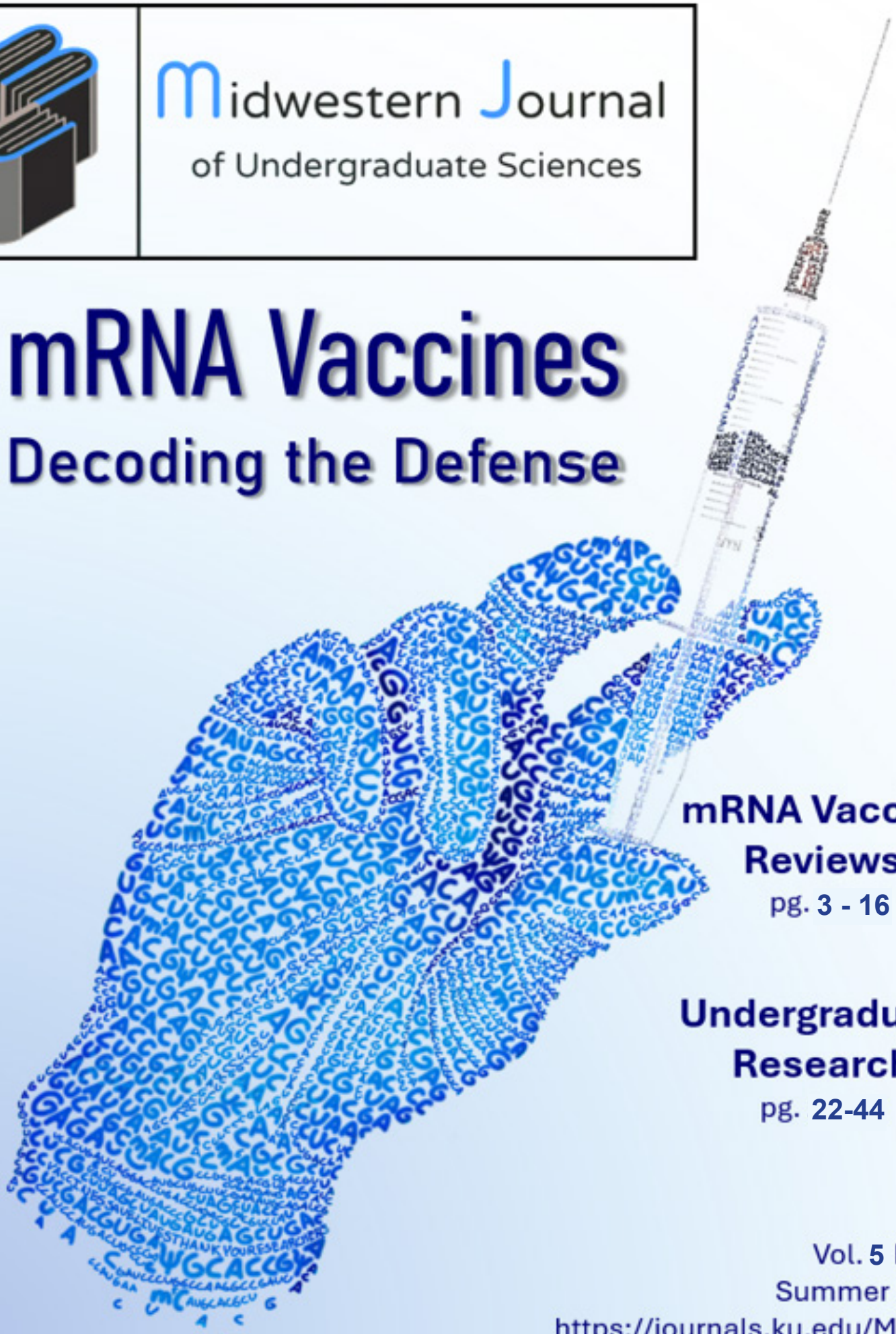
Midwestern Journal of Undergraduate Sciences

mRNA Vaccines

Decoding the Defense



mRNA Vaccine Reviews
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Undergraduate Research
pg. 22-44

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To The Reader,

I am pleased to present this issue of the Midwestern Journal of Undergraduate Sciences. The journal was founded to provide undergraduate researchers with a meaningful venue for work that might not otherwise find a place in the scientific literature, including independent and capstone projects completed outside large research-laboratory settings.

Our annual publication welcomes original research from undergraduate scientists across the natural sciences. Although many submissions arise from senior capstone experiences, the journal also seeks to highlight exceptional work from community-college students and selected master's students conducting independent projects that are not part of a larger laboratory publication.

Each issue also includes student-written mini-reviews that place recent primary research within the broader development of a scientific field. These reviews are organized around a common theme, allowing readers to follow the progression of ideas and discoveries across the issue.

In addition, the journal features classroom-based projects designed for undergraduate science education. These articles provide adaptable models for other instructors, along with examples of student work, reflections on implementation, and evidence of progress toward learning objectives.

We invite students and faculty mentors to consider the Midwestern Journal of Undergraduate Sciences as a home for thoughtful, carefully developed scientific work. Please consult the author guidelines for information about article scope, length, formatting, and submission requirements.

—J. F. Treml



Contributors

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J.F. Treml

Editors

R. Logan
B. Mattingly

Production Editors

E. Messenger
C. Gray
H. Cleveland
E. Cochrane

Creative

P. Kramer
S. Kramer

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A Briefing on the Evolution of Nucleic Acid Vaccines

By JF Treml

The University of Kansas Biotechnology program emphasizes the importance of teaching students to critically evaluate and clearly communicate complex scientific research. Each year, students work in small groups to interpret and present primary research articles arranged chronologically around a common theme. This format allows the class to trace how a field develops over time while giving students repeated practice identifying the key questions, evidence, and implications hidden within complex studies.

This year, we examined the development of nucleic acid vaccines, from early studies of genetic immunization to recent advances in self-amplifying RNA, lipid nanoparticle design, antigen engineering, personalized cancer vaccines, and tolerogenic mRNA therapies. Students followed the field's scientific trail, examining how each new discovery refined the strategy, delivery, and control of mRNA-based medicine. For their final project, students selected a recent publication related to the course theme and prepared a review explaining how the study fits into the larger scientific dossier. The following reviews represent exemplary work completed by students in the course.

The Replication Directive: A Briefing on the Rise of Self-Amplifying RNA Vaccines

By Yenny Feng* and JF Treml*†

Vaccines are the quintessential form of immunotherapy, with mRNA vaccines evolving into the dominant form of new products over the past four decades. Despite their success, a major challenge remains achieving long-term stability. Even though individual vaccine doses are relatively small, large-scale outbreaks, such as the recent SARS-CoV-2 pandemic, led to limitations in global distribution. The number of doses that could be transported and stored simultaneously at low temperature was insufficient compared to the extent of demand. In their 2025 Nature article, Voigt et al. investigated a novel strategy to improve RNA vaccine durability by engineering a self-amplifying RNA (saRNA) platform designed to maintain stability at room temperature, offering a promising path towards more scalable and resilient COVID-19 vaccination. [1]

Voigt et al developed a saRNA vaccine targeting SARS-CoV-2 that induces a strong immune response in mice. This vaccine is formulated with a nanostructured lipid carrier (NLC), making it thermostable and more effective. Researchers showed that the vaccine not only produced cell-mediated immunity, but also established long-lived antibody-secreting plasma cells. Although the study only demonstrated effectiveness in mice, Voigt *et al*. believe it is an important advance in RNA vaccine technology. Lower dose and thermostability are particularly important for low-income areas, where resources are scarse. [1] This approach is important not only for the SARS-CoV-2 pandemic but also for future outbreaks or pandemics.

Across decades, nucleic acid vaccines have undergone continuous enhancement, from DNA encoding proteins to lipid-encapsulated mRNA products.[2] Early in vivo studies showed that injecting DNA expression vectors encoding enzymes such as chloramphenicol acetyltransferase, luciferase, and β-galactosidase into mouse muscle resulted in gene expression without any viral vector (reviewed by Wolff *et al*.)[2] Building on this, plasmid DNA encoding influenza hemagglutinin (HA) was shown to enter host cells, replicate, drive HA antigen expression, and induce strong humoral responses.[3] Parallel work by Tang *et al*. showed that particle bombardment (gene gun) delivery in skin cells produces clustered reporter gene expression and higher antibody titers with less DNA, highlighting the importance of delivery methods.[4] Extending this work beyond influenza, scientists explored gene inoculation of Human Immunodeficiency Virus (HIV) envelope DNA in mice, demonstrating that DNA vaccination can generate both humoral and cellular immunity against non-influenza pathogens.[5]

In addition to DNA vaccines, mRNA vaccines were also explored. Hoerr *et al*., investigated the efficiency of RNA-based vaccines encoding for β-galactosidase (β-gal). Results showed that unmodified naked and protamine-encapsulated mRNA could induce antigen-specific CTL and antibody responses across multiple mouse haplotypes (H-2b, H-2d, and H-2k).[6] In contrast, Martinon *et al*. demonstrated that liposome-encapsulated mRNA encoding viral NP could drive strong CTL responses and antigen expression in vivo; however, this was tested in a single mouse haplotype (H-2d).[7] Later studies validated the effectiveness of sequence-optimized mRNA vaccines encoding the HA influenza virus, showing high neutralizing antibody titers and complete protection in mouse challenge models.[8]

Kariko et al. noticed that the incorporation of modified nucleosides such as pseudouridine, which are present in mammalian RNA but not in prokaryotic nucleic acids, resulted in reduced TLR-mediated cyto-

kine production and prevented innate immune overactivation.[9] A second challenge that arose was the strong immune activation triggered at the injection site, which limited tolerability. To address this, Hassett et al. evaluated different lipid nanoparticle (LNP) chemistries, confirming that LNP composition is the key determinant of both potency and tolerability.[10]

Hassett *et al.*'s findings were timely with the advent of the SARS-CoV-2 pandemic. Nucleoside-modified mRNA encapsulated in LNPs generated strong immunity against SARS-CoV-2 after a single immunization.[11] This approach also increased in vivo expression while minimizing innate immune activation, establishing that chemical modification was essential for achieving antigen production without excessive inflammation.[12] The potency and immunogenicity were determined by the structure of the ionizable lipid within LNPs, revealing that small chemical changes in lipid design produce large-scale differences in expression, cytokine induction, and tolerability.[13] Recently, Voigt *et al.* introduced a COVID-19 saRNA vaccine. The major advance of this study was the development of a thermostable saRNA vaccine formulated with a nanostructured lipid carrier (NLC).[1] Unlike conventional LNP-mRNA vaccines, which require ultra-cold storage, this platform maintains long-term stability at room temperature (**Fig. 1B**), addressing one of the most persistent barriers to global vaccine distribution.[14] The authors showed that lyophilized saRNA-NLC remains chemically intact and biologically potent for at least six months at room temperature, and even longer under refrigerated conditions.[1] This represents an essential improvement over current mRNA vaccines, which rapidly lose activity outside cold-chain conditions.

saRNA vaccines induce strong neutralizing antibody responses and Th1 cellular responses, demonstrating that thermostability does not compromise immunogenicity.[1] This design enabled high antigen expression at extremely low doses, allowing the RNA to replicate within the cell (**Fig. 1A**).1 This effect is vital for a next pandemic response, where manufacturing capacity and cold storage can limit global access. The

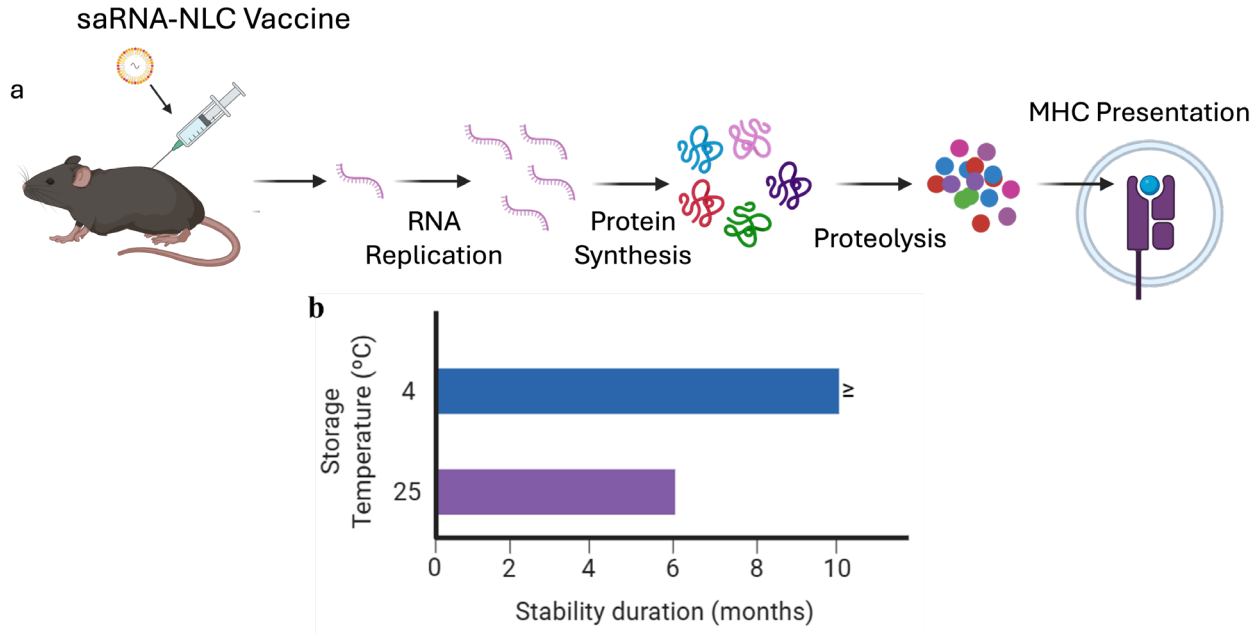


Figure 1 | Mechanism and stability of a self-amplifying RNA (saRNA) vaccine formulated with a nanostructured lipid carrier. (a) Illustration of low-dose saRNA-NLC vaccine initiating immune activation. After intramuscular injection, the saRNA enters host cells where it undergoes intracellular RNA replication, producing multiple copies of the antigen-encoding RNA. The amplified RNA translates into the viral spike protein. (b) Long-term stability of the saRNA-NLC formulation. Created in BioRender. Feng, Y & Treml, J. (2026) https://BioRender.com/b6w5qjk

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study also showed cross-variant neutralization, with measurable activity against multiple SARS/CoV/2 variants, such as alpha, beta, and delta strains, suggesting that the platform remains functional even when the virus evolved.[1]

Overall, the work emphasized manufacturability and adaptability. The NLC formulation is simpler to produce than traditional LNPs, making it more suitable for low-resource settings. By combining saRNA, thermostable formulation, and broad immune activation, this study demonstrates a vaccine platform designed not only for potency but also for global accessibility. It represents a shift from focusing only on immunogenicity to addressing real-world challenges that determine whether vaccines can reach the populations that need them most.

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

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A Matter of State: Destabilizing Postfusion Spike to Preserve the Prefusion Form

By Carson Gray* and JF Treml*†

Nucleic acid vaccines possess several advantages over previous approaches to vaccination and have been an object of study for several decades; only recently, however, has a nucleic acid vaccine had large scale, international use, with the development of a vaccine against SARS-CoV-2 in 2020. mRNA vaccines in particular have garnered significant attention due to their rapid antigen production, generally improved immune response, modularity, and potentially improved safety.[1] In contrast to vaccination with purified protein antigens, mRNA vaccines enable endogenous antigen production, more closely recapitulating viral antigen presentation pathways.[2] The SARS-CoV-2 mRNA vaccine has been demonstrated to produce a more robust cellular immune response compared to the recombinant protein vaccine in murine models, consistent with enhanced MHC class I presentation.[3] mRNA vaccines may also be an improvement over viral platforms in certain contexts. Inactivated viruses have similar issues to purified protein, in that there is no endogenous expression of antigenic material; live attenuated vaccines are a more complete system, however they are prone to inflammation and can in rare cases cause an infection. An advantage of mRNA above almost every other system is that it does not require cell-based pipelines or protein purification during production, making it highly scalable via in vitro transcription-based manufacturing.[4]

A major focus in the current mRNA research landscape is optimization, both in delivery systems and the chemical structure of the RNA itself. Lipid nanoparticles are the only clinically validated delivery mechanism for mRNA vaccines, and work has been done to optimize immunogenicity while making the nanoparticles more tolerable.[5] Additionally, mRNA base modifications have historically been observed to be critical for mRNA stability and expression, especially the presence of pseudouridine which reduces innate immune activation and enhances translational efficiency.[6] Synthesizing these findings is important for generating more effective vaccines, and the continued maturation of mRNA vaccination strategies.[7] An interesting manifestation of this is in rational structure optimization, which has the objective of modifying antigen structure to more effectively display relevant epitopes. The spike proteins of many viruses are metastable, undergoing large structural changes to facilitate membrane fusion.[8] The elicitation of neutralizing antibodies often depends on recognition of the pre-fusion conformation as many neutralizing epitopes become distorted or hidden after the spike transitions to its post-fusion conformation, making stabilization of the pre-fusion structure important for vaccine efficacy. To generate a potent neutralizing response, mRNA vaccines therefore do not necessarily code for the native sequence, but rather a modified form that stabilizes the pre-fusion state.

The SARS-CoV-2 spike is a trimeric class I fusion glycoprotein that changes shape during membrane fusion. The Heptad Repeat 2 (HR2) region plays an important role in this process and has therefore become a focus for engineering mutations that stabilize the spike in its pre-fusion conformation. A landmark publication by Pallesen et al. in 2017 involved a rational double mutation, K986P/V987P, situated at the boundary of HR2 and the central helix hinge in the MERS-CoV spike protein. The simple addition of these two prolines stabilizes pre-fusion conformation by restricting conformational rearrangement of the central helix / hinge region.[9] These mutations are included in the SARS-CoV-2 mRNA vaccine, testifying to the clinical relevance of rational structure optimization of antigens.

