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Immunotherapy using CRISPR-Cas9 systems to treat rheumatoid arthritis with PTPN22 R620W mutations and target PD-1 and CD20

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Abstract
Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that causes joint pain and inflammation. Current treatments for RA leave patients immunocompromised and may have harmful side effects. As part of the mechanism behind RA, overactive T cell signaling to B cells contributes to inflammation. Specific genes, such as *PTPN22*, can play a role in overactive T cell signaling in RA patients' immune systems. The protein encoded by tyrosine phosphatase non-receptor 22 (*PTPN22*), is lymphoid tyrosine phosphatase (LYP), which performs regulatory activities for both T and B cells. In particular, the R620W variant of this protein is associated with RA and may increase the overall sensitivity of an individual's immune system. This review will discuss the mechanisms of CRISPR/Cas9 to disrupt or express specific genes to combat inflammation in RA patients. First, CRISPR/Cas9 can be used to disrupt *PTPN22* in T cells to restore homeostasis. Second, CRISPR/Cas9 can be used to knock in *PDCD1*, which encodes for the PD-1 protein that inhibits cytokine production and T cell proliferation, which may downregulate T cells. T cells play a role in RA by producing proinflammatory cytokines and interacting with synovial fibroblasts and macrophages. Third, CRISPR/Cas9 can be used to target *MS4A1*, the gene that encodes for CD20 on B cells, in order to deplete B cells without affecting T cells. Using CRISPR/Cas9 to either knock out *PTPN22* in T cells or knock in *PDCD1* in T cells and *MS4A1* in B cells could potentially treat and even reverse the symptoms of rheumatoid arthritis for patients affected by the R620W variant of the PTPN22 protein.

Keywords
immunotherapy, rheumatoid arthritis, *PTPN22*, T cells, B cells, CRISPR/Cas9, PDCD1, PD-1, MS4A1, CD20

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Introduction

Characterized by painful swollen joints, rheumatoid arthritis (RA) can cause bone erosion and joint deformity in the long term (1). RA affects ~1% of the population with an estimated 60% heritability (2), (3). Around 1.5 million Americans have been diagnosed with RA, with women being two to three times more likely to get RA than men (4). Various hormones such as estrogen and progesterone play a significant role in this pattern (4). In addition to affecting the quality of life, rheumatoid arthritis is accompanied by a 60% increased risk of a heart attack. The disease can also affect the lungs, heart, vascular system, eyes, skin, and blood (4). RA can be caused by a multitude of factors including environmental, lifestyle, and hereditary (5). Environmental and lifestyle-related factors include smoking, alcohol intake, obesity, and living area (6). The human leukocyte antigen (HLA) genes, part of the major histocompatibility complex (MHC), account for 11-37% of heritability for RA (5). This class of genes encodes proteins on the surface of T cells and is an important part of the immune system (7). Additional genetic factors associated with rheumatoid arthritis include single nucleotide polymorphisms (SNPs) found in genes such as PTPN22 (5). A missense SNP of PTPN22 is present in ~28% of RA patients with Caucasian heritage (3).

Current treatments for RA include nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease modifying anti-rheumatic drugs (DMARDs). NSAIDs and corticosteroids temporarily relieve pain and reduce inflammation but are intended for short-term use. DMARDs function long term to suppress a patient's overactive immune system but are accompanied by various adverse side effects (8). For example, the DMARD called rituximab depletes B cells but also significantly depletes T cells. Although this treatment is effective in suppressing the symptoms of RA thereby stopping the disease's progression, it comes with considerable side effects including high financial cost and immunosuppression (6).

