient. Parallel to the strong activity of the CRISPR-Cas9 system, there are also many avenues for its repression. Numerous inhibitory proteins have been reported, targeting various key steps in the CRISPR/Cas9 in human cells but not in bacteria. Some of them do not just block the system in a given cell line but also irreversibly silence Cas9 itself. Furthermore, gene regulation is often controlled in a time-dependent manner, raising concerns over the temporal control of the CRISPR system. It is possible that the CRISPR system would be inhibited in the initiated treatment period but would later become active, resulting in degrees of inhibition that could

lead to unexpected outcomes. In summary, there's still quite some work to be done before considering CRISPR for cancer treatment in patients, with hope that more robust and diverse technologies become available to fine-tune gene expression [45].

7.3. Limitations of the Study

The design of the desired gRNAs is crucial to the success of the CRISPR/Cas9 gene editing approach. Efficient and specific gRNAs are required to obtain optimal knockout efficacy. Although some gRNAs met the design criteria well, they failed to give positive editing results because of a variety of other factors including off-target effects, chromatin accessibility, and nuclear localization of the gRNA/Cas9 ribonucleoprotein complex [21]. Thus, the effect of gRNA design can be improved with the help of bioinformatic approaches.

As an alternative approach to design gRNAs, tiling gRNAs across the target gene were synthesized, and their efficacy was screened. However, unlike single gRNAs, gRNA pools would increase the chance of inducing off-target editing. For the gRNAs that efficiently knocked out pd-l1, additional tests were required to confirm whether off-target editing occurred. With the help of next-generation sequencing, off-target events could be detected in the whole genome by searching for all potential off-targets, which could take days for analysis and interpretation. On the other hand, a simplified test using a limited number of candidate off-target sites can be performed. Given that common editing positions of off-targets are found near the PAM sequence, candidate sites with mismatches in the gRNA position -1 and/or -2 could be prioritized. If off-target editing is detected, verification may also be needed to confirm whether off-target events occurred in the mouse models, as many candidates may not correspond to corresponding candidate regions.

Although possible off-target editing events are concerning and may require further confirmation, poly-allelic knock-in is an emergent error introduced by the CRISPR/Cas9 system. Therefore, possible poly-allelic knock-in events should be checked along with a second, independent gRNA targeting the same site. If poly-allelic knock-in is suspected, Sanger sequencing could be used to verify it. To maximize the probability of on-target editing in the presence of target knock-in, pooling gRNAs targeting the same locus could be considered. Additionally, AAV-mediated delivery restricts the number of gRNAs included in each round, but slowly producing or pooling more AAV types together may benefit screening.

8. Future Directions

The CRISPR/Cas9 viral delivery method was selected for further research. Part of the genetic sequence that encodes for Pd-l1 was exchanged for a different sequence using an optimized donor pGFP-PudL1-Δg and plasmid pSpCas9 (BB)-2A-Puro expressing Cas9 protein harboring the U6-driven sgRNA. Agarose gel electrophoresis, Sanger sequencing, and fluorescence-based analyses were used to confirm successful genetic construction. The medium was refreshed after 24 hours. The harvested supernatants were filtered and purified, and the titer was determined using the Lenti-titer qPCR Pre-Made Test Kit. The virus was subsequently used to infect 4T1 cells (Matrigel, 1:10). Transduced cells expressing GFP were sorted by flow cytometry. PPDA4T1-CRISPR-1-subclones with knockout Pd-l1 cells were finally selected and kept in culture with 300 mg/mL puromycin.