Recently, Chakraborty *et al*. investigated a new approach to stabilizing the pre-fusion conformation: rather than constricting the helix extension that occurs during the transition process, they destabilized the post-fusion conformation by substituting charged residues in positions that are exposed and buried in the pre-fusion and post-fusion conformations, respectively.[10] Specifically, N969 and I973 were replaced with aspartic acid. This represents a conceptual shift from trapping the pre-fusion state with rigidifying mutations to thermodynamically disfavoring the post-fusion

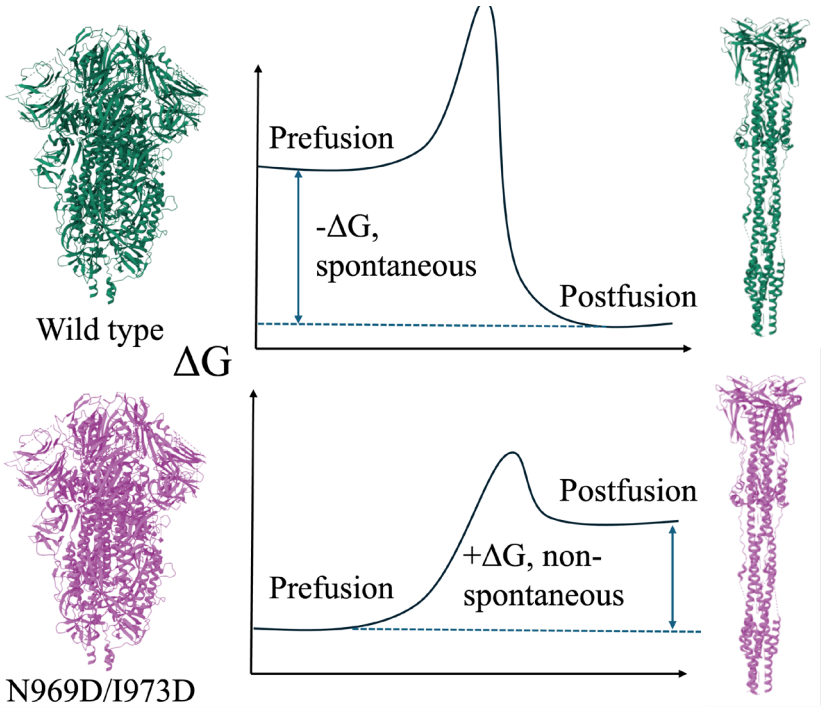


Figure 1 | Stabilizing optimal spike conformation. The introduction of charged residues at positions 969 and 973 destabilize the post-fusion conformation of the SARS-CoV-2 Spike, resulting in a thermodynamically favored pre-fusion conformation.

conformation through targeted electrostatic destabilization (See **Fig 1**).

Hereafter, constructs are named based on their mutations (Spike-#P = number of proline substitutions; Spike-#D = number of aspartic acid substitutions). When expressed in mammalian cells, the Spike-2D ectodomain exhibited significantly higher expression than WT and yields comparable to those of Spike-2P and Spike-6P. A combined construct (Spike-4P-2D), incorporating both proline and aspartate substitutions while reverting the original K986P/V987P mutations, showed similar expression levels. Both Spike-2D and Spike-4P-2D were consistent with pre-fusion stabilization, as assessed by binding to the CR3022 antibody.

Hamsters were vaccinated with all constructs to assess their efficacy in eliciting neutralizing antibodies. All constructs exhibited significant antibody titers upon prime vaccination, and Spike-4P-2D performed similarly to Spike-2P and Spike-6P after a boost vaccination. All were able to effectively neutralize B.1.351 pseudovirus. When animals were challenged with SARS-CoV-2 virus, all vaccinations conferred similar protection in terms of weight and lung histopathology. Taken together, the results of this research suggest that the proposed aspartic acid mutations are an effective means of stabilizing the pre-fusion Spike conformation and can elicit neutralizing antibodies.

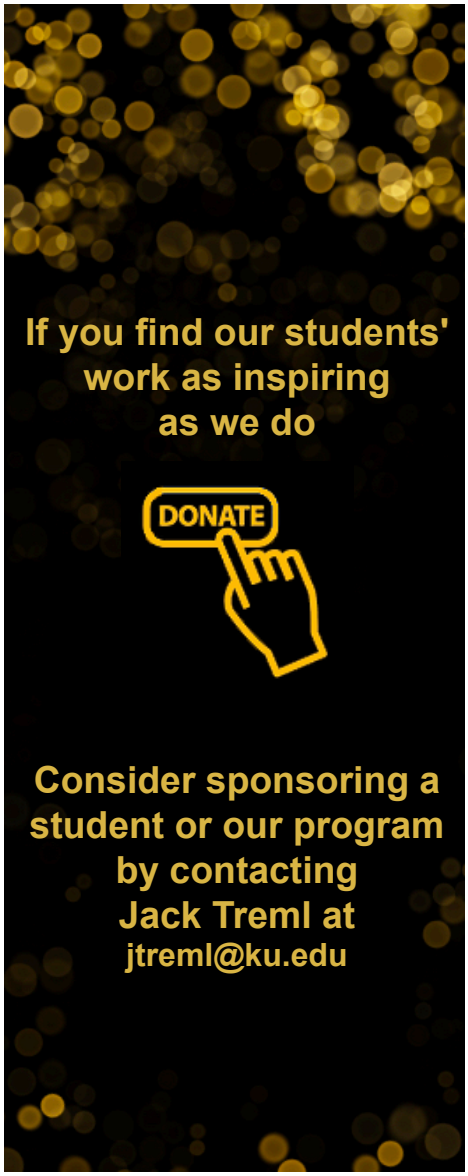
Development of new approaches to stabilize the pre-fusion conformation is an important aspect of mRNA vaccine optimization. Although two different constructs may successfully stabilize the pre-fusion conformation, subtle differences in epitope exposure, expression efficiency, and glycosylation patterns may alter the performance of the vaccine. More broadly, this raises the possibility that charge-based destabilization strategies may be applied to other class I fusion proteins, offering an alternative route to pre-fusion stabilization beyond proline substitution. However, while antibody binding and pseudovirus neutralization support pre-fusion stabilization, high-resolution structural and biophysical measurements would be required to fully establish equivalence between the stabilization strategies.

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

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The Perfect Disguise: Virus-Like mRNA Vaccines Turn Viral Mimicry into Immunity

By Dylan Allender* and JF Treml*†

The rapid success of mRNA vaccines has transformed modern vaccinology, yet their limitations are becoming increasingly clear. Protection against rapidly evolving viral variants is not always complete, and immune responses can decline over time, suggesting that efficacy depends not only on the antigen itself but also on how that antigen is delivered and interpreted by the immune system. Writing in Nature Nanotechnology, Zhang and colleagues present a vaccine platform that seeks to address these challenges by combining antigen delivery, immune targeting, and structural mimicry into a single system[1]. Their virus-like structure (VLS) platform represents a conceptual shift: rather than simply delivering mRNA, it attempts to reproduce key features of viral infection, thereby shaping a more coordinated and durable immune response.

This approach builds on decades of work in genetic vaccination. Early studies demonstrated that direct gene transfer into muscle tissue could result in protein expression within host cells, establishing that the body itself could act as a site of antigen production[2]. Subsequent experiments showed that DNA encoding viral proteins could generate both antibody responses and cytotoxic T cell activity, leading to the concept of genetic immunization, in which nucleic acids serve as instructions for endogenous antigen synthesis.[3-5] This marked a fundamental shift in vaccine design, moving away from the direct administration of proteins and toward programming host cells to produce antigens internally. Such strategies provided the basis for later advances in RNA-based vaccination, which offered similar benefits without the risks associated with genomic integration.

mRNA vaccines expanded this framework but introduced new challenges. Because mRNA functions in the cytoplasm, it avoids nuclear entry and integrates more safely into therapeutic strategies. Early work confirmed that mRNA could induce antigen-specific immune responses in vivo, yet these approaches were limited by the innate immune system's ability to detect foreign RNA. Recognition by pattern recognition receptors such as Toll-like receptors can trigger inflammatory responses that reduce protein translation and limit vaccine effectiveness.[6-8] This barrier was overcome through chemical modification of the RNA molecule itself. Incorporation of modified nucleosides, such as pseudouridine, reduces immune detection while increasing translational efficiency, a breakthrough that enabled the development of modern mRNA vaccines.[9]

At the same time, improvements in delivery systems played a critical role in advancing the field. Lipid nanoparticles were optimized to protect mRNA from degradation and facilitate

cellular uptake, particularly through mechanisms that promote endosomal escape.[10] These innovations made it possible to deploy mRNA vaccines at scale, culminating in highly effective vaccines against SARS-CoV-2 that demonstrated strong antibody and T cell responses in both preclinical and clinical settings.[11,12] However, despite these successes, current mRNA platforms rely largely on passive delivery into cells and do not fully replicate the spatial targeting and immune activation seen during natural viral infection.

Zhang and colleagues address this limitation by designing a delivery system that more closely resembles a virus. Their VLS platform consists of an mRNA-loaded lipid nanoparticle decorated with viral spike protein on its surface, creating a structure capable of engaging host receptors such as ACE2 and DC-SIGN.[1] This receptor-mediated interaction allows the particle to preferentially enter epithelial cells, dendritic cells, and macrophages, which are central to initiating immune responses. By targeting these cells directly, the VLS platform enhances mRNA delivery and antigen expression compared with conventional lipid nanoparticles, effectively shifting from passive distribution to active cellular targeting.

This targeted delivery has important consequences for immune activation. Rather than relying on nonspecific uptake, the VLS system promotes interaction with antigen-presenting cells, leading to a more controlled and balanced activation of innate immune pathways. Experimental data show increased interferon signaling and immune regulator expression without the excessive inflammatory responses often associated with unmodified RNA delivery.[1] This balance is critical, as excessive inflammation can impair antigen expression, while insufficient activation may fail to generate a robust adaptive response. By modulating this balance, the VLS platform creates a more favorable environment for immune priming.

The downstream effects of this improved targeting are evident in vivo. VLS immunization results in greater colocalization of antigen with dendritic cells and macrophages, along with increased activation of these cells. These early innate responses translate into stronger adaptive immunity, characterized by higher antibody titres, improved neutralization of multiple viral variants, and enhanced T cell responses.[1] Notably, these immune responses are more durable than those generated by mRNA or protein alone, suggesting that the coordinated activation of multiple immune pathways contributes to long-term protection.

A key feature of the VLS platform is its ability to deliver both mRNA and protein simultaneously. This dual-delivery strategy allows the immune system to process antigen through multiple pathways. Endogenously produced antigen from mRNA supports presentation via major histocompatibility complex class I pathways, activating $CD8^+$ T cells, while surface-displayed protein provides immediate antigen for uptake and presentation via class II

pathways. This combination enhances communication between different arms of the immune system and may facilitate cross-presentation across diverse dendritic cell populations. In doing so, the VLS platform more closely mimics natural infection, where antigens are encountered in multiple forms and contexts.

The efficacy of this approach is particularly striking. In viral challenge experiments, animals immunized with the VLS platform show minimal viral replication across tissues and are protected against multiple SARS-CoV-2 variants.[1] This suggests that the immune response generated is not only stronger but also more functionally effec-

tive. By integrating innate and adaptive responses, the platform achieves a level of protection that approaches that of natural infection while avoiding its associated risks.

Despite these promising findings, several questions remain. The authors note that the precise physicochemical structure of the VLS particles and the mechanisms governing their interactions with receptors such as DC-SIGN are not yet fully understood[1]. Further investigation will be needed to determine how these interactions influence immune signaling and how they can be optimized. In addition, the complexity of the system may present challenges for large-scale manufacturing and clinical translation, factors that will be critical for its broader application.

More broadly, this work reflects a shift in vaccine design from simple delivery systems to platforms that actively shape immune responses. Early genetic vaccines demonstrated that immunity could be programmed, while later advances refined delivery and molecular stability. The VLS platform builds on these developments by incorporating structural and functional elements of viral infection, enabling more precise control over how the immune system is engaged. This approach has implications beyond infectious disease, particularly in areas such as cancer immunotherapy, where targeted and coordinated immune activation is essential.

Zhang and colleagues therefore offer more than a technical innovation. Their work highlights how combining insights from immunology, virology, and nanotechnology can lead to vaccines that better replicate natural immune processes. As the field continues to evolve, platforms that integrate delivery, targeting, and immune modulation may define the next generation of vaccines, moving closer to the goal of fully engineered immunity.

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*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

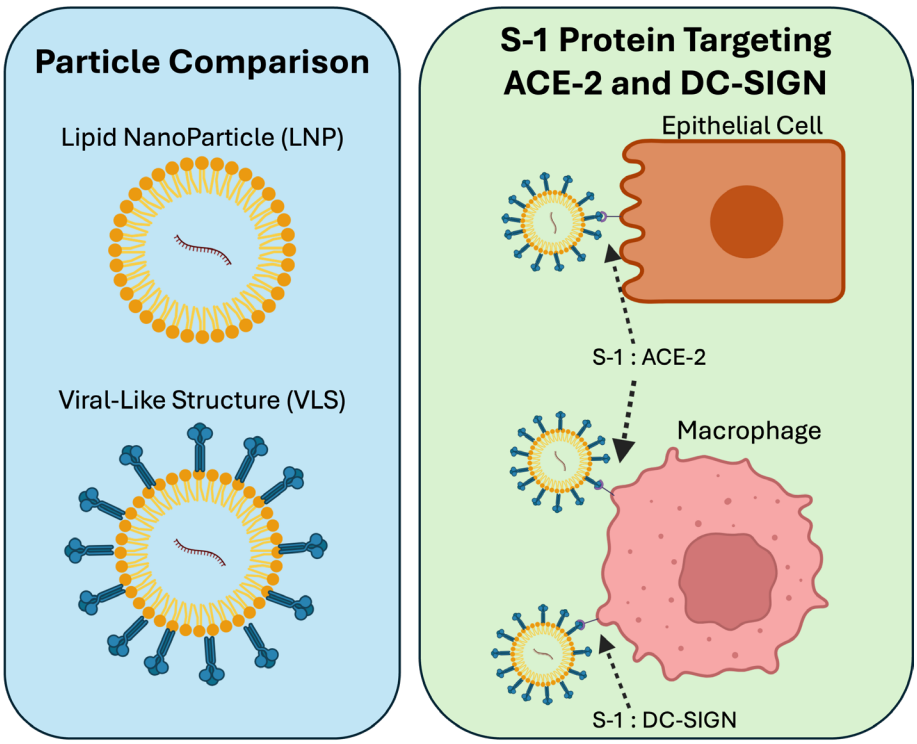


Figure 1 | Viral-Like Structure Interaction with Host Cells. Lipid nanoparticles (LNP) and a viral-like structures (VLS) carrying mRNA. On the left, the LNP is shown as a simple lipid sphere containing mRNA, while the VLS has spike-like S-1 proteins on its surface. The right side shows the S-1 protein on the VLS binding to ACE-2 receptors on epithelial cells and the ability to bind to both the ACE-2 and DC-SIGN receptors on macrophages, allowing the particle to target these cells more specifically. Created in BioRender. Allender, D & Treml, J. (2026) https://BioRender.com/hpspami

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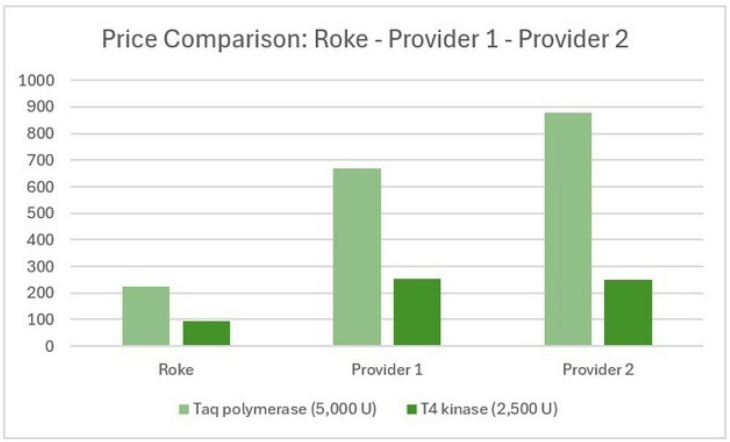
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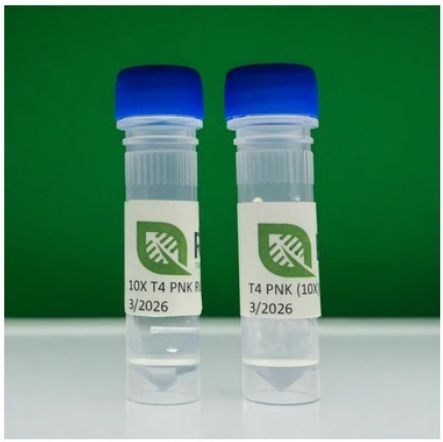
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A Vaccine with Your Name on It: Personalized mRNA Targets Pancreatic Cancer Neoantigens

By Christian Stewart* and JF Treml*†

Genetic vaccines have reshaped modern immunology by replacing traditional administration of weakened pathogens or purified proteins with direct delivery of genetic instructions encoding target antigens. Rather than introducing a preformed antigen, these platforms allow host cells to synthesize proteins internally, leading to endogenous antigen processing and presentation through major histocompatibility complex (MHC) pathways. Early plasmid DNA vaccination studies in the 1990s demonstrated that injected genes could induce both humoral and cellular immune responses.[1] Decades later, improvements in nucleic acid stabilization, vector engineering, and lipid nanoparticle delivery systems culminated in the clinical success of messenger RNA vaccines.[2,3,4] These advances established genetic vaccination as flexible and rapidly adaptable therapeutic mRNA platforms, which were demonstrated during the SARS-CoV-2 pandemic.[5 - 8]

The implications of this approach extend beyond infectious disease. If host cells can be programmed to produce viral antigens, they may also be directed to express tumor-specific targets. This concept is especially relevant in oncology, where therapeutic vaccines targeting non-mutated self-antigens have historically shown limited efficacy due to immune tolerance and weak T cell activation. [9] Tumors arise from somatic tissue and therefore share most of their antigenic repertoire with normal cells. Immune tolerance, tumor heterogeneity, and suppressive microenvironments have repeatedly limited attempts to generate durable anti-cancer immunity.[10]

The major obstacle has been target selection rather than immune stimulation itself. Cancer genomics offered a solution through the discovery of neo-antigens, mutated peptides generated by somatic DNA alteration within tumor cells Because neoantigens arise from tumor-specific somatic mutations and are absent from normal tissues during T cell development, the immune system is not centrally tolerized to these novel targets in the same way it is to self-antigens. As a result, neoantigens can be recognized as foreign and may serve as highly specific immune targets.[9,12] Evidence from pancreatic cancer further supports this concept, as long-term survivors exhibit enhanced neoantigen-specific T cell responses that correlate with improved outcomes.[11]

In their 2023 paper in Nature, Rojas *et al.* investigated whether a personalized mRNA neoantigen vaccine could elicit an effective immune response against tumor cells harboring pancreatic ductal adenocarcinoma (PDAC), a cancer that is typically resistant to immunotherapy and associated with poor survival.[8] In this clinical study, tumors from patients were sequenced to identify individual mutations, results were then used to design mRNA vaccines encoding up to twenty patient-specific neoantigens. These vaccines were given along with anti-PD-L1 immunotherapy and standard chemotherapy to improve T cell activation. The results showed that 50% of patients developed neoantigen-specific T cell responses after vaccination, suggesting that one reason earlier cancer vaccines showed limited efficacy is that many did not target antigens that were sufficiently tumor-specific, immunogenic, and effictively presented to T cells.[8]