Immunosuppression leaves patients susceptible to viral infections such as Covid-19, which is particularly relevant given that infections in general account for a quarter of deaths in people with RA (4). Recently, in 2021, a small molecule inhibitor of PTPN22 called L-1 was generated (9). The study by Ho et al suggests that this therapy augments anticancer immunity; however, this therapy has not been clinically validated for RA, and was created for the purpose of cancer immunotherapy. This therapy is also not currently commercially available. Additional therapies for rheumatoid arthritis include the biologic drug sarilumab, which blocks the production of interleukin-6 (IL-6), a cytokine that plays a critical role in inflammation (10). A study by Burmester et al compares sarilumab monotherapy and adalimumab monotherapy to demonstrate that sarilumab improves physical function and symptoms better than adalimumab. Clearly, there is a need for new therapies for RA. This review discusses CRISPR/Cas9 as a rheumatoid arthritis treatment option that may be long term while avoiding various complications.

CRISPR/Cas9 was discovered as a genome editing tool by Jennifer Doudna and Emmanuel

Charpentier in 2012 (11). CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. When harnessed for gene editing, it allows researchers to alter DNA sequences and, consequently, gene function (11). Cas9 is an endonuclease from Streptococcus pyogenes which binds to DNA, cuts the phosphate backbone, and allows the natural error-prone repair process of non-homologous end joining (NHEJ) to occur, essentially knocking out the targeted gene. In NHEJ, the break ends are directly ligated without the need of a homologous template, resulting in efficient and quick gene editing (12). Guide RNA recognizes the target DNA region and binds with Cas9 to direct the nuclease to a specific location in the genome. The Protospacer Adjacent Motif (PAM), a short DNA sequence of 2 to 6 base pairs, is also needed for Cas9 to bind to a correct sequence (13). Alternatively, Cas9 can activate expression of a gene, requiring HDR with a template to guide repair in a process that is more regulated and inefficient (12). Currently, CRISPR/Cas9 is being tested on animal models to increase genome editing specificity and reduce off-target effects (14). Recently, CRISPR was used for immunotherapy to treat patients with refractory cancer (15). It has numerous uses ranging from cancer treatments and immunotherapy to modifying fruits and vegetables for nutritious purposes.

Protein tyrosine phosphatase non-receptor 22 (PTPN22) encodes protein lymphoid tyrosine phosphatase (LYP), which is involved in regulatory activities for B cells and T cells (16). The protein functions as a negative regulator for T cell activation, but when mutated, the function of the protein may change (16). Specifically, the missense mutation where cytosine is switched for thymine C1858T, results in the amino acid changing from arginine to tryptophan. This R620W polymorphism of PTPN22 protein is a risk allele for rheumatoid arthritis (17), (18), (19). The polymorphism reduces LYP binding with CSK, affecting signaling of T cell receptors (TCRs) and could explain the alterations to the immune system the variant brings (20). Mutations of PTPN22 bring increased production of auto-reactive B cells, as observed in a previous study which reported a 3.4-fold increase in autoreactive B cells in peripheral blood in RA patients compared to the control group (21), (22). RA patients could display enhanced T cell activation when PTPN22 R620W acts as a loss of function mutation in the context of FcyR signaling, as in dendritic cells there is a lack of regulation of immune complex signaling (23). The study by Perry indicates that T cell proliferation is significantly higher with the R620W mutation of the PTPN22 protein than WT (24). There are reports on both gain-of-function and loss-of-function effects on TCR signaling. This review will focus on the gain-of-function effect on TCR signaling. The increased activity of T cells and B cells in the immune system leads to inflammation in the joints. Interactions between T cells, B cells, pro-inflammatory cytokines, and synovial fibroblasts, the main stromal cells of the joint synovium found in the synovial lining, contribute to inflammation in RA patients (25). The Cas9 endonuclease knicks the DNA backbone in the gene to allow NHEJ to repair the DNA strand, leading to insertions and deletions (indels) in the PTPN22 gene, ultimately deleting the gene

from patients' genome. Patients would not need to take DMARDS or other treatments as the knockout of the PTPN22 gene may augment immunosuppression as T cells are downregulated. However, the symptoms of RA might be alleviated. This review discusses the use of CRISPR/Cas9 to knock out the variant PTPN22 to decrease the reactivity of the immune system and thus decrease overall inflammation in rheumatoid arthritis.