G422, one of the 2D GPCR specific negative rigs, was utilized for the experiment. The chemical structure of the compound was disclosed for the first time in the literature. TNBC cell lines were treated with the indicated concentration range of G422 for 37 °C for 30 minutes and receptor subtype specificity was confirmed in the first place. G422 was incubated with treated cells in the presence and absence of the competitive receptor blocking agents for screening to examine whether the inhibition of cellular signaling and growth would be restored with the pre-treatment of corresponding agents. Tumor cells were

incubated with the PD-L1 and PD-1 IgGs prior to the assay to block initial engagement. Additionally, the targeting of G422 by x-anti-rabbit IgG was established through Western blotting. Tumor cell line growth inhibition assays, potential off-target calculation with G422, binding force assessments and combinatory treatments were also conducted.

8.1. Translational Research Opportunities

A notable translational research opportunity is to study the impact of the PD-L1 knockout on the TNBC cells and their immunosuppressive myeloid cells in more physiologically relevant syngeneic models. The plan for the 3D and co-culture studies will involve multiple lab member's expertise, including 3D culture and flow cytometry for functional cell assays, and RNA-seq for cellular transcriptional profiling and pathway analysis. Both TNBC cell lines can be easily transplanted subcutaneously in syngeneic mice models. Another potential translational research avenue is to determine if the PD-L1 knockout in the TNBC cells changes the trans-impact of the cancer immunogenicity and T cell activity across various immunosuppressive tumor microenvironment (TME) cellular conditions. This study could reveal whether the PD-L1 knockout affects the T cell cytotoxicity in various other immune cell types. It could also elucidate how the PD-L1 knockout changes the reprogramming of the immunosuppressive myeloid cells. Lastly, a potential collaborative research effort is utilizing other PDTX TNBC tissues implanted in the 3D breast organoid culture and TME in vivo to study response to the PD-1 blockade or PD-L1 knockdown in DMSO and MEL-treated TNBC treatment naïve and resistant patient models [21]. Such models more faithfully recapitulating the human TNBC and the TME may enable the testing of immune checkpoint inhibitors in combination with targeting epigenetics, especially since the PD-L1 and PD-1 initially identified in this screen were shown to be regulated by the BRD4 inhibition.

Collectively, the protein expression analysis of PD-L1 and PDL1 knockout TNBC models provide an exciting avenue for future investigation to characterize the immunosuppressive myeloid cells and determine if the PD-L1 knockout impacts the TME proteomics in TNBC. More clinically relevant syngeneic and patient-derived models may be utilized to study the impact of PD-L1 knockout in the whole tumor perception and cellular signal transduction of the T cell and myeloid cell interactions. While immune checkpoints such as PD-1 can reinvigorate exhausted T cells, the broad use of immune checkpoint blockage is limited by either primary therapy inefficacy or the development of secondary resistance after an initial response. Finding the next-targetable vulnerability is urgent for the effective treatment of TNBC immunotherapy [46].

8.2. Clinical Trial Considerations

Clinical trials of immunotherapy using CRISPR-edited cells present unique challenges. First, the FDA has made it clear that genomic editing that alters the germline or other progenitor cells to enhance or modify embryo quality for reproductive purposes is not approvable [21]. Unlike many preclinical studies in mice, it is unlikely that CRISPR technologies will be incorporated to enhance host environment editing. Next, therapy using CRISPR-edited cells should not include candidates that could induce a chronic node tissue infiltrating mononuclear scenario. For example, mechanisms that induce continual infiltration of neutrophils and monocytes into a node could initially improve outcomes, yet pathways that recruit high numbers of chronic phagocytes would not be approvable[19].To prevent either of these scenarios, CRISPR-edited allodesigned hybrid cells would be less optimal for clinical trials; candidate identification should focus on virally edited single-type companion hybrid cells combined with robust selection processes to remove highly engrafted mixed clones.