The effectiveness of this vaccine relies on its ability to generate a targeted T cell response against tumor-specific neoantigens. After administration, the mRNA vaccine is taken up by antigen-presenting cells, where the encoded neoantigens are translated into proteins and processed into peptide fragments. These peptides are then presented on MHC molecules, particularly MHC class I, which activates $CD8^+$ cytotoxic T cells. Rojas et al. showed that their vaccine induced strong de novo T cell responses, meaning these responses were newly generated rather than pre-existing. In responding to patients, multiple T cell clones expanded significantly, in some cases making up a large proportion of circulating T cells. These cells also displayed functional markers such as interferon-γ, granzyme B, and perforin, which are all associated with cytotoxic activity and tumor cell killing. Together, these findings suggest that the vaccine is not only capable of activating T cells but also generating a functional and sustained anti-tumor immune response.[8]

To further characterize the immune response, Rojas *et al.* analyzed the expansion offunctionality found in vaccine-induced T cell population. T cell analysis showed that vaccine-expanded T cell clones increased from undetectable pre-vaccination levels to as much as 10% of all circulating blood T cells in responding patients. This expansion was not limited to a single clone, but rather a true polyclonal immune response. This finding is consistent with earlier workshowing that mRNA neoantigen vaccines can induce broad, multi-specific T cell responses against tumors.[13] In addition, these expanded T cells displayed a strong cytotoxic phenotype. Single-cell sequencing revealed that they were primarily $CD8^+$ T cells expressing key effector molecules such as perforin, granzyme B, and interferon-γ, all of which are associated with direct tumor cell killing.[8] Functional assays further confirmed that these cells produced cytokines and degranulated upon antigen re-exposure, demonstrating that they were not only present, but functionally active against the vaccine-targeted neoantigens. Importantly, these T cell populations were also shown to persist over time and expand following booster vaccinations, suggesting the formation of long-term immune memory, a feature that was also observed in other neoantigen vaccine studies.[14]

Beyond immune activation, the study also evaluated whether this T cell response was translated into meaningful clinical outcomes. At a median follow-up of 18 months, patients who developed vaccine-induced T cell responses showed improved recurrence-free survival compared to those who did not respond. Specifically, responders had not yet reached a median recurrence time, while non-responders experienced recurrence at a median of 13.4 months. This suggests that the immune response generated by the vaccine plays a role in controlling residual disease after surgery, supporting findings from other neoantigen vaccine studies that link T cell response to improved clinical outcomes.[13,14] A particularly notable example involved a patient who developed a small liver lesion after

vaccination that was initially suspected to be metastatic disease. However, biopsy revealed a dense infiltration of vaccine-induced T cells, and the lesion later resolved, suggesting that these immune cells may have actively eliminated metastatic tumor cells.[8]

The findings represent an important shift in cancer immunotherapy, demonstrating that even tumors traditionally considered immunologically unresponsive can be targeted through precise selection of the correct tumor-specific antigen targets and personalized vaccine design. Rather than demonstrating that mRNA vaccines can activate the immune system, Rojas *et al.* provide evidence that precise neoantigen targets can overcome the fundamental limitations that have historically constrained cancer vaccine efficacy.[8] This distinction is critical, as it shifts the focus of immunotherapy from generalized immune stimulation to targeted immune recognition. As sequencing technologies and predictive algorithms improve, the ability to identify and prioritize immunogenic neoantigens will likely determine the success of future therapeutic strategies. In this context, personalized mRNA vaccines represent not simply a new treatment modality, but a broader framework for integrating tumor genomics into immune-based cancer therapy.

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*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

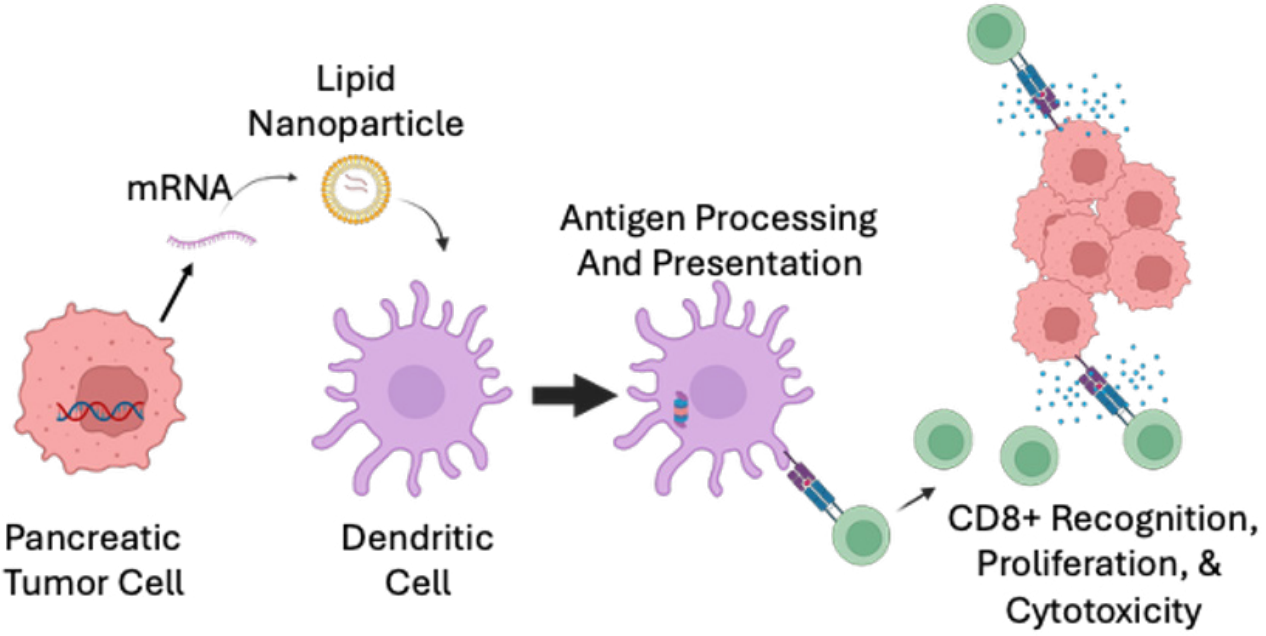


Figure 1. Mechanism of personalized mRNA neoantigen vaccination in PDAC. Tumor-specific neoantigens are encoded into mRNA, delivered to antigen-presenting cells via lipid nanoparticles, and presented on MHC class I to activate $CD8^+$ T cells. This induces clonal expansion and tumor cell killing by granzyme B and perforin. Created in BioRender. Stewert, C. & Treml, J. (2026) https://BioRender.com/hpspami

The mRNA That Came In from the Cold: Foiling Aldehyde Sabotage in Lipid Nanoparticles

By Arianna Theleman* and JF Treml*†

Nucleic acid vaccines have received increasing research attention over the past 40 years.[1] Traditional vaccines often require lengthy development, manufacturing, and regulatory timelines. Many traditional vaccines use live-attenuated or inactivated viruses, or selected viral proteins, to elicit protective immune responses. Although these approaches are effective, developing, manufacturing, and approving a new vaccine can require considerable time and resources that are all in short supply during a global pandemic such as that caused by the SARS-CoV-2 virus. The COVID-19 pandemic accelerated the development and widespread adoption of mRNA vaccine technology.

In the late 1980s and early 1990s, researchers began investigating whether nonreplicating DNA and RNA could direct protein production in vivo. Direct injection of gene-encoding mRNA or DNA into mouse skeletal muscle produced detectable protein without a specialized delivery system. Subsequent studies showed that vaccination by this method elicited antigen-specific immune responses.[2-5] However, unmodified mRNA could activate innate immune pathways, causing inflammation and limiting mRNA stability, translation, and the desired antigen-specific response.[6,7] Karikó and Weissman addressed this problem by incorporating modified nucleosides that reduced innate recognition of the mRNA while preserving translation of the encoded antigen.[8,9]

As researchers improved the stability and immunological properties of mRNA, attention shifted toward delivery systems that could protect the molecule and promote efficient cellular uptake. Initial work focused on liposomes as delivery vehicles that could protect mRNA from degradation.[10,11] Lipid nanoparticles (LNPs) gained attention because they could protect mRNA, promote cellular uptake, and improve delivery efficiency.[12]

Together, these advances established mRNA as a promising vaccine platform capable of generating effective and durable immune responses. When the COVID-19 pandemic emerged, decades of prior research made it possible to develop and manufacture mRNA vaccines rapidly.[13-16] However, one major issue remained: the limited thermostability of mRNA vaccines, a challenge Hashiba et al. sought to improve with their research published in Communications Biology in 2024.[17] Pandemic responses require the rapid production of vaccines that remain stable and effective during storage and distribution. Early formulations of some COVID-19 mRNA vaccines required ultralow-temperature storage to limit mRNA degradation, complicating transportation and creating barriers for healthcare facilities without specialized freezers. Hashiba et al. designed an LNP capable of maintaining mRNA stability at 4 °C, a major step forward in making vaccines accessible for all. The researchers developed an LNP formulation containing the ionizable lipid CL15F. They compared this CL15F formulation with formulations containing CL4F, ALC-0315, or SM-102. To assess delivery efficiency, the researchers measured expression of luciferase-encoding mRNA. They found that the CL15F formulation produced greater levels of bioluminescence compared that containing CL4F. They also tested the CL15F formulation's ability to deliver vaccine that resulted in antigen response in mice, and found it was significantly stronger than the two clinical lipids. These results indicated that the CL15F formulation performed at least as well as the comparison formulations in the measured delivery and immunogenicity assays.

To assess storage stability, the researchers gave separate groups of mice formulations that had been stored for 0, 1, 3, or 5 months at −80 °C or 4 °C. After five months at −80 °C, the CL15F and comparison formulations retained similar in vivo activity. After storage at 4 °C, however, only the CL15F formulation retained substantial activity through five months.

An additional factor affecting the shelf stability of mRNA vaccines is the formation of aldehyde-containing impurities within LNP formulations. These reactive impurities can interact with mRNA and reduce its translational activity by obstructing ribosome progression (See **Figure 1**). To compare aldehyde impurity levels in CL4F and CL15F formulations, researchers used a fluorescence assay based on NBD-H, which becomes fluorescent after reacting with aldehyde carbonyl groups. The assay showed that CL15F formulations contained significantly lower levels of aldehyde impurities than the CL4F formulation. This difference is important for long-term mRNA stability. Hashiba et al. also examined how aldehydes react with mRNA to form covalent adducts, in which an aldehyde-derived chemical group becomes attached to the mRNA. They found that higher aldehyde concentrations were associated with greater adduct formation and a corresponding loss of mRNA activity.

Despite several limitations, the study showed that LNP structure can influence the long-term thermostability of mRNA formulations. Not all CL15F-based formulations produced equivalent results, and the loss of mRNA activity could not be attributed entirely to aldehyde impurities. Nevertheless, the findings suggest that both lipid structure and aldehyde-containing impurities influence storage stability. These findings may guide future efforts to improve the stability and accessibility of mRNA vaccines and therapeutics.

Future mRNA therapies have the potential to treat cancer, genetic disorders, and autoimmune diseases. These treatments, however, cannot become broadly accessible care options until a stable and consistent way to store and transport them is found. Continued improvements in the storage stability and delivery of mRNA formulations will allow these types of treatments to reach all the people who need them, rather than remaining as novel ideas in deep-freeze.

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*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

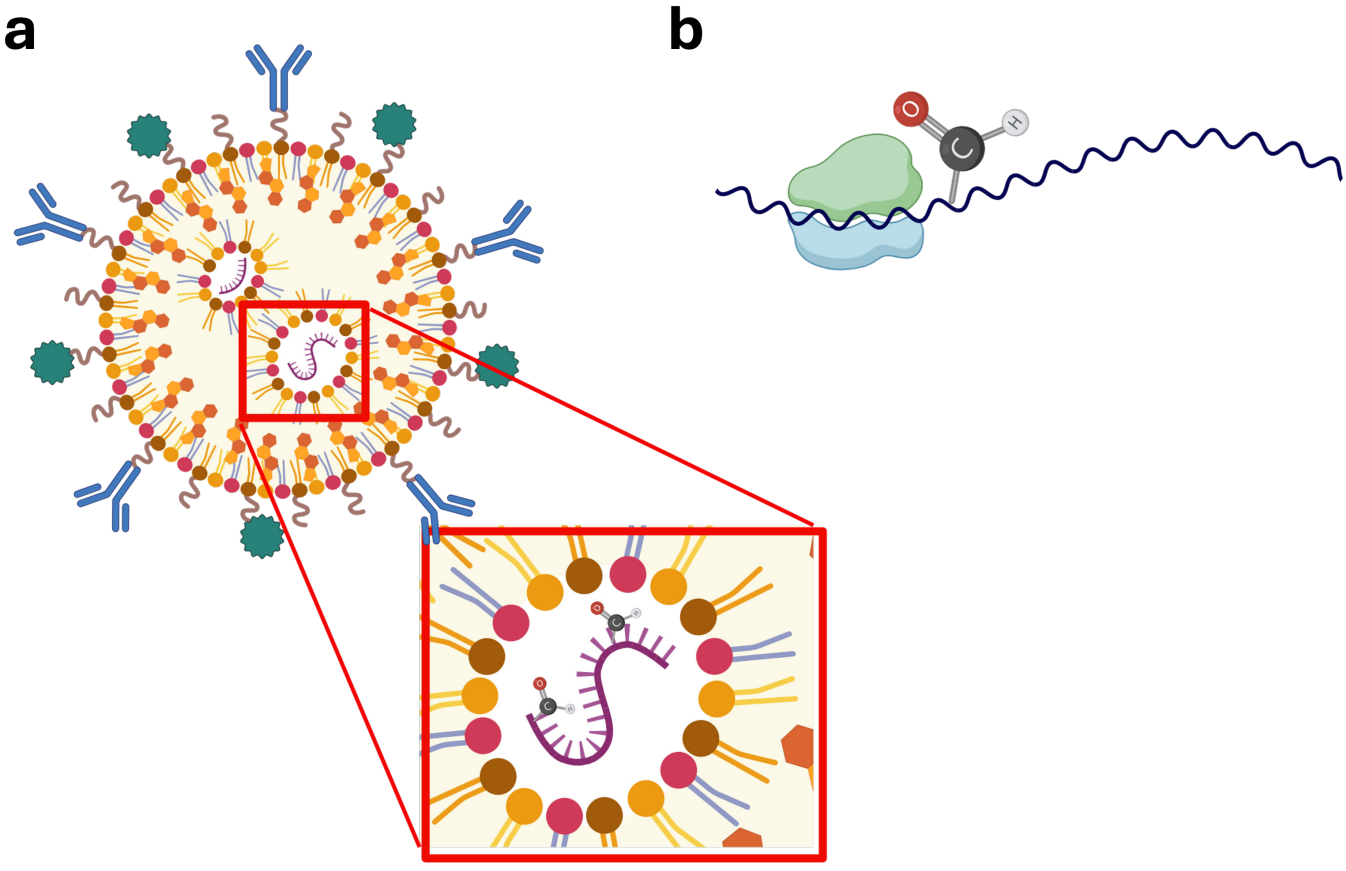


Figure 1 | Aldehyde Impurities Inhibiting Translation. a. An LNP-mRNA formulation with inset highlighting aldehyde impurities binding to the mRNA. b. An aldehyde residue binding to the mRNA in the coding sequence blocks translation by the ribosome. Created in BioRender. Theleman, A. & Treml, J. (2026) https://BioRender.com/c02h3wy

A Message of Tolerance: Turning Down the Heat on Allergic Asthma

By Jaden Fox* and JF Treml*†

Asthma affects over 300 million people around the world, and while current therapies control airway inflammation and relieve symptoms to improve patients' quality of life, they don't address the immunological defects that drive allergic asthma. In allergic asthma, the immune system fails to maintain tolerance to harmless environmental antigens.[1] It misidentified otherwise harmless substances as a danger and triggers airway inflammation, the overproduction of mucus, and bronchial hyperresponsiveness. Although current therapies like corticosteroids and targeted biologic therapies have greatly improved disease management, these treatments mainly suppress downstream inflammation and usually require lifelong administration. Most current therapies do not fully correct the underlying immune dysregulation responsible for allergic asthma.

In recent years, mRNA–lipid nanoparticle technology has emerged as a powerful platform for nucleic acid delivery as a powerful platform for vaccination and immune activation.[2–4] The applications of mRNA systems extend beyond the induction of protective immunity against infectious diseases. Rather than activating immunity, mRNA-LNP systems can also be engineered to dampen immune responses and promote.[5] This work reframes mRNA therapies as tools for immune re-education. The central challenge in developing such a therapy against asthma is to selectively reprogram the immune system to tolerate allergens without causing broad systemic immunosuppression. Although existing therapies effectively control airway inflammation, they fail to eliminate immune memory. Furthermore, current allergen immunotherapies require extended treatment, vary in efficacy, and may produce adverse reactions. As a result, only about 5% of people with allergic rhinitis, asthma, or both in the United States undergo this treatment.[4] To overcome these limitations, Wang *et al*. tested a spleen-targeted approach using tolerogenic mRNA–LNPs incorporating long-chain saturated fatty acids to reprogram allergen-specific immunity and suppress allergic asthma through tolerance mechanisms.[5,6] This work represents a new approach to using programmable mRNA therapeutics to address the upstream immunologic cause rather than simply managing downstream symptoms.

Previous work with an mRNA–LNP vaccine platform incorporated the long-chain saturated fatty acid stearic acid into the lipid nanoparticles to produce stearic acid–doped LNPs (SA-LNPs). Stearic acid alters the physicochemical properties of the particles and had previously been shown to promote preferential targeting of the spleen and splenic dendritic cells.[5] The SA-LNPs were used to co-deliver nucleoside-modified ovalbumin (OVA) mRNA and celastrol, an anti-inflammatory compound that suppresses dendritic cell activation.[7] Using luciferase mRNA, fluorescently labeled nanoparticles, and LNP formulations doped with natural long-chain saturated fatty acids, the researchers showed that the modified particles preferentially accumulated in the spleen, with particularly high uptake by splenic dendritic cells.[5] Uptake of the tolerogenic mRNA–LNPs by splenic dendritic cells was significantly greater than uptake by other immune-cell populations. Importantly, the addition of celastrol did not impair mRNA delivery efficiency, nanoparticle stability, or protein expression. These findings indicate that the tolerogenic modification preserved the delivery capabilities of the LNP while changing its immunological behavior.

The next set of experiments examined whether the mRNA-LNPs could induce a tolerogenic phenotype among DCs. Bone marrow-derived DCs generated in vitro were exposed to nanoparticles and then stimulated with lipopolysaccharide, which would normally induce inflammatory maturation. DCs treated with control mRNA–LNPs lacking celastrol showed increased expression of costimulatory molecules, consistent with immunogenic activation. In contrast, the DCs treated with celastrol-loaded tolerogenic mRNA-LNPs reduced the expression of activation markers and maintained the DCs in an immature, tolerogenic state. RNA sequencing revealed that celastrol fundamentally changed the transcriptional state of treated cells, promoting immune regulation while blocking inflammatory signals. DCs treated with sLNP-OVA/celastrol or sLNP-celastrol produced higher levels of anti-inflammatory cytokines IL-10 and TGF-b while reducing pro-inflammatory cytokines IL-6 and IL-1b (Figure 1A). Western blot analysis showed inhibition of the NF-kB signaling pathway through reduced phosphorylation of IKKB and p65 (Figure 1B), providing evidence that celastrol actively rewired intracellular inflammatory signaling within antigen-presenting cells.