A strategy for treating rheumatoid arthritis is using CRISPR/Cas9 in the context of immunotherapy. Current immunotherapies suppress various aspects of the immune system; CRISPR/Cas9 can target specific aspects without directly interfering with the entire immune system. Helper T cells play a crucial role in adaptive immunity and help activate B cells to produce antibodies (26). T cells, specifically CD4+ T helper cells, secrete cytokines. Pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, and IL-17 stimulate inflammation (27). Abnormally high levels of TNF, which play major roles in cellular survival, proliferation, differentiation, and death, are found in autoimmune inflammatory diseases such as rheumatoid arthritis (28). TNF-alpha inhibitor therapy, such as the monoclonal antibody adalimumab, can be used for the treatment of RA (10). T cells play a role in the pathogenesis of rheumatoid arthritis and subsequently, depletion of T cells or an anti-CD4+ monoclonal antibody is an effective treatment for inflammation in RA patients (29).

Furthermore, B cells can be targeted with CRISPR/Cas9 systems as a treatment for rheumatoid arthritis. B cells play an integral role in adaptive immunity as the source of rheumatoid factors and anti-citrullinated protein antibodies (ACPAs) (30). Rheumatoid factors are autoantibodies against the Fc region of immunoglobulin G (IgG), which result in the immune system attacking healthy cells (31). Rheumatoid factors are found in 80% of RA patients (30). A study by Matsumoto et al in 2002 demonstrates that the infusion of IgG autoantibodies to glucose-6-phosphate isomerase (GPI) led to the rapid development of synovitis in mice in a KRN/NOD model (32). Synovitis is present in rheumatoid arthritis and is the inflammation of the synovium, the membrane that lines joints. B cells assume a central role in the pathogenesis of rheumatoid arthritis and, subsequently, the depletion could be an advantageous aspect in RA therapy. Current commercially available therapies specifically target markers such as CD19, CD20, and CD22 on the surface of B cells. Current therapies such as rituximab and ocrelizumab target CD20, while epratuzumab targets CD22 (33). These therapies have side effects including fever, dizziness, chills, and heartburn. CD20, encoded by the gene MS4A1, has been shown to be highly expressed on the surface of B cells (34). CD20 plays a role in B cell activation, differentiation, and cell cycle progression from G1 to the S phase (34). Depletion of B cells can be implemented to treat rheumatoid arthritis.

Immunotherapy: Targeting PTPN22 and PDCD1 in T cells

T cells play various roles in the pathogenesis of rheumatoid arthritis as seen through the

interactions between T cells, B cells, pro-inflammatory cytokines, and synovial fibroblasts (35). Synovial fibroblasts were found to be activated by a diverse range of cytokines and in turn, promote inflammation and joint destruction in rheumatoid arthritis (25). A study by Krzesicki examines the adhesion of peripheral T cells to synovial fibroblasts stimulated with pro-inflammatory cytokines such as TNF. The study indicates synovial hyperplasia is profoundly affected by pro-inflammatory cytokines, which act by controlling the T cells through the upregulation of adhesion molecules on synovial fibroblasts. Further activation occurs during contact between T cells and fibroblast-like synoviocytes, and consecutively produces various cytokines including TNF (36), (37). Enhanced activation of T cells leads to increased interactions between synovial fibroblasts and production of proinflammatory cytokines, subsequently triggering a cycle of inflammation in rheumatoid arthritis patients. Moreover, B cells contribute to the inflammatory signaling cascade in synovial joints (38). The R620W variant of PTPN22 protein leads to enhanced T and B cell function and overall reactivity of the immune system and has shown to be a risk allele associated with rheumatoid arthritis (4), (18), (21), (23). Due to the pro-inflammatory effects on T cells from the SNP in PTPN22, depletion of the gene may be key to halt the inflammatory signaling cascade (39). A study by Anderson et al in 2019 examines the effects of the disruption of PTPN22 in primary human CD4+ T cells. The study concluded that PTPN22-deficient human CD4+ T cells display a decreased response to TCR stimulation. Moreover, CRISPR/Cas9 knock-out of PTPN22 in T cells increases expression of PD-1 (40). Programmed cell death protein 1 (PD-1; PDCD1) plays a major role in inhibiting cytokine production and T cell proliferation and subsequently, additional copies of PD-1 will diminish T cell populations (41), (42). PD-1 is a protein expressed on the surface of various immune cells, including T cells, encoded by the gene PDCD1, which acts as an immune checkpoint receptor (41), (42). PD-1 regulates the action and function of T cells during development, activation, and effector phases (42). In patients with overactive T cells, one can use CRISPR/Cas9 to target PD-1. Knocking in PDCD1 in T cells, using CRISPR/Cas9 systems, may downregulate the production of T cells. This process may restore T cell homeostasis, decreasing inflammation in rheumatoid arthritis patients without leaving them totally immunosuppressed. Both methods of knocking out PTPN22 and knocking in PDCD1 may result in a downregulation of T cells which in turn decreases inflammation in rheumatoid arthritis patients.