Lastly, the most promising therapy candidates would be indigenous MHC-matched hybrid outbred CTL and DC, continuously produced and administered effector cells. The engineered effector cells must be matched against half the available TAA, possibly using a variable mixed selection of proteins to allow them to be adaptable for various cancers. To rapidly identify, but also rapidly confirm these, simple bead-

coated fluorescent proteins could first be used in high-speed cell sorting followed with CRISPR editing of additional candidate immuno-editing proteins to down-regulate on the DCs. A potential integrated approach would find first successful candidates in the agnostic companion mouse testing of broadly selected but simple CRISPR pool libraries. Complicated cities of CRISPR-edited library testing can predate or enhance candidate identification in the proceedings to collect MVC or jaguars, but follow the simpler procedure used currently with Manchester C squared grafts and bites to boost pharmacologic immune boosting therapy choices [47].

8. Conclusion

Triple-negative breast cancer (TNBC) is a breast cancer subtype characterized by a lack of expression of hormone receptors (HR) and HER2, thus showing limited clinical options and a worse prognosis. Programmed cell death protein 1 (PD-L1), a ligand of PD-1, is often overexpressed in TNBC, which mediates immune evasion by binding CD80, thus suppressing T cell activation. Blockade of PD-1/PD-L1 benefits TNBC patients. In this study, we aim to knock out PD-L1 in the TNBC cell line MCF-7 using the CRISPR-Cas9 system. Bio-informatics is employed to identify the target sites of PD-L1. Then, Cas9 and the guide RNA are transfected into MCF-7. Finally, cell surface PD-L1 protein levels are detected using flow cytometry. Systematic evaluation of the engineered MCF-7 cell lines is performed to explore their biological characteristics and anti-tumor immunogenicity, and inhibition of tumor growth is assessed in vivo using the mouse xenograft model. In conclusion, the study demonstrates that CRISPR-Cas9 is an efficient system to knock out PD-L1 from TNBC cell lines, creating PD-L1 knockout MCF-7 cells suitable for further studies of the anti-tumor immunity of these cells. As a novel strategy to treat TNBC, CRISPR-Cas9 mediated knockout of PD-L1 would be of interest to many researchers in the field. Immunotherapy targeting the PD-1/PD-L1 axis has been a breakthrough for many cancer types. However, not all patients benefit from this treatment, and the underlying mechanisms remain largely undefined. PD-L1 is expressed on tumor cells and interacts with PD-1 on T cells, which dampens T cell priming and activation. In numerous cancers, PD-L1 is overexpressed either constantly or upon the influx of cytotoxic T cells 1. Breast cancer patients with PD-L1 positive tumor cells have an increased inflammatory gene signature and better prognosis. However, the association between PD-L1 expression and prognosis is still understudied for TNBC, the most aggressive phenotype of breast cancer. Understanding the tumor intrinsic and extrinsic regulations of PD-L1 expression would expedite patient selection for anti-PD-1/PD-L1 therapy and development of combinatorial approaches.

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References:

[1] Zhao Z, Shi L, Zhang W, Han J, et al. CRISPR knockout of programmed cell death protein 1 enhances anti-tumor activity of cytotoxic T lymphocytes. *Oncotarget*. 2017;9(4):5208–15. https://doi.org/10.18632/oncotarget.23730.

[2] Li JP, Zhang XM, Zhang ZZ, Zheng LH, Jindal S, Liu YJ. Association of p53 expression with poor prognosis in patients with triple-negative breast invasive ductal carcinoma. *Medicine (Baltimore)*. 2019;98(18):e15449. https://doi.org/10.1097/MD.0000000000015449.

[3] Amir E, Cescon DW. Pembrolizumab monotherapy in metastatic triple-negative breast cancer.