After establishing tolerogenic DC induction, Wang et al. investigated whether the altered antigen-presenting cells could generate regulatory T cells (Tregs). In co-culture experiments, bone marrow-derived DCs treated with tolerogenic vaccine induced higher numbers of $CD4^+$ $Foxp3^+$ Tregs compared with standard mRNA-LNP formulations. The Tregs also expressed elevated levels of Foxp3, IL-10, and TGF-α, indicative of a suppressive phenotype. In vivo experiments showed that vaccination increased OVA-specific Treg accumulation in the spleen and lungs. This suggests that immune tolerance initiated in the spleen could influence other sites of allergic inflammation, linking systemic immune reprogramming to local suppression of airway pathology.

Finally, Wang tested whether the tolerogenic vaccine could therapeutically suppress allergic asthma in mice sensitized and challenged with the OVA allergen. Histological analysis of the lung tissue following treatment showed marked reductions in peribronchial inflammation and mucus secretion in treated mice. Flow cytometry revealed decreased eosinophil infiltration in the lungs, and bronchoalveolar lavage fluid contained fewer inflammatory cell populations including eosinophils, neutrophils, and lymphocytes. Additional immunofluorescence and flow cytometric analysis demonstrated fewer inflammatory monocytes and greater Treg infiltration in lung tissue following treatment. Together, these experiments in this mouse model that spleen-targeted tolerogenic mRNA-LNP vaccination not only altered systemic immune signaling but also alleviated the cardinal pathological features of experimental allergic asthma. This supports the idea that antigen-specific immune tolerance can be

therapeutically induced through programmable mRNA nanomedicine.

This article extends the potential for applications of mRNA therapeutics and allergic disease by showing that mRNA-LNP systems are programmable platforms that can be engineered to induce antigen-specific immune tolerance. By demonstrating that spleen-targeted delivery can reprogram DCs towards a regulatory phenotype, promote Treg responses, and reduce features of allergic airway inflammation in vivo. The study expands the versatility of mRNA therapeutics, which can be formulated either to stimulate protective immune responses or to promote antigen-specific immune tolerance.[8,9] This presents new possibilities for treating allergic asthma and other autoimmune diseases rather than simply suppressing their symptoms.

*University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

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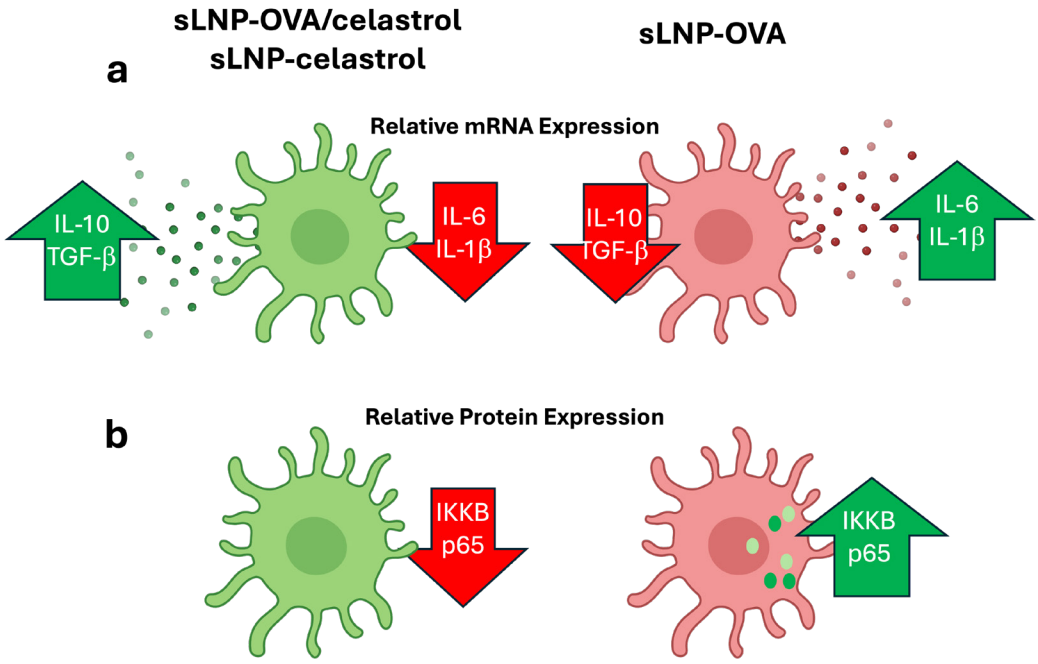


Figure 1 | Comparative analysis of relative mRNA and protein expression in bone marrow derived DCs. Cells were treated with sLNP-celastrol, sLNP-OVA/celastrol vs. sLNP-OVA A) Relative mRNA expression of anti-inflammatory cytokines IL-10 and TBF-B, and pro-inflammatory cytokines IL-6 and IL1-B in bone marrow-derived DCs. B) Relative protein expression of NF-kB pathway proteins, IkkB and p65.


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The mRNA Case File: A Chronological Reading List in Genetic Vaccine Development

JF Treml

This chronological reading list builds a strong background of primary literature in the field of nucleic acid - based vaccines. It traces the key evidence behind genetic vaccination from early DNA transfer to modern mRNA vaccine design. Together, these papers reveal how successive advances in antigen expression, immune sensing, delivery, and clinical testing built the field as it exists today.

SECTION I Early DNA Vaccine Origins & Molecular Immunology Foundations (Weeks 1–4)

1.Wolff et al., 1990 Direct DNA transfer

a.Wolff et al., 1990 — Direct gene transfer into mouse muscle in vivo, a.Science, 247(4949 Pt 1):1465–1468
This paper sets a baseline: i.m. injected DNA can express proteins in muscle cells in vivo. Very simple methods make it a good first primary article for students.

Additional reading: Liu, 2011. DNA vaccines: an historical perspective (Gene Therapy)

2.Ulmer et al., 1993 DNA immunization protects against influenza

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Additional reading: Donnelly et al., 1997. DNA vaccines: progress and challenges

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An early demonstration that DNA vaccines work for non-influenza antigens, with accessible techniques including plasmid injection, ELISA, western blot, and lymphocyte proliferation assays.

Additional reading: Liu, 2003. DNA vaccines: a review

4.Tang et al., 1992 Gene gun delivery of DNA

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Introduces gene gun DNA delivery — simple concept, minimal complex methods. Shows that delivery method matters.

Additional reading: Ulmer & Donnelly, 1998. DNA delivery systems

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5.Martinon et al., 1993 mRNA in liposomes induces CTLs

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Additional reading: Weissman & Karikó, 2015 — Overview of mRNA therapeutic development

7.Petsch et al., 2012 Protective efficacy of in vitro transcribed mRNA vaccines against influenza in mice

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Optimized mRNA synthesis and antigen expression. Introduces more modern formulation but still avoids advanced ionizable lipid chemistry. Acts as a mid-semester difficulty bump.

Additional reading: Kramps & Elbers, 2017. mRNA vaccine technology (Curr Opin Immunology)

SECTION III Innate Immune Sensing, Modified Nucleosides, & Delivery Innovations (Weeks 8–11)

8.Karikó et al., 2005 Pseudouridine reduces innate sensing

a.Karikó K et al. (2005). Immunity. 23(2):165-75. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA.

Foundational molecular immunology and nucleoside chemistry that makes RNA vaccinations a leading model.

Additional reading: Kawai & Akira, 2010 — Overview of innate immune sensing pathways (TLRs, RLRs)

9.Karikó et al., 2008 Incorporation of pseudouridine into mRNA yields superior translation and reduced immune activation

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Builds directly on the 2005 paper with more mechanistic detail. Demonstrates improved translation, cytokine reduction, and increased protein expression. Adds depth.

Additional reading: Mauger et al., 2019 — RNA structure and translation efficiency

10.Hassett et al., 2019 Optimization of lipid nanoparticles for RNA delivery

a.Hassett et al., 2019. Mol Ther Nucleic Acids. 2019 Apr 15;15:1-11.Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines

Provides a pre-COVID view of LNP optimization. Introduces controlled variation of ionizable lipids, PEG-lipids, etc. Ideal preparation for the clinical-era work.

Additional reading: Hou et al., 2021 — Lipid nanoparticles for mRNA delivery (Nat Rev Mater)

SECTION IV COVID-19 Era Design, Immunogenicity, and Clinical Outcomes (Weeks 12–15)

11.Laczkó et al., 2020 Cell and Antibody responses

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Prototype pathogen preparedness, spike modification rationale.

Additional reading: Graham, 2020 — Review on the prototype pathogen preparedness program
& Saunders et al., 2020 — "COVID preclinical vaccine design" summary commentary

13.Walsh et al., 2020 Pfizer–BioNTech Phase

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Early human data with dose finding

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Core preclinical study explaining why BioNTech selected BNT162b2 over BNT162b1.
Deep insight into antigen selection, immune profiling, and comparative immunology.
Excellent precursor to clinical trials (Baden).

Additional reading: Sahin & Türeci, 2021 — Commentary on BioNTech vaccine design

15.Baden et al., 2021 — Moderna Phase 3

a.Baden et al., 2021. N Engl J Med. 2021 Feb 4. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine.384(5):403-416

Additional reading: McGill et al., 2021 — Vaccine efficacy review commentary
& Polack et al., 2020 (Pfizer Phase III)

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Often times undergraduate science students' laboratory exposures are limited to single-day (or otherwise encapsulated) experiences. It is common for students to be given comprehensive, stepwise protocols that provide little encouragement to fully understand why each step is performed. When the experiment is completed and a lab report is turned in, students are left with only a vague understanding of whether their lab 'worked' or not, but don't understand why, nor are they offered the opportunity to revisit any of their work.

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In the KU Edwards Campus Biotechnology program, we have developed a senior capstone research curriculum based on the experience of a graduate student working on their independent research topic. The curriculum consists of one semester of research and planning and one of hands-on experimental work. Students independently conduct a research project of their choice and gain exposure to valuable aspects of scientific research such as budgeting and inventorying their own reagents, scheduling with Gantt charts, and troubleshooting. Throughout the year, weekly lab meetings are designed to keep students moving forward and give them practice in presenting updates on their work. In students' own words, these meetings cultivate an environment where students are inspired by and invested in not only their own capstone projects but those of their classmates as well.

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Investigating the Impact of O-GlcNAc Regulatory Elements on the Gene Expression of ATF4

Nathaly Wember-Portillo[1], S. Thomas[1], M. Daggett[1], JF Treml[1 †], B. Mattingly[1], M. Parker[2], and C. Slawson[2]

Regulation of mitochondrial structure and function plays a vital role in cellular processes. The organelle facilitates control of energy metabolism, immune signaling, and apoptosis with the help of post-translation modifications (PTMs). PTMs are covalent modifications that occur during or after protein synthesis and can rapidly alter protein activity, localization, stability, or interactions. Mitochondrial stress can activate the integrated stress response (ISR), a cellular mechanism that regulates protein synthesis to maintain homeostasis. If mitochondria are impaired by stress it can lead to cascading effects linked to diseases such as neurodegenerative disorders, cancer, and others. During the ISR, eukaryotic translation initiation factor 2 alpha (eIF2α) is phosphorylated, regulating the downstream synthesis of numerous proteins. Among these, eIF2α phosphorylation promotes the translation of stress-related proteins, including activating transcription factor 4 (ATF4). Chronic ATF4 upregulation is observed in several disease states, many of which are also associated with mitochondrial stress and dysregulated O-GlcNAcylation, the covalent attachment of O-linked N-acetylglucosamine (O-GlcNAc). O-GlcNAcylation is an example of a PTM that occurs on the serine or threonine residues of thousands of proteins. O-GlcNAc transferase (OGT) is responsible for the addition of the sugar while O-GlcNAcase (OGA) removes the same sugar, potentially changing the activity of the protein. This dynamic maintains the cellular O-GlcNAc homeostasis, as well as regulation of mitochondrial function. Critically, O-GlcNAc homeostasis is believed to be indispensable to maintaining a healthy cell and preventing diseases such as cancer and neurodegenerative disorders. Our aim is to use a 'dead' CRISPR Cas9, 'dCas9', construct with either OGT or OGA fusions to target their activity specifically to promoter-associated proteins or chromatin at the ATF4 promoter. The SH-SY5Y neuroblastoma cell line will be used to determine how targeted OGT or OGA activity affects ATF4 expression during the ISR. This creates a model with the ability to observe the interaction between O-GlcNAc and ATF4 and how it may influence changes in gene expression. This study aims to understand the role that OGT and OGA play during a stress response and how the disruption of O-GlcNAc can impact mitochondrial function.

Current models of cellular function emphasize the dynamic interplay among metabolism, environmental conditions, and regulatory pathways. Within this complexity lies the mitochondrion, a crucial organelle in biological processes for maintaining cellular homeostasis. It is known for its role in generating adenosine triphosphate (ATP) and involvement in metabolic pathways. It also has a highly coordinated protein import and assembly where the majority of mitochondrial protein are encoded by nuclear DNA.[1] Defects in mitochondrial gene expression can lead to dysfunction in diseases affecting muscular and neuronal tissues.[2]

Mitochondrial proteins participate in regulating apoptosis, cellular signaling pathways, and homeostasis, which is important for maintaining cellular health and responding to stress.1 The mitochondrial integrated stress response (ISR) is a fundamental cellular adaptation mechanism with protective and pathological roles.[3] It functions to modulate protein synthesis, convert energy, and direct resources during times of metabolic stress. While this process decreases production of housekeeping proteins, it also increases synthesis of stress-response proteins. A multi-protein factor, eukaryotic initiation factor 2 (eIF2α), is central to the ISR for regulating protein synthesis.[4] When eIF2 α is phosphorylated by kinases such as Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK) and General Control Nonderepressible 2 (GCN2), translation of most mRNAs is repressed, while translation of activating transcription factor 4 (ATF4) is increased.[5] This transcription factor is vital for regulating genes related to metabolism and facilitates a coordinated stress response by control of a range of adaptive genes involved in amino acid transport and redox homeostasis to promote cell survival. However, under persistent stress ATF4 promotes a switch to cell-cycle arrest and potentially apoptosis.[6] The balance between this transcription factor's pro-survival and pro-apoptotic effects

[1]University of Kansas, Edwards Campus, Biotechnology
[2]University of Kansas Medical Center
†Corresponding Author jtreml@ku.edu

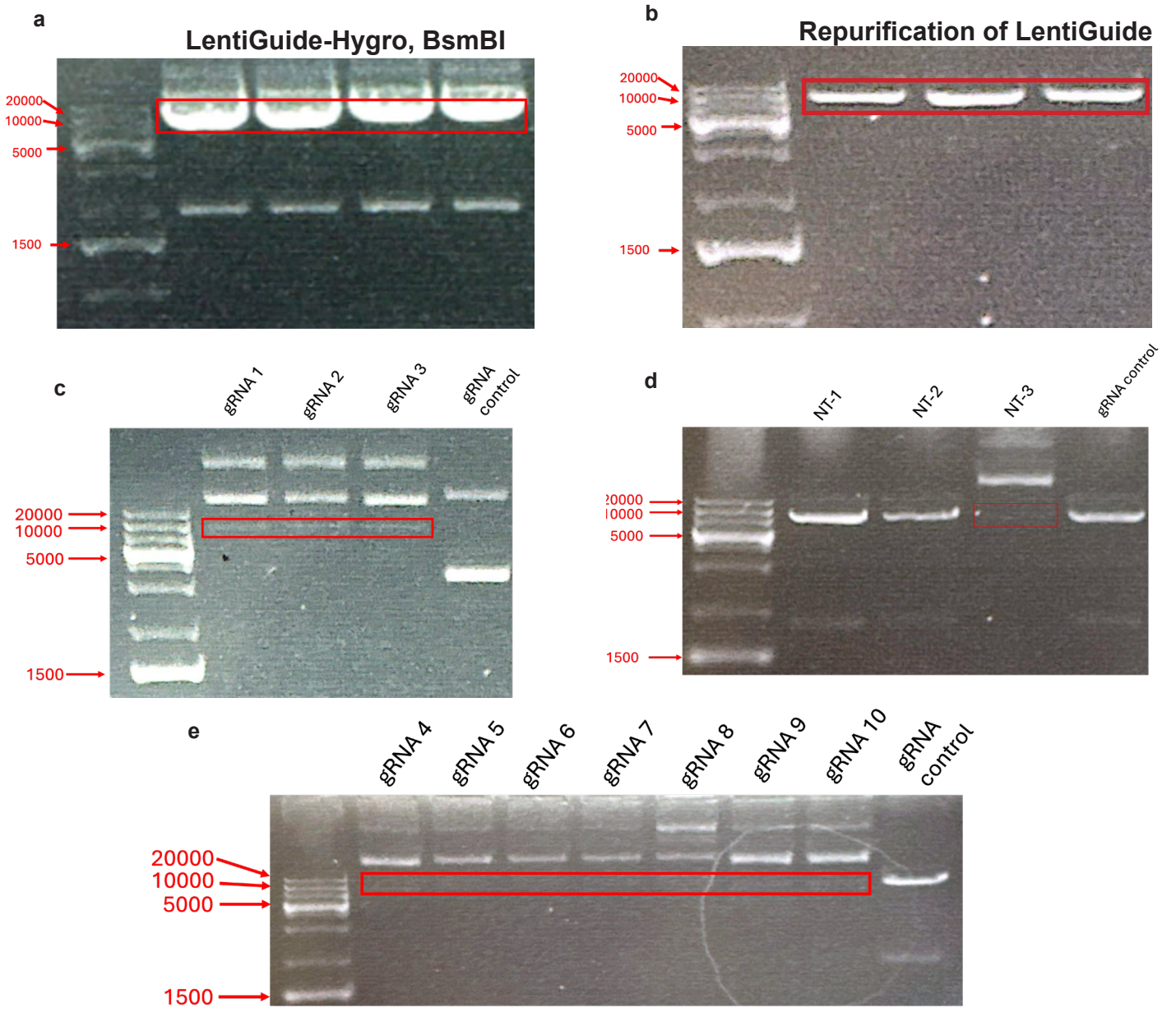


Figure 1 | Target guide sequence cloning using a lentiviral vector. a, The LentiGuide vector was digested with the restriction enzyme, BsmBI V2 nand products were run on a 1% agarose gel. All lanes are the same sample. The top band (highlighted in red) was gel purified. **b,** The purified sample from Fig.1a was re-cut with BsmBI V2 and re-purified. **c-e**, Target guide sequence cloning plasmids, gRNA 1-10, NT 1-3, and gRNA control, were isolated by miniprep, digested with BsmBI V2 and run on a 1% agarose gel. Sucessful clones were identified by restriction pattern including the (very faint) ~8,000 bp band.

depends on regulation by post translational modifications (PTMs).[7]

PTMs are chemical changes that occur during protein synthesis that can change the stability, activity, and structure of proteins. Mitochondrial PTMs are used to regulate cellular homeostasis, respond to dynamic metabolic and environmental changes, and their dysregulation has been associated with several diseases.[8] They also regulate a number of processes including epigenetics, gene expression, translation, and protein degradation.[9] Glycosylation is a kind of PTM that involves the addition of a sugar to a protein. A specific kind of glycosylation PTM involves O-linked N-acetylglucosamine modification (O-GlcNAcylation). O-GlcNAcylation occurs intracellularly and is catalyzed by two enzymes. O-GlcNAc transferase (OGT) adds O-GlcNAc to a serine or threonine amino acid residue of a protein whereas O-GlcNAcase (OGA) removes it.[10,11] This PTM has the ability to modify thousands of proteins, including those directly within the mitochondria. Additionally, it functions directly as a cellular nutrient and stress sensor that has impacts on cellular physiology and disease progression. This sugar has been linked to diseases such as neurodegeneration, cancers, diabetes, and has been associated with mitochondrial dysfunction.[9] Studies involving O-GlcNAc can be quite difficult because complete knockout of OGT or OGA can be lethal.[12]

To approach this obstacle, ATF4 can serve as a model target to observe what is occurring with O-GlcNAc during a stress response and as a model for genetic regulation via O-GlcNAc. ATF4 and O-GlcNAc both share metabolic sensor roles, where they are involved in metabolism,

stress response, cellular homeostasis, and mitochondrial function. ATF4 promotes transcription of activating transcription factor 5 (ATF5), which is also regulated by eIF2α and includes an ATF4 promoter. 13–15. ATF5 transcription may provide an additional mechanism of measuring ATF4 activity. This construct will carry enzymes OGT or OGA to the ATF4 promoter region during a stress response, where they may interact with the transcription factor or other cis-regulatory elements. Regulation of ATF4 will be assessed by qPCR and Western blot as a measure of the effect O-GlcNAcylation has during a stress response.