Immunotherapy: B cells and anti-CD4+

The overexpression of B and T cells plays several critical roles in the pathogenesis of rheumatoid arthritis (30), (43), (44). Many treatments are geared towards depleting these immune cells to improve a patient's RA condition. For example, the monoclonal antibody rituximab targets CD20 to deplete B cells in rheumatoid arthritis patients (34). The open study by Edwards and Cambridge investigated the effects of rituximab in five patients with refractory rheumatoid arthritis. The patients received standard doses for 12-17 months of rituximab cooperatively with doses of cyclophosphamide and oral prednisolone. Three

patients demonstrated a 70% response to the treatment while the other patients had a 50% response at 26 weeks. The authors found that rheumatoid factor levels declined to normal levels while immunoglobulin levels declined marginally. Importantly, peripheral blood B cells were undetectable in all patients after the infusion with cell numbers remaining low for 6 months and patients demonstrated significant synovitis reduction. The findings from this study suggest that B cell depletion is an effective therapy for rheumatoid arthritis. In addition to the depletion of B cells, rituximab induces T cell depletion in RA patients (43). A study examines peripheral blood cells, including various T cell populations, NK cells and B cells before and during the first course of rituximab. Specifically, CD4+ T cells were strongly affected, showing at week 12 the average percentage of the cell population decline was 37%. However, this decrease in T cells occurs much later than the depletion of B cells. In contrast, at week 2, total B cell depletion was demonstrated. The CD4+ T cell decline of 80% during the overall rituximab treatments portray that rituximab not only depletes B cells but also T cells over time. B and T cell roles in the pathogenesis of rheumatoid arthritis may be closely associated because of the functions of B cells, including as B cell antigen presenting cells (APCs) abilities or cytokine secretion capabilities (43). Furthermore, T cell activation is mediated by B cells (22). Previous studies have shown that targeting CD4+ T cells can improve inflammation and symptoms of RA. A study by Choy et al examined 24 rheumatoid arthritis patients to determine the effects of a humanized IgGI anti-CD4 monoclonal antibody, 4162W94. The treatment resulted in clinical improvement of rheumatoid arthritis, displaying anti-CD4 antibodies as a possible treatment for inflammation in RA. Additionally, Abatacept, a CTLA-4-Fc protein that inhibits CD4+ activation by APCs has been approved for treatment (43). Treatment with abatacept led to improved rheumatoid arthritis condition (45). In tandem, these studies demonstrate the viability of T cell and B cell depletion as a successful treatment for rheumatoid arthritis.

Treatment Delivery

A cost effective, accessible treatment for rheumatoid arthritis could be to deliver CRISPR/Cas9 systems in peripheral blood to specifically target T and B cells. Possible future delivery methods may be able to utilize nanoparticles as an efficient and safe vehicle to deliver CRISPR components into human cells. However, current research by Carl June's lab details a strategy for isolating immune cells and conducting CRISPR/Cas9 editing ex vivo in a small clinical trial (46). In addition, viral vectors can be implemented as a delivery vehicle for gene editing. A study by Blaese et al implemented retroviral-mediated delivery to knock in gene ADA in T cells to combat severe combined immunodeficiency (SCID). After treatment ended, expression of ADA persisted in T cells showing viral vectors as a suitable delivery method (47). Although viral vectors may provide successful results, they are accompanied by drawbacks such as low insert capacity and random insertion disrupting normal genes (48). Using the isolation of immune cells method, the PTPN22 gene can be more efficiently targeted, when a patient has the C1858T variant, in T cells to diminish expression.