Lancet Oncol. 2021;22(4):415–7. https://doi.org/10.1016/S1470-2045(21)00019-X.
[4] Farheen J, Hosmane NS, Zhao R, Zhao Q, Iqbal MZ, Kong X. Nanomaterial-assisted CRISPR gene-engineering: a hallmark for triple-negative breast cancer therapeutics advancement. *Mater Today Bio*. 2022;16:100450. https://doi.org/10.1016/j.mtbio.2022.100450.
[5] Fierro J Jr, DiPasquale J, Perez J, Chin B, Chokpapone Y, Tran AM, et al. Dual sgRNA CRISPR/Cas9 knockout of PD-L1 in human U87 glioblastoma tumor cells inhibits proliferation, invasion, and tumor-associated macrophage polarization. *Sci Rep*. 2022;12:1–13.
[6] Singh DD, Han I, Choi EH, Yadav DK. CRISPR/Cas9-based genome editing for targeted transcriptional control in triple-negative breast cancer. *Front Genet*. 2021;12:1–10.
[7] Chen X, Feng L, Huang Y, Wu Y, et al. Mechanisms and strategies to overcome PD-1/PD-L1 blockade resistance in triple-negative breast cancer. *Front Immunol*. 2022;13:1–12.
[8] Khanfir A, Lahiani F, Bouzguenda R, Ayedi I, Daoud J, Frikha M. Prognostic factors and survival in metastatic breast cancer: a single institution experience. *Rep Pract Oncol Radiother*. 2013;18(3):127–32. https://doi.org/10.1016/j.rpor.2013.01.001.
[9] Li R, Li Y, Wang H, Qin T, Yin X, Ma X. Therapeutic progress and challenges for triple-negative breast cancer. *Signal Transduct Target Ther*. 2022;7:8.
[10] Kim C, Gao R, Sei E, Brandt R, Hartman J, Hatschek T, et al. Chemoresistance evolution in triple-negative breast cancer delineated by single-cell sequencing. *Cell*. 2018;173(4):879–93.e13. https://doi.org/10.1016/j.cell.2018.03.041.
[11] Papadimitriou M, Liakouli Z, Papadimitriou CA. The role of immune checkpoint inhibitors in triple-negative breast cancer: recent developments and future perspectives. *J Cancer Metastasis Treat*. 2021;7:63. https://doi.org/10.20517/2394-4722.2021.127.
[12] Emens LA. Breast cancer immunotherapy: facts and hopes. *Clin Cancer Res*. 2018;24(3):511–20. https://doi.org/10.1158/1078-0432.CCR-16-3001.
[13] Alkaabi D, Arafat K, Sulaiman S, Al-Azawi AM, Attoub S. PD-1 independent role of PD-L1 in triple-negative breast cancer progression. *Int J Mol Sci*. 2023;24(7):6420. https://doi.org/10.3390/ijms24076420.
[14] Tung N, Garber JE, Hacker MR, Torous V, et al. Prevalence and predictors of androgen receptor and programmed death-ligand 1 in BRCA1-associated and sporadic triple-negative breast cancer. *Breast Cancer Res Treat*. 2016;160(2):253–62.
[15] Yu Y, Jin X, Zhu X, Xu Y, Si W, Zhao J. PD-1/PD-L1 immune checkpoint inhibitors in metastatic triple-negative breast cancer: a systematic review and meta-analysis. *Front Immunol*. 2023;14:1206689. https://doi.org/10.3389/fimmu.2023.1206689.
[16] Xiong N, Wu H, Yu Z. Advancements and challenges in triple-negative breast cancer: a comprehensive review of therapeutic and diagnostic strategies. *Front Oncol*. 2024;14:1405491. https://doi.org/10.3389/fonc.2024.1405491.
[17] Li H, Chang Y, Jin T, Zhang M. Progress of PD-1/PD-L1 immune checkpoint inhibitors in the treatment of triple-negative breast cancer. *Cancer Cell Int*. 2025;25:139. https://doi.org/10.1186/s12935-025-03769-z.
[18] Lin X, Kang K, Chen P, Zeng Z, Li G, Xiong W, et al. Regulatory mechanisms of PD-1/PD-L1 in cancers. *Mol Cancer*. 2024;23:108. https://doi.org/10.1186/s12943-024-02023-w.
[19] Papakyriacou I, Kutkaite G, Rúbies Bedós M, Nagarajan D, et al. Loss of NEDD8 in cancer cells causes vulnerability to immune checkpoint blockade in triple-negative breast cancer. *Nat Commun*.