Materials and Methods

Target guide sequence cloning

To insert the sgRNA sequence into the LentiGuide-Puro (lentiGuide-Puro was a gift from Feng Zhang (Addgene plasmid # 52963 ; http://n2t.net/addgene:52963 ; RRID:Addgene_52963)), the Target Guide Sequence Cloning Protocol by Zhang Lab was used.[16,17] The puromycin resistance region in the vector was previously replaced with hygromycin resistance via Gibson assembly. Gel purification was performed on the 8000 bp band after the LentiGuide plasmid was cut with BsmBI V2 (NEB, Cat No. R0739L Lot No. 10315738) in NEBuffer r3.1(NEB, Cat No. B6003S Lot No. 10281919). The Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat No. D4007 Lot No. 267352) was used for the gel purifications. To phosphorylate the oligos, 1 µL of 10X T4 DNA Ligase buffer (NEB, Cat No. B0202S) containing 1 mM ATP was added to oligonucleotides and T4 Polynucleotide Kinase (NEB, Cat No. M0236S). The LentiGuide backbone was ligated with the oligos using T4 DNA Ligase (NEB, Cat No. M0202S) and transformed into High Efficiancy NEB 5-alpha Competent *E. coli* using their protocol (NEB, Cat No. C2987H).

Plasmid DNA isolation from bacterial cultures

Single colonies were selected from transformed bacteria on Luria-Bertani (LB) Miller agar plates (Fisher Scientific, Cat No. BP1425-500 Lot No. 106557) with 100 µg/mL of ampicillin. Colonies were inoculated in LB liquid media with ampicillin overnight in a shaking incubator at 250 rpm at 37 °C. Plasmid DNA was isolated from bacterial cultures using either a QIAprep Spin Miniprep Kit (Qiagen, Cat No. 27106 Lot No. 56903375) or ZymoPURE II Plasmid Maxiprep Kit (Zymo Research, Cat No. D4203-A Lot No. 269176).

Gel electrophoresis and restriction enzyme digests

All agarose gels used were made at 1% with TopVision Agarose (Thermo Scientific, Cat No. R0491 Lot No. 3374282) and 0.5x Tris/Boroate/EDTA (TBE) buffer. All gel electrophoresis utilized GeneRuler 1 kb Plus Ladder (Thermo Scientific, Cat No. SM1333 Lot No. 00835607) and Gel loading Dye Purple 6x (NEB, Cat No. B7024A Lot No. 10308510) for samples. Gels were run for approximately 1-1.5 hrs. at 80 volts. OGT- and dOGT-dCas9 plasmids were digested with ClaI (NEB, Cat No. R0197S Lot No. 0361101) and KpnI (NEB, Cat No. R0142S Lot No. 0531010) with CutSmart Buffer (NEB, Cat No. B6004S Lot No. 10021126). OGA and dOGA constructs were digested with EcoRV (NEB, Cat No. R0195S Lot No. 0441101) and NEBuffer r3.1 (NEB, Cat No. B7003S Lot No. 0151502)

Cell culture

The SH-SY5Y neuroblastoma cell line was cultured in DMEM with 4 mM of D-(+)-Glucose (Sigma, Cat No. G7021-1KG Lot No. 080M01759V), 15 mg/L phenol red (Acros Organics, Cat No. 143-74-8 Lot No. A0266455), 44 mM sodium bicarbonate (Fisher Scientific Cat No. S233-3 Lot No. 170369), 1% GlutaMAX 100x (Gibco, Cat No. 35050-061 Lot No. 2360316), 10% Fetal Bovine Serum (GeminiBio, Cat No. 100-106 Lot No. A324000), and 1% penicillin–streptomycin (Gibco, Cat No. 15140-122 Lot No. 250552). Additional reagents for passaging cells included PBS (phosphate buffered saline) and 0.25% Trypsin-EDTA 1x (Gibco, Cat No. 25200-056 Lot No. 3136922).

Results

Target guide sequence cloning using a lentiviral vector

To deliver and express the single guide RNA (sgRNA) for the CRISPR-dCas9, a lentiviral vector, LentiGuide-hygromycin, was used.[17] The LentiGuide backbone was isolated by digestion with BsmBI V2 followed by running the entire reaction across several wells of an agarose gel (**Fig. 1a**). The digest produced two bands, one ~8,000 bp and one ~2,000 bp piece, where the 2 kb piece was an inconsequential portion of the vector and was discarded. The linearized ~8,000 bp band was extracted and gel purified. The isolated fragment was re-digested with BsmBI V2 to ensure that the initial digestion was complete and run on a second gel and gel-purified a second time (**Fig. 1b**). The target guide sequence cloning was preceded by phosphorylation and annealing of the oligos. In parallel reactions, the oligo duplex, a control duplex, and a water control were ligated to the digested LentiGuide and transformed into DH5α. Single colonies were selected and grown overnight in LB supplemented with antibiotic and miniprepped the following day. gRNA-LentiGuide samples were digested with BsmBI V2 (**Fig. 1c-e**). The large bands running slower than the 20,000 bp ladder band, represent uncut, supercoiled forms of the plasmid. The smaller, faint band, can be seen at the expected ~8,000 bp size. The 'no-gRNA' control sample included no oligo duplex during the ligation step. Colonies that grew from the control samples were a result of a re-ligated plasmid since there was no oligo duplex. Ten different gRNA samples were cloned and sequenced to ensure that the gRNA was successfully inserted into the LentiGuide-hygromycin.

Linear plasmid map of dCas9 constructs and restriction enzyme digests

The CRISPR-dCas9 constructs were previously designed and constructed by Dr. Matt Parker (manuscript in preparation and **Fig. 2a**). Constructs were made on a PUC19 backbone with a puromycin resistance selection marker. OGT-dCas9 had a control, dOGT-dCas9, which mirrored OGT but included a single amino acid change at the amino acid 558 position, in the OGT region, resulting in a histidine-to-phenylalanine substitution. OGA-dCas9 also had a control, dOGA-dCas9, with a modification at the amino acid 174 position, in the OGA region, of an aspartate-to-alanine substitution. To verify the constructs a restriction digest using ClaI and KpnI was performed. OGT -and dOGT -dCas9 plasmids showed the expected bands at 8055, 2757, and 1794 bp. OGA- and dOGA -dCas9 plasmids had expected bands at 7431, 3303, and 1512 bp which were seen on the gel as well (**Fig. 2b**).

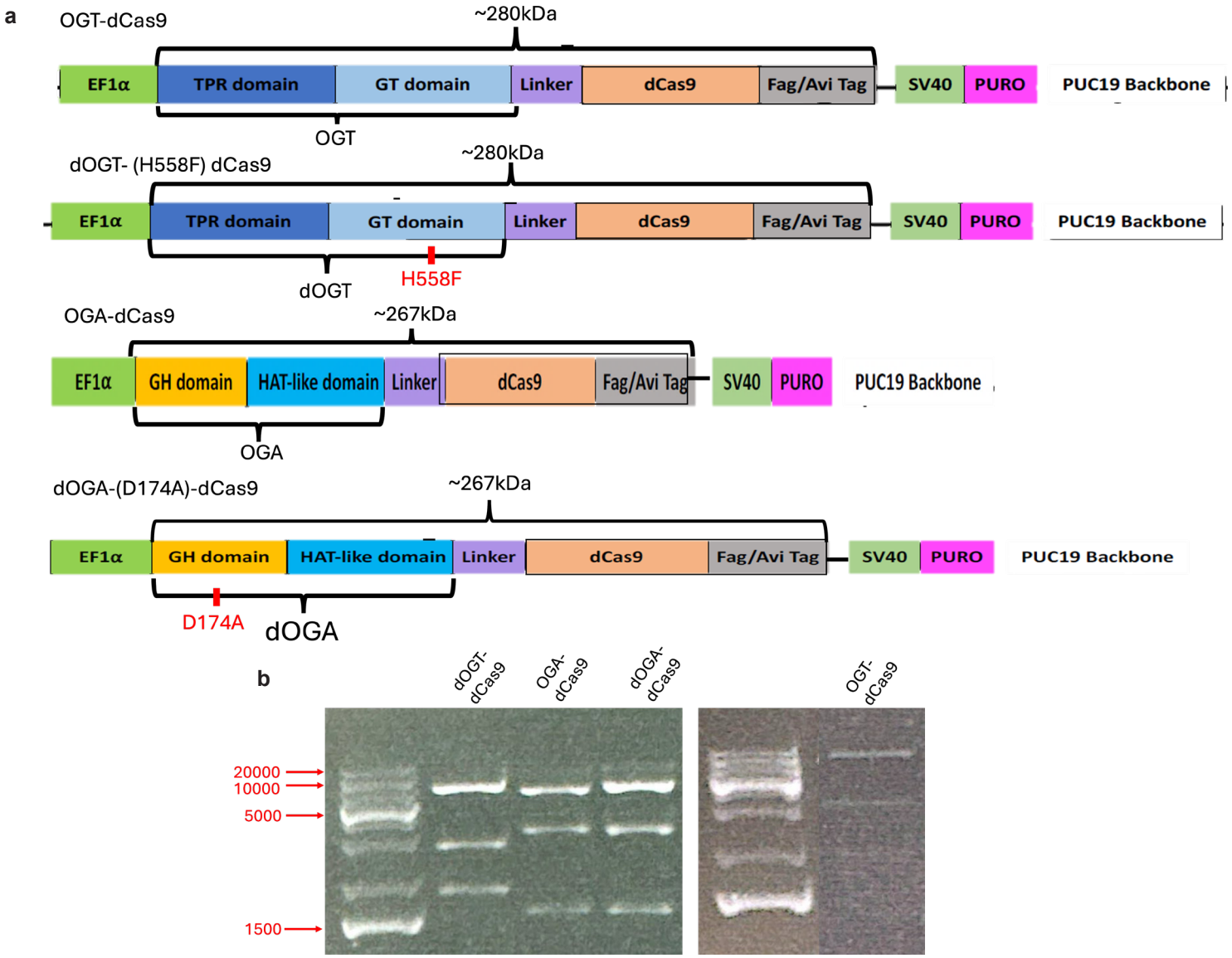


Figure 1 | Linear plasmid map of dCas9 constructs and restriction enzyme digest. a, Linear maps of plasmid dCas9 constructs OGT, dOGT, OGA, and dOGA. **b,** dOGT-dCas9 was cut with enzymes ClaI and KpnI with expected bands at 8055, 2757 and 1794 bp. OGA-dCas9 and dOGA-dCas9 were cut with EcoRV with expected bands at 7431, 3303, and 1512 bp. OGT-dCas9 was also cut with ClaI and KpnI restriction enzymes, with expected bands at 8055, 2757 and 1794 bp.

Sanger sequencing of dCas9 plasmid constructs

Restriction digests were performed on putative positive clones to rule out obviously flawed constructs. However, the OGX/dOGX constructs each produced identical restriction digests, therefore, to further verify that the CRISPR dCas9 plasmid constructs had the correct mutations and DNA sequence, they were sent for Sanger sequencing. An alignment was performed on the OGA portion of OGA-dCas9 against the wild type enzyme. Both constructs should have had a GAT, aspartic acid, at the 174th amino acid, which was confirmed by direct sequencing. At the same position, the dOGA-dCas9 was constructed to include an inactivating mutation, GCT, encoding alanine, which sequencing also confirmed (Fig. 3).

Similarly, OGT-dCas9 includes a CAT codon encoding histidine at the 558th amino acid position of the OGT region. The alignment of sequencing results showed that the OGT-dCas9 had a TTT codon encoding phenylalanine at this position, meaning the 'OGT-dCas9 construct' was a mislabeled dOGT-dCas9 construct. dOGT-dCas9 alignment also included a TTT at the mutation site, confirming that the dOGT-dCas9 construct was labeled correctly (**Fig. 3**). Another mutation was found upstream of both OGT and dOGT -dCas9 at the 498th amino acid where a second histidine to alanine mutation was found (**Fig. 3**). To confirm, multiple aliquots of the same construct were sequenced, and the same mutation was found. This 'mutated' 498th amino acid of OGT matched the OGT RefSeq from NCBI, revealing that the difference found from the original sequence data was due to an allelic variant. Given this, the 498th amino acid was accepted and not changed.

Discussion

Previous research of OGT and OGA have examined the cellular effects of its deletion. Complete OGA knockouts have shown re-

duced OGT expression and undetectable OGA levels.[18] Knockout of OGT in mice have found substantial defects in termination of liver regeneration with increased liver injury.[19] CRISPR-Cas9 has been utilized to create these knockouts and investigate substrate function. Using a CRISPR-dCas9 system provides a new tool for observing O-GlcNAcylation in cells. This project was intended to provide preliminary data to measure the influence of O-GlcNAc regulatory elements' effect on the gene expression of ATF4. The gRNA was successfully inserted into the LentiGuide-hygro vector and is ready for transfection. To transfect with the gRNAs, the CRISPR -dCas9 plasmid constructs must also be set. Based on the restriction enzyme digest of the CRISPR -dCas9 constructs, the resulting band sizes are as predicted, and the expected components are present. From the sequencing of the O-GlcNAc regulatory elements in the CRISPR -dCas9 plasmid constructs, OGA and dOGA have the correct sequences at the mutation site. The construct labeled as OGT-dCas9 was determined to be mislabeled dOGT-dCas9 constructs and a correct OGT-dCas9 was not located. An additional base mismatch at the 498th amino acid was also discovered but was indicative of a 'normal' allelic variant. Therefore, the dOGT-dCas9s is ready for transfecting, but the OGT version will need to be remade. This verification was crucial to ensure differences in gene expression are truly based on the regulatory element. Although this prevented the project from proceeding with transfection, it was necessary to verify these constructs before they could produce confounding results. There are several methods available to rectify the mutation or recreate the construct entirely. With the validated OGT construct in hand, transfection of SH-SY5Y cells may proceed.

Future Directions

To continue this project, a new OGT construct must be made via Gibson assembly or site-directed mutagenesis. Either method will correct the error to generate a new CRISPR-dCas9 construct. When the construct is verified by full plasmid sequencing, H-SY5Y cells can then be transfected by electroporation. To select transformants after electroporation, media will be supplemented with both hygromycin and puromycin will be used. The LentiGuide vector contains hygromycin resistance, which took the place of the puromycin resistance gene in the original construct. Using both antibiotics, only cells that have both plasmids will survive. Additionally, direct confirmation of O-GlcNAc labeling will be necessary to confirm that the model is performing as expected. After selection of stable cell lines, samples can be collected for comparative qPCR and western blots. qPCR of the ATF4 transcripts will allow for an initial quantification of the impact of O-GlcNAc-mediated regulation. Western blots will be used to assay the relative changes in protein abundance of OGT, OGA, and ATF4. Through these two assays, differences in gene expression can be measured based on the regulatory element being integrated into the cell. The possibility that these constructs may lead to unexpected gross alterations in cellular health remains, although ATF4-/- and ATF5-/- animals are viable, suggesting that dysregulation of this pathway will not be toxic to cells.

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A special thank you to my professors Dr. Jack Treml, Dr. Brendan Mattingly, Dr. Melissa Daggett, and Dr. Sonia Thomas, for their guidance during this project. I am also grateful to Dr. Chad Slawson for his mentorship and providing space and resources for me to conduct my capstone project in his lab at the KU Medical Center, as well as Dr. Matt Parker for providing reagents used in this project.