Moreover, CRISPR/Cas9 could knock-in additional copies of the PDCD1 gene which would down-regulate T cell activity (42). CRISPR/Cas9 can knockout MS4A1, the gene that encodes CD20 in humans, to deplete B cells (49). Since, CD20 plays a role in B cell activation, differentiation, and cell cycle progression from G1 to the S phase, deletion of the gene that encodes for CD20 may hinder the activity of B cells (34). Once the immune cell is isolated and CRISPR mechanisms are triggered, PTPN22 is depleted or PDCD1 is expressed in T cells and MS4A1 is knocked out in B cells, respectively. The process of NHEJ will repair the B cell DNA as the gene was knocked out and will work similarly to knockout PTPN22 in T cells. In contrast, the more complicated process of HDR will repair the T cell DNA as the PDCD1 gene will be expressed. Using CRISPR/Cas9 to target specific genes in immune cells allows for cost-effective and efficient modification of an individual's genome to treat rheumatoid arthritis.

Discussion

The C1858T variant of PTPN22 has been documented to be a genetic risk factor for rheumatoid arthritis in different ethnicities. PTPN22 is not associated as a risk factor for Japanese and African populations but has been consistently shown as a risk allele for patients with North European ancestry (50), (51). The meta-analysis study by Nabi and colleagues in 2016 examined rheumatoid arthritis patients of different ethnicities to determine if the polymorphism is a risk allele. The variant had no statistical significance or risk for rheumatoid arthritis in Asian patients. However, rheumatoid arthritis susceptibility is associated with the C1858T variant in Caucasian populations (52). The CRISPR treatments detailed in this review based on the C1858T polymorphism may have a more significant effect on rheumatoid arthritis patients with North European ancestry. Creating this treatment may be costly as the addressable market is small (only 5-10% of the North American population has the R620W mutation), but the benefit is personalized medicine, where treatments are tailored to individual patients. In this case, the treatments detailed in this review would likely benefit the North American populations with the R620W mutation in the PTPN22 protein.

There have been opposing results on the role of the mutation of PTPN22 in immunity. The study by Orozco suggests that R620W mutation in PTPN22 might be beneficial in immunity (53). The mutation may promote antiviral and anti-tumor immune responses, resulting in a lower likelihood of cancer. In mice studies, mice expressing the mutation have less tumor burden and clear viral infections faster than its WT counterpart. According to this study, the knockout of the PTPN22 mutation and knockin of PDCD1, as this review suggests, may lead to increased vulnerability to infectious disease and susceptibility to carcinogenesis, due to the antitumor effect the variant brings. Conversely, the study by Maine et al specifically portrays the PTPN22 knockout mice showing improved clearance of LCMV cl13 infection compared to WT cells (54). In addition, there have been conflicting results documented on the function of PTPN22 variants, but the differences might not relate to PTPN22 activity (55). It may be related to