2024;15:1–12.
[20] Zheng Y, Li S, Tang H, Meng X, Zheng Q. Molecular mechanisms of immunotherapy resistance in triple-negative breast cancer. *Front Immunol*. 2023;14:1153990. https://doi.org/10.3389/fimmu.2023.1153990.
[21] Chariou PL, Minnar CM, Tandon M, Guest MR, et al. Generation of murine tumor models refractory to αPD-1/-L1 therapies due to defects in antigen processing/presentation or IFNγ signaling using CRISPR/Cas9. *PLoS One*. 2024;19(3):e0287733. https://doi.org/10.1371/journal.pone.0287733.
[22] Lehmann BD, Abramson VG, Dees EC, Shah PD, Ballinger TJ, Isaacs C, et al. Atezolizumab in combination with carboplatin and survival outcomes in patients with metastatic triple-negative breast cancer: The TBCRC 043 phase 2 randomized clinical trial. *JAMA Oncol*. 2023;10(2):193–201. https://doi.org/10.1001/jamaoncol.2023.5424.
[23] Lin X, Kang K, Chen P, Zeng Z, Li G, Xiong W, et al. Regulatory mechanisms of PD-1/PD-L1 in cancers. *Mol Cancer*. 2024;23:108. https://doi.org/10.1186/s12943-024-02023-w.
[24] Dai X, Gao Y, Wei W. Post-translational regulations of PD-L1/PD-1: mechanisms and opportunities for combined immunotherapy. *Semin Cancer Biol*. 2021;85:246–52. https://doi.org/10.1016/j.semcancer.2021.04.002.
[25] Pilon-Thomas S, Mackay A, Vohra N, Mulé JJ. Blockade of PD-L1 enhances the therapeutic efficacy of combination immunotherapy against melanoma. *J Immunol*. 2010;184(7):3442–9. https://doi.org/10.4049/jimmunol.0904114.
[26] Zhang F, Wang SS. Narrative review on advancing breast cancer treatment: harnessing the power of PD-1/PD-L1 inhibitors for improved patient outcomes. *Front Oncol*. 2023;13:1–12.
[27] Guo Y, Wang Z, Zhou H, Pan H, Han W, Deng Y, et al. First-in-human study of GFH018, a small molecule inhibitor of transforming growth factor-β receptor I, in patients with advanced solid tumors. *BMC Cancer*. 2024;24:444. https://doi.org/10.1186/s12885-024-12121-4.
[28] Dong E, Yue XZ, Shui L, Liu BR, Li QQ, Yang Y, et al. IFN-γ surmounts PD-L1/PD-1 inhibition to CAR-T cell therapy by upregulating ICAM-1 on tumor cells. *Signal Transduct Target Ther*. 2021;6:20. https://doi.org/10.1038/s41392-020-00452-5.
[29] Annunziato S, Kas SM, Nethe M, Yücel H, Del Bravo J, Pritchard C, et al. Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. *Genes Dev*. 2016;30(12):1470–80. https://doi.org/10.1101/gad.279190.116.
[30] Chow RD, Chen S. Cancer CRISPR screens in vivo. *Trends Cancer*. 2018;4(5):349–58. https://doi.org/10.1016/j.trecan.2018.03.002.
[31] Rosenblum D, Gutkin A, Kedmi R, Ramishetti S, Veiga N, Jacobi AM, et al. CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy. *Sci Adv*. 2020;6(47):eabc9450. https://doi.org/10.1126/sciadv.abc9450.
[32] Wang Y, Shelton SE, Kastrunes G, Barbie DA, Freeman GJ, Marasco WA. Preclinical models for development of immune-oncology therapies. *Immuno-Oncol Insights*. 2022;3(8):379–98. https://doi.org/10.18609/ioi.2022.41.
[33] Allison KH, Hammond MEH, Dowsett M, McKernin SE, Carey LA, Fitzgibbons PL, et al. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. *J Clin Oncol*. 2020;38(12):1346–66. https://doi.org/10.1200/JCO.19.02309.
[34] Sreevalsan S, Döring M, Paszkowski-Rogacz M, Brux M, et al. MLLT6 maintains PD-L1 expression and mediates tumor immune resistance. *EMBO Rep*. 2020;21(12):e51397.