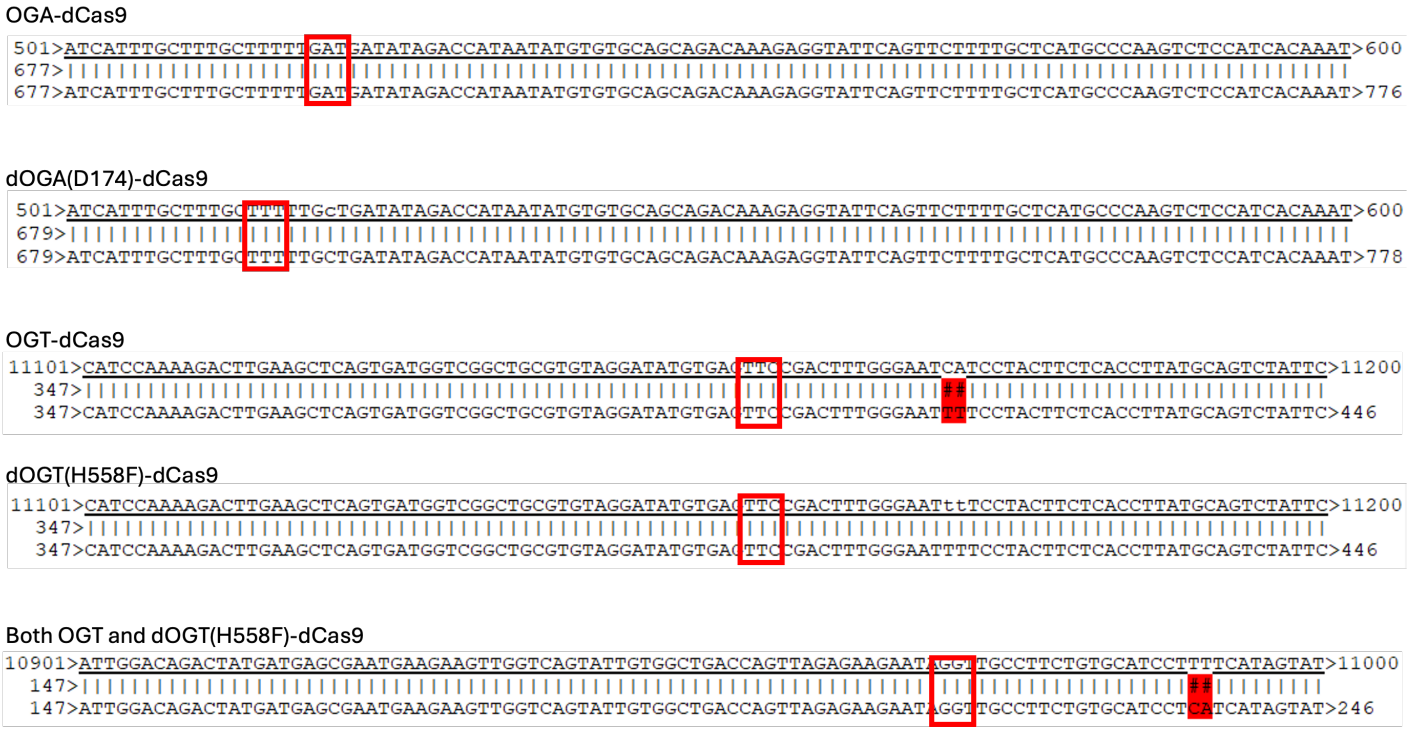


Figure 3 | Sanger sequencing of dCas9 plasmid constructs. Primers were made previously for sanger sequencing and OGT or OGA regions were sequenced. OGT and dOGT –dCas9 had an additional mutation found at the 498th amino acid within the OGT region.

Author Biography

Nathaly is an emerging scientist in the Biotechnology Program at KU Edwards. She has always had a passion for science and research, as well as strategic problem-solving. She is eager to continue learning and expand these interests.

Author Contributions

N.WP. contributed to the design & execution of the work and wrote the manuscript. M.P. providing reagents used in this project. C.C. provided mentoring, funding, reagents, and space. B.M., J.F.T. M.D., and S.T. contributed mentorship and writing.

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Differential oxidation of APE1 suggests oxidation-associated modulation of endonuclease activity

Carson Gray[1], Gopikaashri Chidambaranathan[2], Nate Tolla[3], B. Mattingly[1], M. Daggett[1], S. Thomas[1], B. Freudenthal[4], and JF Treml[1†]

Apurinic/apyrimidinic endonuclease 1 (APE1) is an essential enzyme in the base excision repair (BER) pathway, hydrolyzing the phosphodiester backbone upstream of abasic sites. Beyond its canonical repair role, APE1 also acts as a redox-dependent regulator of multiple transcription factors, leading to its alternative designation, redox effector factor 1 (Ref-1). Given its dual roles in genome maintenance and transcriptional control, APE1 dysregulation has been strongly implicated in cancer and other diseases. When enacting its redox function, APE1 undergoes a redox cycling mechanism which has been proposed to involve the formation of intramolecular disulfide bonds. Of note, the nucleophilic cysteine is not solvent accessible, and the other redox active cysteine residues are too distant for covalent interaction in its native conformation. Consequently, a conformational shift may be required to accommodate the chemistry of substrate reduction. We propose that APE1's endonuclease role may be influenced by oxidation states that emerge from its function as a redox regulator, potentially leading to distinct redox-dependent functional states. To investigate this possibility, we exposed APE1 to mildly oxidizing conditions for various lengths of time and quantified its relative endonuclease activity. Interestingly, over a short period of H^2O^2 treatment, APE1 consistently exhibited higher relative endonuclease activity; however, longer periods of treatment resulted in inconsistent endonuclease activity across experimental groups. These results indicate that APE1 activity is associated with mild H^2O^2 treatment conditions and may increase under short exposure times, although oxidation state was not directly measured and conditions are inferred from prior studies of similar H^2O^2 exposure.

Oncogenesis arises due to a complex combination of interrelated factors, but frequently involves some form of DNA damage.1 One of the most prevalent sources of such damage is oxidative stress, which occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of cellular antioxidant systems, resulting in the accumulation of reactive species that can damage nearby biomolecules, including DNA and proteins.2 These interactions can give rise to a variety of DNA lesions, including oxidized bases and abasic sites, which, if left unrepaired, can compromise genomic integrity and contribute to carcinogenesis.1,2

Cells possess sophisticated mechanisms by which they recognize and repair DNA damage. One such mechanism is the base excision repair (BER) pathway, which can accurately identify and replace singular damaged or incorrect nucleotides. In mammals, the damaged base is removed by a damage-specific glycosylase, followed by backbone hydrolysis by APE1 to generate a 3′-hydroxyl and an internal 5′-deoxyribose phosphate terminus, which is subsequently removed before the missing nucleotide is replaced by DNA polymerase β. Finally, DNA ligase III seals the nick at the 3' end of the new base.3 This pathway is vital to the development and survival of organisms, as evidenced by the blastocyst-stage lethality of murine APE1 knockout models.4 Furthermore, it has been found that loss of Ape1 after birth in the neural tissue of mice leads to the rapid and widespread development of brain tumors.5 Interestingly, APE1 is overexpressed in certain cancers, potentially serving a protective role, and there is interest in developing drugs that target APE1 for cancer treatment.6,7 Taken together, these observations suggest that APE1 has a complex and context-dependent role in cancer development. High-resolution structural and kinetic data demonstrate the specificity with which APE1 recognizes and cleaves its substrate, and it has been found that several pathological mutations of APE1 have the effect of reducing its endonuclease activity.8,9 Working towards a more complete understanding of the role of APE1 in oncogenesis is thus of high importance and relevance.

While APE1 has traditionally been associated with the BER pathway, it is also known to modulate the expression of several genes by reducing inactivating disulfide linkages in their transcription factors, including AP-1.10 APE1 enacts this function through a redox cycling mechanism that has been proposed to involve intramolecular disulfide bond formation; Cys65, acting as the nucleophilic cysteine, attacks one of the sulfur atoms involved in the substrate disulfide bond, leading to the formation of a mixed disulfide bond between APE1 and the substrate. To release the activated substrate, a resolving cysteine, potentially Cys93 or Cys99, may attack Cys65, allowing the substrate to dissociate and leaving

[1]University of Kansas, Edwards Campus, Biotechnology
[2]Washburn Rural High School
[3]Mill Valley High School
[4]University of Kansas Medical Center
[†]Corresponding Author jtreml@ku.edu

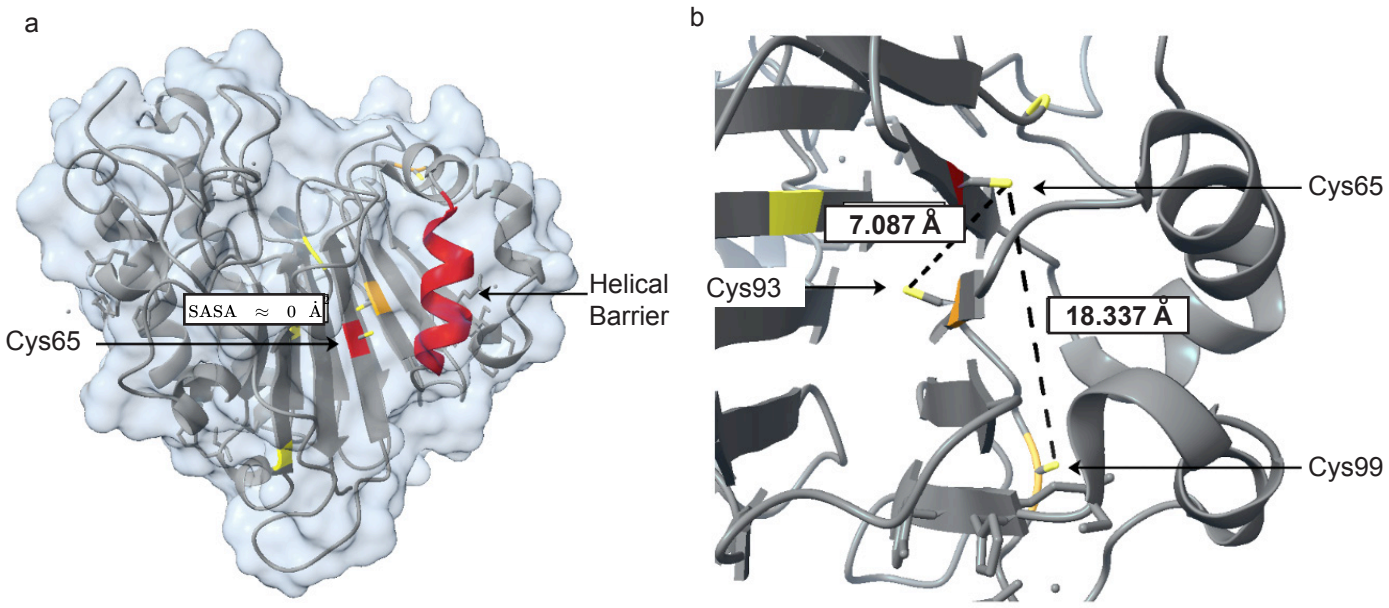


Figure 1 | APE1 Structure and Cystidine locations
(a) Characterization of Cys65 solvent accessibility and adjacent helical barrier.
(b) Distances between Cys65, Cys93, and Cys99

APE1 in an oxidized disulfide state.11,12 A growing body of research has demonstrated the multifunctionality of APE1 in regulating cellular physiology under oxidative stress, including mitochondrial function, neural survival, and cell cycle progression.13–15

In the native state of APE1, Cys65 is positioned within an interior β-sheet, with Cys93 located on the opposite side of the sheet face. Cys99 is located on a connecting loop relatively nearby. These structural features complicate mechanistic interpretations of its redox function, as Cys65 is not accessible to the solvent, and Cys93 and Cys99 are too distant to form covalent interactions with it. There is evidence to suggest that APE1 can assume a partially unfolded state, which may allow Cys65 to act as a nucleophile and form covalent interactions with the resolving cysteines.16 This suggests that APE1 may exist in an equilibrium between its native reduced form and alternate conformational states potentially associated with redox activity.

We hypothesized that APE1 may exhibit altered endonuclease kinetics under oxidizing conditions relative to untreated protein. Our data indicate that APE1 endonuclease activity is increased following short exposure to mildly oxidizing conditions, suggesting that APE1 activity may be correlated to its oxidation state. While oxidation state was not directly confirmed, we believe this work prompts further investigation into whether APE1 endonuclease activity is modulated by its redox-regulatory function.

Materials and Methods

Bacterial Expression of APE1

pET28HIS-hAPE1 was a gift from Primo Schaer (plasmid # 70757 ; http://n2t.net/addgene:70757 ; RRID:Addgene_70757), which was used to produce His-tagged APE1 in a bacterial expression system. Kanamycin (K1377-1G, Sigma-Aldrich) used at a working concentration in all media at 50 µg/mL. All bacterial cultures were grown in Luria–Bertani (LB, 12780052, Invitrogen) broth or agar supplemented with kanamycin unless otherwise specified. Individual bacterial colonies were inoculated into 10 mL LB medium containing kanamycin and incubated at 37 °C with shaking (200 rpm) for 3–5 h. For larger starter cultures (10–40 mL), a single colony was inoculated and incubated for 4–6 h. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.55–0.6; growth beyond this point was avoided to ensure optimal protein expression. One milliliter of each culture was centrifuged at 16,000 x g for 5 min, and the resulting cell pellet was stored at –20 °C. The remaining culture was either used immediately for inoculation of expression cultures or stored at 4 °C overnight.

Starter cultures were used to inoculate larger expression cultures in LB medium containing kanamycin, using the following ratios: 1 mL starter culture per 50 mL, 5 mL per 250 mL, and 20–40 mL per 1–1.5 L culture. Cultures were incubated with shaking at 37 °C until reaching an OD_{600} of 0.5–0.6.

Expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, 15529019, Invitrogen) at 20 °C and incubated for 12–18 h with shaking.

Cells were harvested by centrifugation at 3,500 × g for 20 min at 4 °C and either processed immediately or stored at –20 °C.

Cell pellets were resuspended in His-tag lysis buffer [50 mM Tris–Cl (pH 8.0), 1 mM imidazole, 100 mM NaCl, 0.1 mM EDTA] at a ratio of 2–10 mL buffer per 20–100 mL of culture, 10–25 mL buffer per 100–500 mL, or 25–50 mL buffer per 1–2 L culture. Immediately prior to use, phenylmethylsulfonyl fluoride (PMSF, AB14103210G, Abcam) was added to 1 mM and β-mercaptoethanol(21985023, Gibco) to 10 mM.

Cells were lysed by addition of lysozyme to a final concentration of 0.5–1 mg/mL, followed by incubation on ice for 30 min with gentle mixing. Samples were sonicated on ice (3 × 2 min bursts) and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was filtered (0.45 µm) and used for affinity purification.

Nickel–nitrilotriacetic acid (Ni–NTA, PI88221, ThermoScientific) resin was gently resuspended, and 1–2 mL of slurry was added to a chromatography column. The resin was equilibrated by washing with 5 column volumes (CV) of water, followed by 5–10 CV of lysis buffer. Clarified lysate

was applied to the column and allowed to pass through by gravity. The resin was washed with 10 CV of wash buffer [50 mM Tris–Cl (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, and 1 mM PMSF].

Bound protein was eluted with 5 CV of elution buffer [50 mM Tris–Cl (pH 8.0), 50 mM NaCl, 300 mM imidazole, 0.1 mM EDTA, and 1 mM PMSF]. Eluted fractions were analyzed by SDS–PAGE, and fractions containing APE1 were pooled. Imidazole was removed by dialysis against storage buffer [50 mM NaCl, 1 mM dithiothreitol (DTT), 0.05 mM EDTA, and 10% glycerol].

Dialysis tubing was pre-equilibrated in buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl). Samples were dialyzed against 100 volumes of replacement buffer at 4 °C with three buffer changes over ~20 h (2 h, 2 h, and overnight). Protein concentration was determined by spectrophotometry, and samples were concentrated to 2–3 mg/mL.

Preparation of Duplex DNA Substrates

Oligonucleotides were stored at 100 µM in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). For duplex formation, complementary oligonucleotides were mixed to final concentrations of 12 µM (template) and 10 µM (FAM-labeled THF abasic construct, ThermoScientific) in TE buffer. Samples were annealed by heating to 98 °C and cooled to 25 °C at 1 °C/min in a thermal cycler. Aliquots were frozen at -20 °C for later use.

Controlled Oxidation and Endonuclease Digestion

Reactions were performed in 2x base excision repair (BER) buffer [40 mM HEPES–KOH (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin]. Hydrogen peroxide (1 mM final; 0.034% w/w) was freshly prepared from a 30% stock.

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Purified APE1 (10 µM) was pre-incubated with 1 mM H_2O_2 for 0, 5, 15, or 30 minutes at room temperature. Following oxidation, excess H_2O_2 was quenched with catalase prior to activity assays. 10 µL duplex DNA (400 nM) was mixed with 20 µL 2X BER buffer. 10 µL of APE1 (20 nM) was added to each reaction, and the mixture was incubated at 37 °C for 8 minutes before being quenched with 40 µL of 0.5 M EDTA at pH 8 and placed on ice. For each run, an additional reaction was prequenched for a negative control, and another was allowed to go to completion (40 minutes). 160 µL of loading dye (98% formamide, 0.25 mg/mL bromophenol blue) was added to each sample before proceeding to electrophoresis.

Denaturing PAGE and Detection

Reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) using 20% denaturing gels containing 8 M urea in 1× TBE buffer. Prior to loading, the gels were pre-run at 300 V for 30 minutes, each lane was syringe-rinsed with 1x TBE, and samples were heated to 85 °C for 4.5 min and snap-chilled on ice.

Electrophoresis was performed at 300 V until the bromophenol blue dye front reached approximately 80% of the gel length (~1.5 hours). The gels were gently agitated in 1X TBE for 30 minutes before analysis. Band intensities were quantified by densitometry using a LI-COR imaging system.

Kinetic Data Analysis and Statistical Significance

When analyzing the kinetics gels, three bands were present. Substantial evidence suggested that the two bands with the lowest electrophoretic mobility corresponded

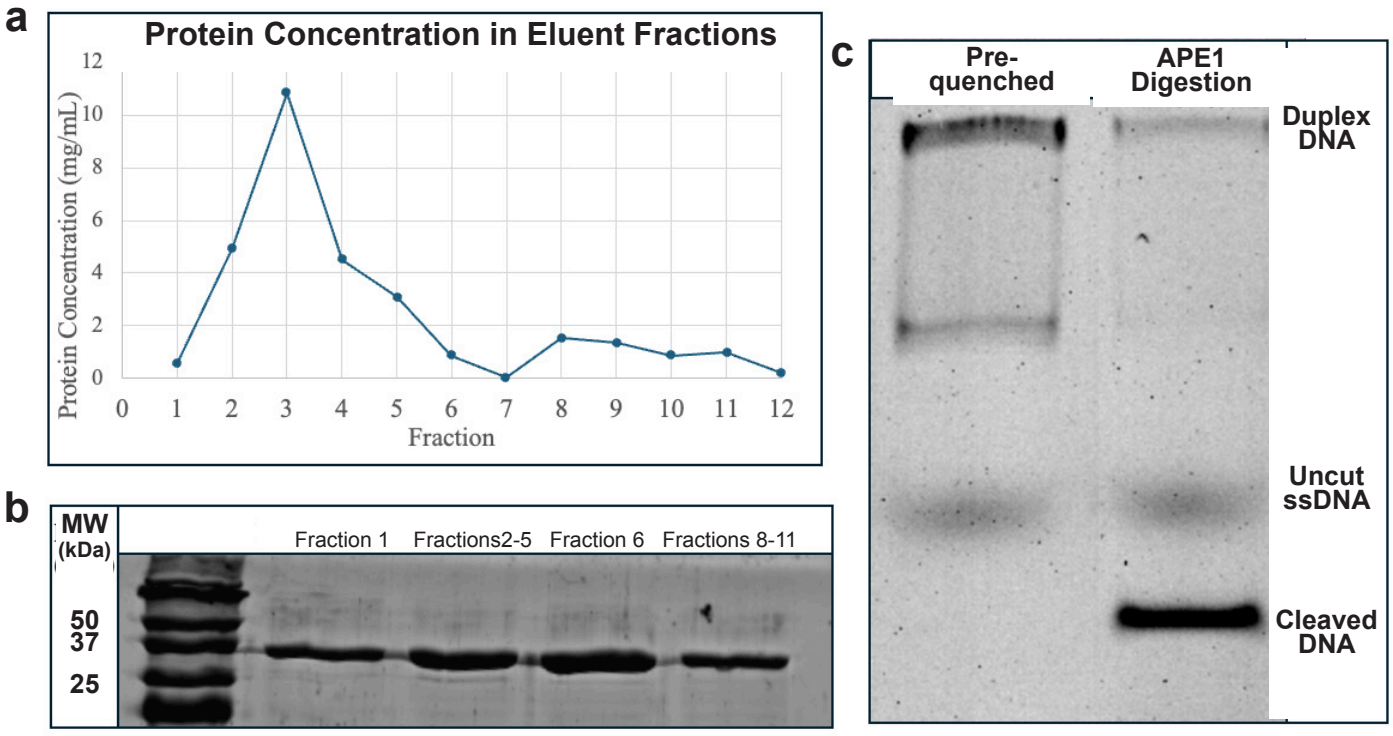


Figure 2 |APE1 Purification. (a) Concentration of APE1 fractions eluted from affinity column as determined by absorbance at 280 nm. (b) Coomassie-stained SDS-PAGE gel showing samples from fraction 1, fraction 6, and grouped samples of fractions 2-5 and 8-11. (c) PAGE gel of FAM-labeled DNA pre-quenched versus APE1 digested.