interactions between proteins or splice variations. In mice studies, the protein variant R620W appears to be loss-of-function mutation on TCR signaling. For example, the study by Knipper et al found that PTPN22-deficient mutant murine T cells resulted in higher lymphopenia-induced proliferation than WT cells, demonstrating mice studies showing a loss of function mutation (56). In contrast, human studies have demonstrated a gain-of-function mutation on TCR signaling (55). This review highlights the gain-of-function effect on TCR signaling and how to potentially treat rheumatoid arthritis patients with this variant. The variant increases T and B cell function and the overall reactivity of the immune system (18), (22), (23). Overactive T cells result in a signaling cascade in synovial joints which leads to inflammation in rheumatoid arthritis patients. The inflammatory signaling cascade consists of synovial fibroblasts, pro-inflammatory cytokines, B cells, macrophages and other cells stimulated by T cells (40). To treat inflammation in patients with the R620W variant, CRISPR/Cas 9 can be implemented to restore T cell homeostasis. CRISPR knockout of PTPN22 in T cells results in increased expression of PD-1 (40). In addition, knocking in PDCD1 may downregulate T cells and restore homeostasis in patients with the R620W variant and overactive T cells. An additional immunotherapy strategy to treat rheumatoid arthritis is utilizing CRISPR to target B cells. Targeting the CD20 marker on B cells has been documented to decrease B cell activity in rheumatoid arthritis. This is seen through the monoclonal antibody treatment called rituximab (57). Utilizing CRISPR to target the CD20 marker may produce similar results in a cost-effective and simple manner. B and T cell roles are interconnected in the pathogenesis of rheumatoid arthritis (44). For rheumatoid arthritis patients with the R620W variant, using CRISPR to knockout PTPN22 or knock in PDCD1 may return T cell homeostasis, reducing inflammation in synovial joints. Likewise, implementing CRISPR/Cas9 to target MS4A1 may deplete B cell numbers and treat inflammation in rheumatoid arthritis patients. These approaches would likely require an ex vivo delivery method, where one isolates patient T cells and re-infuses the CRISPR engineered T cells. CRISPR treatments could be delivered as often as twice per year due to the discovery that diminished T and B cells after treatment have been documented to remain in peripheral blood for around 6 months (58). The treatments would target mature B or T cells, since both lineages arise from the bone marrow hematopoietic stem cells. Gene editing in such precursor cells could have other negative off-target effects.

Future directions concerning RA research could specifically target the arthritic joints, cartilage and synovium. A possible delivery method would consist of an approach termed as selective organ targeting (SORT). This method uses various lipid particles engineered to edit tissue with a supplemental SORT molecule (58). These modified nanoparticles can be used to target specific organs or tissues and therapeutically relevant cells with CRISPR/Cas9 systems (59). The lipid nanoparticles (LNPs) are small enough to travel through the bloodstream and enter cells (59). Each SORT LNP has specific proteins that help identify and deliver to specific tissues.

Treatment of rheumatoid arthritis would have SORT targeting cartilage as the disease affects primarily the joints. The LNPs will carry the gene editing cargo, Cas9 mRNA and target-specific guide RNA that become activated inside the cell (59). This process would be happening in vivo as the SORT molecule and modified lipid nanoparticle would be injected into a patient. The 2018 study conducted by Wang examined LNP delivering siRNA as a cartilage therapeutic tool in a rat model of surgery-induced osteoarthritis. Osteoarthritis affects the joints; however, it is a degenerative joint condition as opposed to an autoimmune condition. The data indicated LNPs can deliver siRNA into the cytoplasm of chondrocytes with almost a 100% transfection rate. The study indicates that LNPs cannot function in the synovial membrane of the rat model and is a tool that can specifically be used for cartilage (60). As rheumatoid arthritis affects the cartilage in cases of chronic inflammation, this method can be used in severe cases of the disease. This delivery method could substantially improve a RA patient's condition as it can specifically target the immune cells or even affected tissue. This future work could direct research focus from the immune cells to the joints and cartilage itself.

The C1858T variant of PTPN22, encoding for the R620W mutation in the PTPN22 protein, enhances T and B cell expression in rheumatoid arthritis patients. The inflammatory signaling cascade stimulated by T cells could be halted through the downregulation of T cells. Likewise, B cell interactions in synovial joints characterizes rheumatoid arthritis inflammation. The immunotherapy strategy of implementing CRISPR/Cas9 systems to target PTPN22 and PDCD1 to decrease T cells and MS4A1 to decrease B cells may be a cost-effective, accessible treatment to decrease inflammation in rheumatoid arthritis patients with the C1858T variant of North European ancestry.

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