https://doi.org/10.15252/embr.202051397.
[35] Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov*. 2018;8(9):1069–86. https://doi.org/10.1158/2159-8290.CD-18-0367.
[36] Zhao Z, Shi L, Zhang W, Han J, Zhang S, Fu Z, et al. CRISPR knockout of programmed cell death protein 1 enhances anti-tumor activity of cytotoxic T lymphocytes. *Oncotarget*. 2017;9(4):5208–15. https://doi.org/10.18632/oncotarget.23730.
[37] Zhang B. CRISPR/Cas9: a robust genome-editing tool with versatile functions and endless application. *Int J Mol Sci*. 2020;21(14):5111. https://doi.org/10.3390/ijms21145111.
[38] Chen S, Sun H, Miao K, Deng CX. CRISPR-Cas9: from genome editing to cancer research. *Int J Biol Sci*. 2016;12(12):1427–36. https://doi.org/10.7150/ijbs.17421.
[39] Lu Y, Xue J, Deng T, Zhou X, Yu K, Huang M, et al. A phase I trial of PD-1 deficient engineered T cells with CRISPR/Cas9 in patients with advanced non-small cell lung cancer. *J Clin Oncol*. 2018;36(15_suppl):3050. https://doi.org/10.1200/JCO.2018.36.15_suppl.3050.
[40] Le Naour A, Rossary A, Vasson MP. EO771: is it a well-characterized cell line for mouse mammary cancer model? Limit and uncertainty. *Cancer Med*. 2020;9(21):8074–85. https://doi.org/10.1002/cam4.3295.
[41] Karn T. High-throughput gene expression and mutation profiling: current methods and future perspectives. *Breast Care (Basel)*. 2013;8(6):401–6. https://doi.org/10.1159/000357461.
[42] He C, Han S, Chang Y, Wu M, Zhao Y, Chen C, et al. CRISPR screen in cancer: status quo and future perspectives. *Am J Cancer Res*. 2021;11(4):1031–50.
[43] Laurent M, Geoffroy M, Pavani G, Guiraud S. CRISPR-based gene therapies: from preclinical to clinical treatments. *Cells*. 2024;13(10):800. https://doi.org/10.3390/cells13100800.
[44] Mout R, Ray M, Lee YW, Scaletti F, Rotello VM. In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and challenges. *Bioconjug Chem*. 2017;28(4):880–4. https://doi.org/10.1021/acs.bioconjchem.7b00057.
[45] Davidson AR, Lu WT, Stanley SY, Wang J, Mejdani M, Trost CN, et al. Anti-CRISPRs: protein inhibitors of CRISPR-Cas systems. *Annu Rev Biochem*. 2020;89:309–32. https://doi.org/10.1146/annurev-biochem-011420-111224.
[46] Zheng Y, Li S, Tang H, Meng X, Zheng Q. Molecular mechanisms of immunotherapy resistance in triple-negative breast cancer. *Front Immunol*. 2023;14:1153990. https://doi.org/10.3389/fimmu.2023.1153990.
[47] Flörcken A, Kopp J, van Lessen A, Movassaghi K, Takvorian A, Jöhrens K, et al. Allogeneic partially HLA-matched dendritic cells pulsed with autologous tumor cell lysate as a vaccine in metastatic renal cell cancer: a clinical phase I/II study. *Hum Vaccin Immunother*. 2013;9(6):1217–27. https://doi.org/10.4161/hv.24149.

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