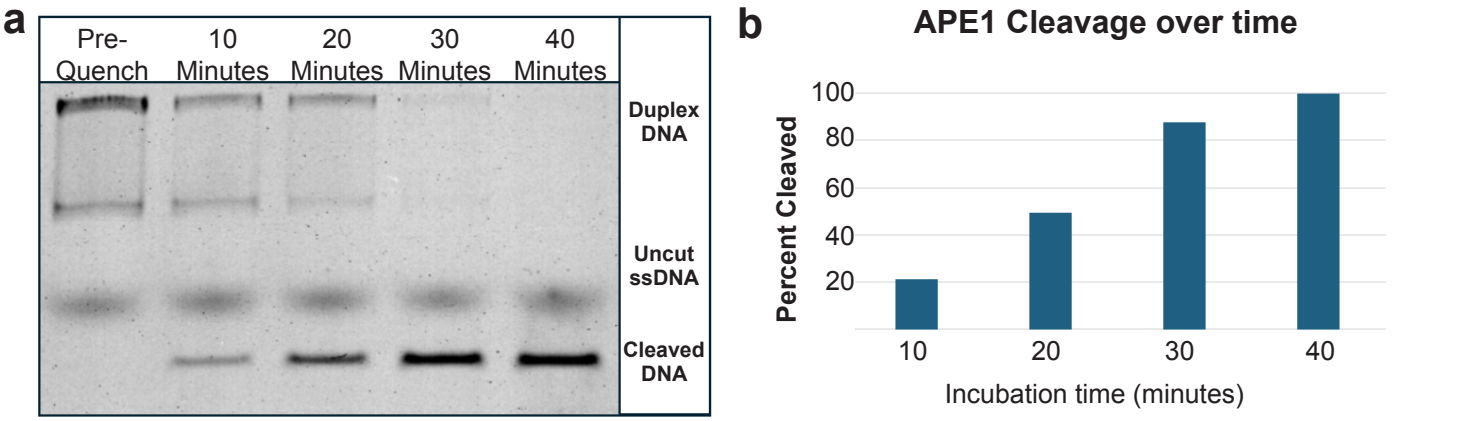


Figure 3 | APE1 Cleavage over Time. (a) Denaturing DNA PAGE of duplexed FAM-labelled oligos incubated with APE1 for various time points. The highest band is believed to be uncut duplex DNA, the middle band single stranded uncut DNA, and the bottom-most band cleaved single-stranded DNA. (b) Percent cleavage of APE1 digestion time points.

to single-stranded or duplex uncut FAM-labeled oligonucleotides, while the band of highest electrophoretic mobility corresponded to single-stranded cleaved oligonucleotides. For gel quantification, band intensities were measured using defined regions of interest (ROIs) corresponding to cleaved and uncleaved species, with identical ROIs applied across all lanes for consistency. Background signal was estimated from equivalent ROIs in regions lacking specific bands and subtracted from corresponding experimental intensities. The intensities of the uncleaved and cleaved ROIs was used to determine total signal, and the proportion of cleaved substrate was calculated as the cleaved intensity divided by the sum of cleaved and uncleaved intensities. This value was then normalized to the corresponding non-oxidized control to obtain relative activity. Statistical significance was assessed using a one-sample t-test comparing normalized relative activity values to an expected mean of 1, with p-values calculated using Microsoft Excel. The t-statistics were obtained using the standard one-sample t-test equation, where n represents the number of independent experimental replicates:

$$t = \frac{\bar{x} - 0.1}{S/\sqrt{n}}$$

Results

APE1 Expression

APE1 was expressed in a bacterial system and purified using Ni-based chromatography. Purified fractions were analyzed using PAGE, and concentrations were determined spectrophotometrically. All tested fractions produced bands indicating a protein with a molecular weight slightly less than 37 kDa, which is consistent with APE1 (Mr = ~35.5 kDa).

To ensure that the purified APE1 was catalytically active, a THF abasic analogue-containing FAM-labelled duplex DNA strand was incubated at 100 nM with 5 nM APE1 at 37 °C for 40 minutes. This was compared to a prequenched solution containing the same components. Of the three bands present, the two with lower electrophoretic mobility decreased as the intensity of the higher-mobility band increased . The uncut DNA may not fully denature under the given conditions, leading to the formation of two bands; however, because the bands disappear after longer incubations (See **Fig. 2**), the cut DNA is likely to fully denature.

Estimation of Enzyme Kinetics

An identical reaction was quenched after varied lengths of incubation to establish the period of kinetic linearity. The time points tested were 10, 20, 30, and 40 minutes. The reaction appeared to reach completion by 40 minutes, and cleavage appeared to be approximately linear within the 0–30-minute range. To ensure that the enzyme maintained constant velocity, 8 minutes was chosen to be the experimental incubation time for comparison across differentially oxidized species. The APE1-dependence of results and quenching efficacy was established using a prequench control and a fully cleaved control were included in every experiment.

APE1 was incubated in hydrogen peroxide for 0, 5, and 15 minutes before being quenched with catalase. After incubation with the model substrate, reactions were quenched and subjected to denaturing PAGE. FAM-based fluorescent intensity was quantified at regions of interest corresponding to uncut and cut fragments. Cleavage percentages were calculated for all groups and were normalized to the 0-minute oxidation control. A statistically significant increase in endonuclease activity was observed following 5 minutes of H_2O_2 treatment, while there was no significance found in the 30-minute group (n = 3).

Jurkat E6.1 T cells experience quantitatively different signaling after exposure to acidic environments

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Discussion

Fractions collected during APE1 purifications all exhibited a band corresponding to

APE1's molecular weight, suggesting that APE1 was successfully purified from the bacterial expression system. After dialysis, the solution was tested for APE1 endonuclease activity by adding a FAM-labeled THF-analogue model substrate. When the products were run by PAGE the solution was found to produce cleaved substrate. Furthermore, percent cleavage increased with incubation time prior to an EDTA quench, suggesting that the system is time-dependent and reliably quantifiable. Taken together, these results suggest that we were able to successfully purify active APE1 and could quantify its endonuclease activity.

When the endonuclease activity of H_2O_2-treated APE1 was compared with that of untreated APE1, the 5-minute time group consistently exhibited a roughly 20% increase in activity compared to the untreated APE1. This was surprising, as most enzymes tend to become less active in oxidizing conditions. However, since APE1 experiences oxidation because of its canonical redox activity, these results may reveal oxidation-dependent regulation of APE1 function. Specifically, APE1's endonuclease activity may be enhanced under mildly oxidizing conditions. We speculate that this effect may be related to its physiological redox states. Although the redox state of APE1 was not directly measured, our conditions were based on previous work that analyzed disulfide formation over time using tandem MS/MS.17 While still speculative, this would be consistent with the increased biological need for DNA repair due to oxidation-related accumulation of nucleotide damage.

Because APE1's redox state was not directly measured and residual DTT was present, the redox environment may have differed among groups. Furthermore, H_2O_2 may impact protein structure and DNA binding through mechanisms independent of specific cysteine oxidation. Given these limitations, Further work is needed to confirm APE1 oxidation state and determine its relationship to endonuclease activity . While limited in scope, these results may provide early evidence for an unexplored aspect of APE1 biology.

Future Directions

As noted throughout this manuscript, our work was limited by the inability to directly measure oxidation state alongside the observed changes in endonuclease kinetics. The most important next step would be to couple the kinetics assay with a method to directly assess APE1 redox state (e.g., thiol-specific labeling or mass spectrometry-based oxidation mapping). Direct measurement would resolve this important source of uncertainty in future work. Additionally, even if a relevant redox state was definitively connected to altered endonuclease activity, this does not necessarily establish biological relevance. If the change is only moderate, or if the rate of another step dominates the overall rate of BER, it may not influence DNA repair. To establish physiological relevance, an inhibitor specific to APE1's redox function (without affecting endonuclease activity) could be used to assess changes in DNA repair efficiency in vitro.

Author's Biography

Carson Gray is passionate about the molecular basis of biology, with a particular interest in the structure and physics of biomolecules. He has been admitted to the IGPBS program at KU Medical Center, where he plans to obtain his PhD in biochemistry or a related field.

Author Contributions

C.G. contributed to the design & execution of the work and wrote the manuscript. G.C. contributed to the protein structure figures and and related work. N.T. contributed to enzyme kinetics and related work. B.M.,, M.D., S.T., B.F., and J.T. contributed mentorship and reagents.

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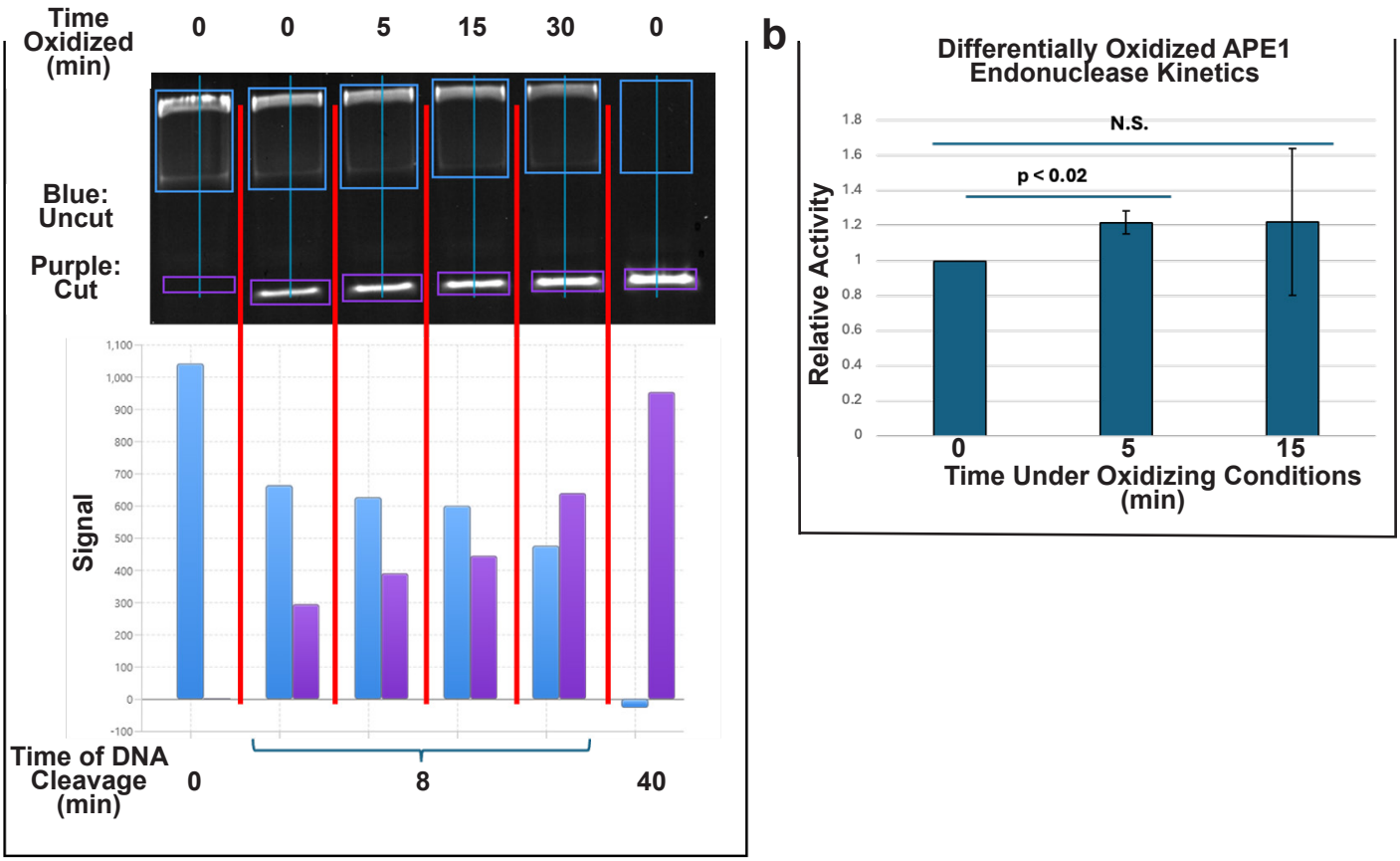


Figure 4 | Enzyme Kinetics. a Representative image of kinetics data showing AOI for uncut (blue) and cut (purple) DNA fragments. Fluorescent signal of these bands were first used to calculate percent cleavage, and were compared to the 0 min oxidized 8 min cleaved control to determine relative activity. **b** Relative activity of APE1 oxidized for 0, 5, and 15 min. 5 min oxidized APE1 exhibited a statistically significant enhancement of endonuclease activity ($p < 0.01$), while 15 min oxidized APE1 lacked statistical significance.

Gut Feeling or Good Science: The Impact of Probiotics on Inflammation

Arianna Theleman[1], S. Thomas[1], and JF Treml[1†], M. Daggett[1] , B. Mattingly[1]

Probiotic use has been promoted for numerous health benefits, however, there is limited research supporting the claims for reduced inflammation. We set out to determine if there was a correlation between probiotic exposure and inflammatory signaling in immune cells, comparing the changes in pro-inflammatory and anti-inflammatory cytokine production. Pre-inflamed macrophages were treated with the probiotic strain *Lactococcus lactis*, isolated from a probiotic yogurt culture and identified using 16S rRNA sequencing, to determine the immune response produced when introduced to the probiotic. RNA was isolated from macrophages and cDNA was generated to detect a change in cytokine transcription through RT-qPCR, and ELISA was used to measure secreted cytokines associated with inflammation to assess the relative abundance in response to the addition of a probiotic. Results were analyzed to determine if there was a change in the inflammatory response of immune cells when presented with a probiotic finding that there may be time dependent modulatory changes in inflammatory cytokine signaling.

Pseudoscience has been of great interest to researchers and has captivated individuals through highly desirable claims of healing and relief for hundreds of years. However, some of these claims come with exaggerated benefits and false scientific legitimacy, and these 'quick-fix' products undermine the initiatives of long-term behavioral health. Products that have been widely popular in recent past include ear candles, DMSO, and feet pads, to name a few. These products were marketed for their abilities to remove toxins from the body, help relieve pain, and even possibly cure cancer, although there was never any solid scientific evidence to back these claims. It is easy to look at these products and trends and laugh, but what about products that may be more difficult to distinguish. They aren't obvious scams, but there is also not a lot of evidence to support them either.

Microbiome health has been a focus of many studies in recent years, and researchers have found that healthy and balanced microbiomes can lead to improved digestion and nutrient absorption, help reduce inflammation, support immune systems in identifying and fighting off harmful pathogens, and even has associations with mental health issues when unbalanced.[1,2] One product that has been highly popularized on the market for its potential benefits to gut health, reduced inflammation, improved immunity and mental health boosts are probiotics[3,4] Probiotics are live microorganisms that provide benefits to their host.[5,6] Probiotics claim to help with restoring microbiomes to normal levels, improve symptoms of inflammation by lowering/improving cytokine signaling, *etc*.[7,8] They are found in so many of the products available for us to consume; yogurt, supplements, kombucha, kefir, and many more.[9,10] While science does support the importance of a healthy microbiome, how much does the consumption of probiotics actually help? Are these benefits due to the probiotic, or an already healthy microbiome?[11]

One of the more popular claims for probiotic use is to reduce inflammation. In circumstances of chronic inflammation, serious damage can occur due to high levels of cytokine signaling and probiotics are said to help reduce these pro-inflammatory signals.[12] However, minimal research provides a lack of supporting evidence to these claims of reduced inflammation. The results that these studies have published are inconsistent and lack statistical significance.[13,14,15] The goal for this research project was to look at the modulatory effects probiotics have on inflammatory signals released by immune cells when interacting with each other in the same environment by measuring the production of inflammatory cytokines by macrophages. By culturing macrophages, immune cells that contribute substantially to inflammatory responses in the gut, and measuring their cytokine release after exposure to a probiotic under inflammatory conditions, we aim to determine whether the probiotic produces measurable anti-inflammatory effects. If the claims are true, it is expected to see a statistically significant reduction of pro- inflammatory cytokines, as well as an upregulation of anti-inflammatory cytokine signaling reflecting a reduced state of inflammation. Further testing will be conducted to test the cells' response to the probiotic introduced in different forms, i.e. live, heat killed, or an acellular culture supernatant. It is of interest to determine if there is a change in the benefit received when the probiotic is dead versus alive, and to understand if this impacts the immune response produced by our macrophages.

[1]University of Kansas, Edwards Campus, Biotechnology

[†]Corresponding Author jtreml@ku.edu

Materials and Methods

Growth of Probiotic from Serial Dilutions of yogurt

Growth plates were made using Lactobacilli MRS Agar (Becton, Dickinson, Lot 5293921) or LB agar media (Fisher Scientific, Lot 242143). For the MRS plates

ultrapure water was added and media was autoclaved before plates were poured. The LB plates were made with LB agar and ultrapure water, adjusted to pH 7.2, then autoclaved using the liquid 15 setting on the autoclave. Once media finished in the autoclave, it was removed, allowed to cool slightly, then plates were poured aseptically to about 20 mLs of media per plate, cooled overnight and left to sit for 48 hours before use. Probiotic yogurt samples were diluted by a 10-fold dilution series from 1:10 to 1:100000. 100 µL of each dilution was added to an agar plate and aseptically spread across surface with glass spreader. These plates were then incubated at 37 °C for 48 hours. Colonies were picked and streak plates made to isolate bacteria strains. Repeat process of making streak plates 2-3 times to be sure that isolated colonies only contain one bacterial strain.

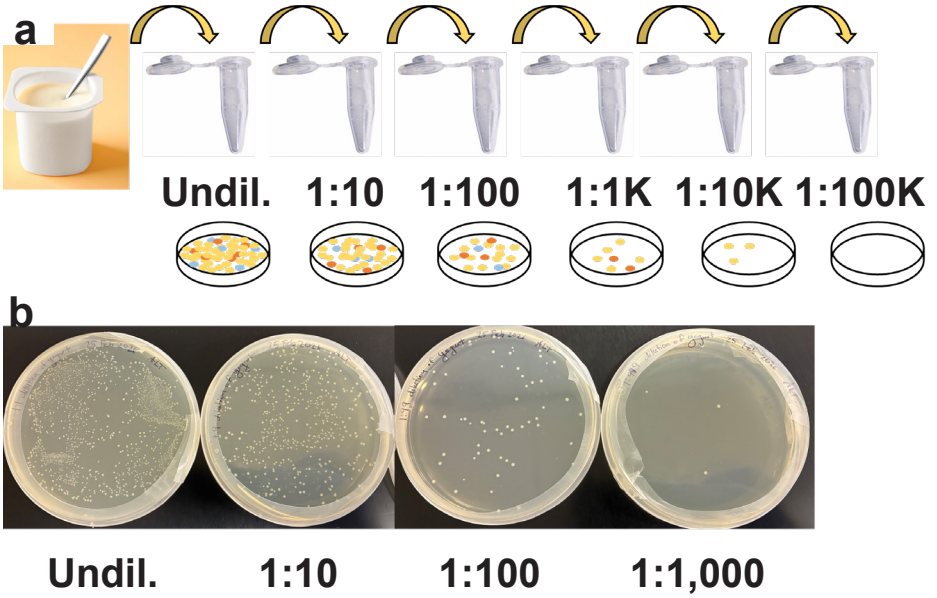


Figure 1 | Fishing experiment serial dilution. Probiotic candidates were isolated from a yogurt sample to be tested and later identified. **a,** A serial dilution from a sample of yogurt was performed and samples were plated for colony growth **b,** Plates depicted are LB media grown for 48 hours.

PCR Amplification of 16S rRNA gene for probiotic identification

Using colonies from streak plates, isolate DNA using a DNA Isolation protocol. Pick a colony of bacteria and resuspend in 1 mL of dH_2O. Centrifuge for one minute at 12000x g, saving pellet and discarding the supernatant. Add 200 µL InstaGene Matrix (BioRad, batch# 64573327) to the pellet and incubate at 56 for 30 minutes. Vortex cells at high speed for 10 seconds, and incubate at 100 °C for 8 minutes. Vortex again at high speed for 10 seconds and then centrifuge for 3 minutes at 12000x g. DNA is in the supernatant.

Once DNA is purified run PCR using 10 µL of GoTaq Green Mastermix (Promega, Lot 0000127680), 1 µL of 27FHT & 1942RHT primers (amplifies the 16S rRNA gene), 6 µL of DNA in InstaGene supernatant, 3 µL of dH_2O. The thermocycling reaction utilized a 56 °C annealing temperature to amplify product.

Gel electrophoresis to verify amplification of 16S rRNA PCR product and cytokine amplification

Electrophoresis was performed using a 1.2% agarose gel (Fisher Scientific, Lot 188373) in TBE (prepared by Dr Daggett) against a 1 kb plus DNA ladder. Samples were run for one hour at 100V and visualized with UV.

Growth and differentiation of THP-1 Monocytes

Sterile 1640 media (Gibco, Lot 3267238) with 10% Fetal Bovine Serum, and pen strep (Gibco, Lot 268394) was used to grow THP-1 monocytes in T-25 or T-75 flasks, starting at ~$8x10^5$ cells/mL (passing density) at 37 °C in 5% CO2, and were passed every 2-3 days into a new flask with fresh media.

To differentiate the THP-1 monocytes to M0 macrophages, 100ng/mL PMA (Sigma, Lot MKCL1145) was added to cells at $0.5x10^6$ cells/mL complete RPMI media. Cells were allowed to rest for 4 hours at 37 °C to allow for adherence to the flask. Stimulating media was removed and cells were gently washed in room temp or slightly warmed PBS pH7.4 (Gibco, lot 2412443). Cells were then provided fresh RPMI complete media and incubated for 24 hours. To further differentiate the M0s to M1 macrophages, media was removed and fresh media with LPS was added to a final concentration of 250 ng/mL. Cells were allowed to grow an additional 48 hours to differentiate.

RNA Isolation and purification

RNA was isolated using the Monarch Total RNA Miniprep kit (Monarch, #T2010S, Lot 10214460). Cells were pelleted and RNA isolated for cDNA generation. Nanodrop RNA to test for purity and to determine concentration.

cDNA generation

cDNA was generated using iScript super mix (BioRad, Batch 64622404). A no-RT control was included to test for the presence of DNA. Reverse transcription was performed using the cDNA thermocycler program: Priming 25 °C x 5' > Reverse transcription 46 °C x 20' > RT inactivation 95 °C x 1'

qPCR

qPCR was performed using SYBR Green qPCR Master mix (Fisherbrand, Lot 2794602 and Lot 3058486), 1 µL of the corresponding primers (F/R), 1 µL of cDNA and 8 µL deionized H2O to a total of 20 µL per well. Using the cDNA generated previously, qPCR was performed for beta actin (as a housekeeping gene), TNF-α, IL-1β, IL-6 (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine). Cytokine primers were designed on Benchling, purchased from Integrated DNA Technologies, and reconstituted to contain both forward and reverse primers at a final concentration of 200 pmol/µL. Reaction was run following the qPCR program:

Initial denaturation (1 cycle)	
	95 °C x 5'
Amplification	(30 cycles)
	denaturing
	94 °C x 30"
	annealing
	55 °C x 30"
	extension
	72 °C x 90"

Plate Read
Final extension (1 cycle)
Melt Analysis

Culturing M1 macrophages with *Lactococcus lactis*

M1 macrophages were grown with the *L. lactis* by seeding M1 cell into wells at a minimum density of $0.5x10^5$ cells per mL in RPMI media (no additives). For each condition, probiotic cells were added in a 10:1 [probiotic:M1] ratio. For the heat killed condition, *L. lactis* was boiled for 8 minutes in the thermocycler prior to adding to M1 cells. The supernatant condition contained probiotic conditioned media spun down for 3 minutes at 14000x g to pellet bacteria, only supernatant was collected. All conditions were incubated at 37 °C for 4 hours before removing cells for the initial RT-qPCR, or for 24 hours before pulling media for ELISA and cells for RT-qPCR.

Quantibody ELISA Array

The ELISA array was completed following the protocol as written in the RayBiotech Quantibody Human Inflammation Array Manual, Catalog #: QAH-INF-1 (RayBiotech, Lot 03102610051). Starting protein concentrations were calculated after running a BCA Protein Assay for normalizing protein concentrations. Samples were incubated for three hours at concentrations of 1:10 and 1:10000 dilutions. Detection antibody cocktail was allowed to incubate for two hours, and Cy3 dye-conjugated streptavidin was incubated for 1 hour. All wash steps were performed as instructed in the manual. ELISA slide was shipped back to RayBiotech for imaging and fluorescence results were sent back for analysis.

Results

16S Sequence data confirms isolated probiotic as *Lactococcus lactis*

A goal of this study was to identify one or more probiotic strain(s) present in the probiotic yogurt product to test with macrophages and measure the changes in immune response signaling of cytokines. To do so, a 16S PCR and sequencing reaction was performed. Samples were initially isolated following the serial dilution scheme as depicted in **Fig. 1**, and were further isolated using a streak plate method to confirm each candidate colony was a single strain of bacteria. These colonies were then grown and DNA isolated and amplified using PCR to amplify the 16S rRNA gene (~1500 bp). The amplification products were run on a 1.2% gel (**Fig. 2a**) to verify amplification of the product, and select samples were sent off for sequencing. The returned sequencing results were then analyzed and identified. The colony assayed in lane B resulted in sequencing results (**Fig. 2b**) and the sequence was run through NCBI Blast, resulting in a match to *Lactococcus lactis*. This colony was utilized for further testing with the macrophages.

Addition of LPS differentiates M0 macrophages to M1s

In keeping with the inflamed environment expected of the gut of an individual suffering from chronic inflammation, macrophages were successfully differentiated from the THP-1 monocytes utilizing Phorbol 12-myristate 13-acetate (PMA), which was determined visually as non-adherent THP-1 cells differentiate into adherent M0s, LPS was added to the cell media to further differentiation into M1s, which are macrophages that have increased pro-inflammatory signaling. To verify that the addition of LPS did cause the M0s to shift to M1s, an RT-qPCR analysis was run to compare the production of mRNA template for the cytokines TNF-α, IL-1β, IL-6 and IL-10. All cytokines except IL-6 (due to lack of amplification) were seen to be upregulated in the M1 macrophage condition (**Fig. 3c**) compared to M0 controls. This provided confidence to adopt the differentiation protocol and test the M1 cells with the probiotic strain.

Culturing M1s with *L. lactis* appears to create a time dependent modulatory effect on cytokine mRNA transcription.

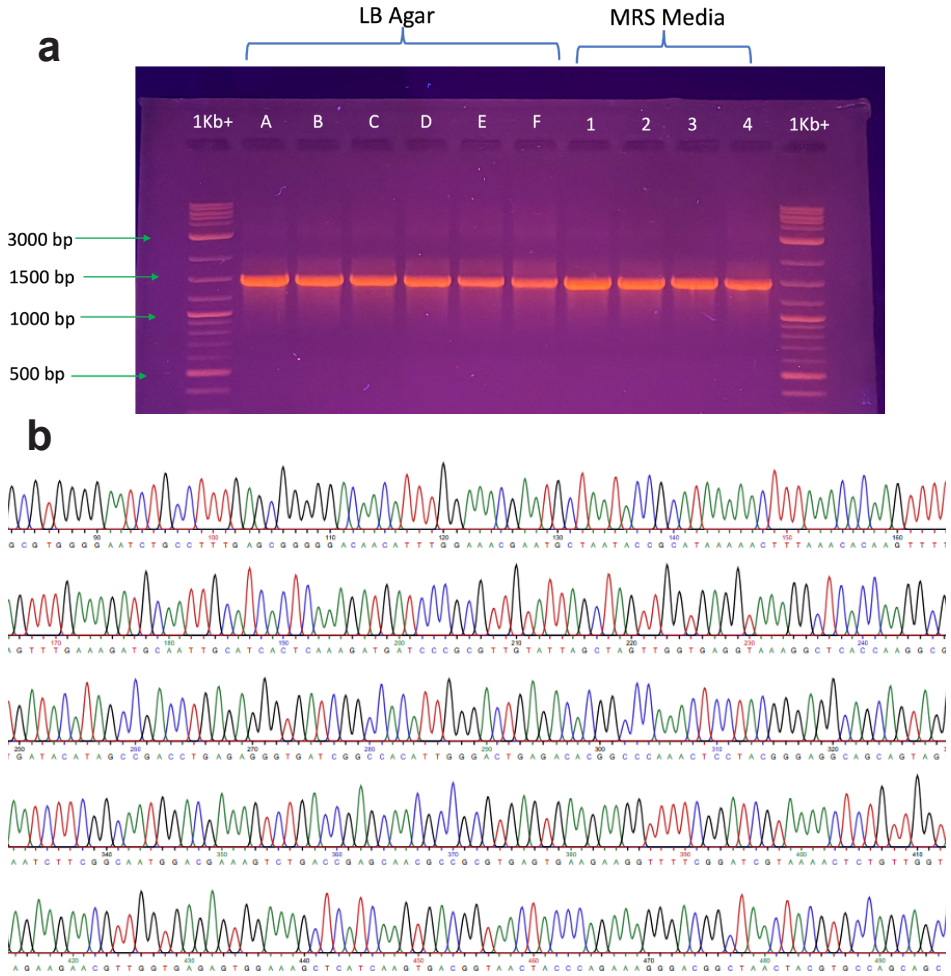


Figure 2 | DNA Sequencing of 16S rRNA. DNA amplification of 16S rRNA was run to identify probiotic strain. **a**, Gel of PCR amplification product showing samples from both LB agar and MRS media plates to verify amplification. **b**, Part of a sequencing readout received from sample B of the gel.

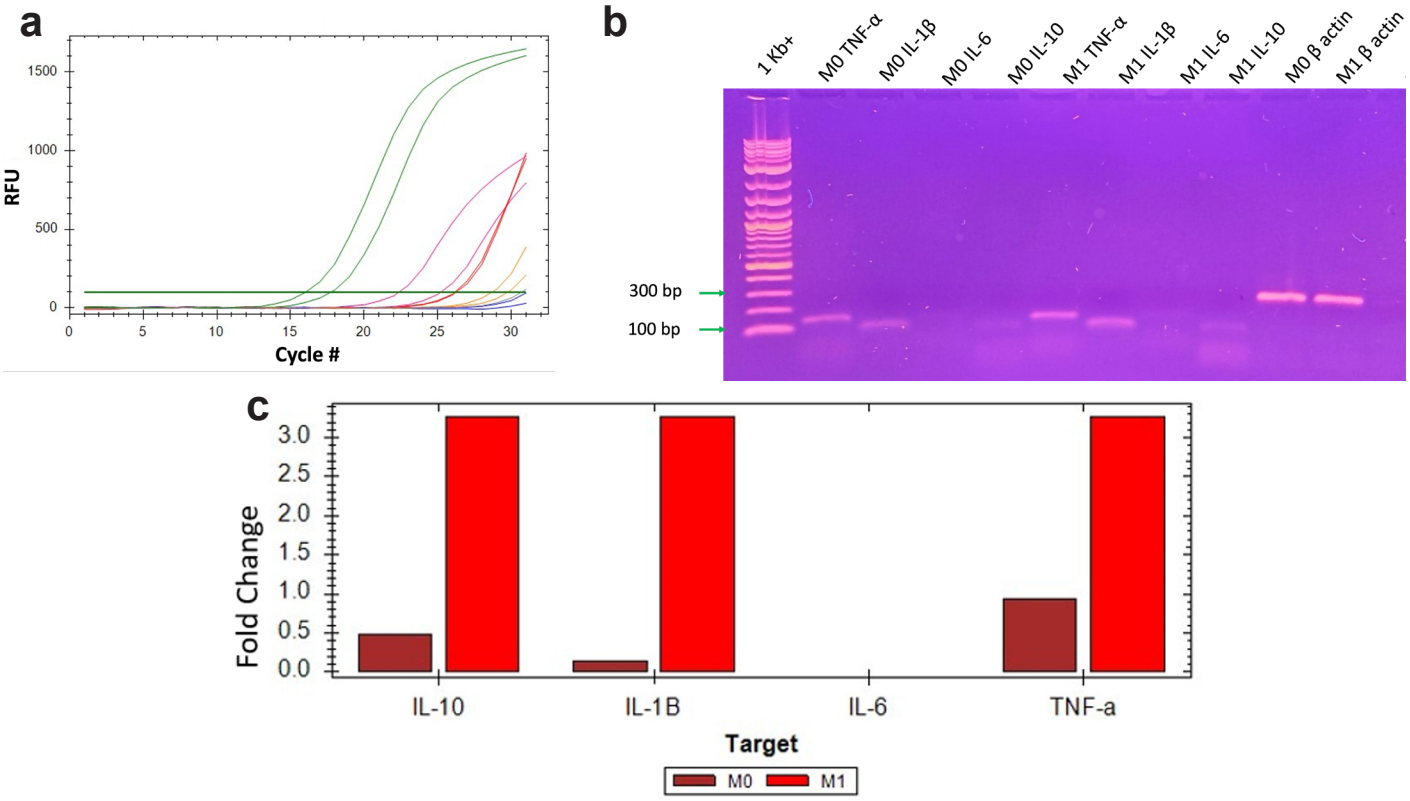


Figure 3 | qPCR validation for M0 differentiation to M1 Determination of cytokine mRNA transcript production change between M0s and M1s to verify that the differentiation protocol was successful. **a**, Amplification curve of the M0s vs M1 macrophages testing β Actin, IL-1β, IL-6, IL-10 and TNF-α. **b**, Gel of the amplification products in A to visualize bands made in amplification. (C) Fold change analysis of M0s to M1s using a ΔΔCT analysis to compare the expression of cytokine mRNA.

Once it was confirmed that the macrophages were successfully differentiated to M1s, modeling an inflamed environment, we cocultured these cells with the probiotic *L. lactis*. Several conditions were tested to determine if signaling changed based on how the probiotic was presented to the immune cells. Various conditions in which the probiotic was presented alive, in which the probiotic had been heat killed, and using only the supernatant of media that *L lactis* had grown in were assessed. The supernatant-treated condition, however, was determined to be contaminated, and the results are not considered in Fig. 4a, and are more accurately considered as a condition of unknown probiotic concentration. After incubating for a total of 4 and 24 hours, M1 cells were harvested and the RNA purified and RT-qPCR was run. The cytokine mRNA transcription results suggested a time-dependent modulatory effect of the co-culture. Based on the fold change analysis seen in Fig. 4a, there appears to be a down regulation of IL-1β after 24 hours compared to the 4-hour time point in both the live and heat killed conditions, and an upregulation of IL-10 at 24 hours in the live probiotic condition. Fig. 4b shows that transcript numbers of IL-10 and IL-1β change in response to the bacteria compared to the M1 control with no probiotic added. Transcripts for both cytokines exhibit up regulation compared to the condition with no probiotic added. however, in the live probiotic condition, the fold change seen in IL-10 appears to have a significant up regulation of anti-inflammatory signaling, but further replicates need to be run for statistical significance to be determined.

Discussion

Probiotics have been promoted to provide various health benefits to their host, especially in helping to modulate inflammatory signals, reducing inflammation. Through the preliminary findings of this research probiotics are seen to have possible modulatory effects on the cytokine signaling of macrophage cells changing the production and output of mRNA template for several cytokines involved in inflammatory signaling. This change in the production of cytokines may also be time dependent as seen in the RT-qPCR data showing that as incubation time increases, macrophages exhibit reduced transcription of the pro-inflammatory signaling of IL-1β and increase the transcription the anti-inflammatory cytokine, of IL-10. The results received from the ELISA Quantibody Array were inconclusive, and will need to be repeated to see if the cytokine protein levels change in accordance with the transcriptional changes. While not obtaining enough data to establish statistical significance, the preliminary findings are encouraging. Seeing a potentially beneficial change in inflammatory signaling due to the probiotic helps strengthen further research on probiotic impacts to inflammation.

Future Directions

This study suggests that there may be beneficial modulatory effects seen in the inflammatory signaling of macrophage cells when exposed to *L. lactis*, however, further testing with increased sample replicates will need to be completed to demonstrate whether these findings are supported by statistical analysis. Further testing of this strain and other probiotic strains, as well as dosing requirements, will help in determining the true benefits probiotic products may have for their consumers and may establish confidence in the use of probiotics to improve health in ways that don't re-

quire medications with potentially harmful side effects. Future work to determine the specific modulatory effects of probiotics will help physicians better understand and prescribe probiotics as potential behavior health changes in diet that can help to treat various conditions in which inflammation is a chronic issue.

Acknowledgements

I would like to extend many thanks to Drs Jack Treml, Melissa Daggett, Brendan Mattingly, and Sonia Thomas for their help and support throughout the work of this project to answer questions and provide insight that helped guide my research in the right direction. I would also like to thank Dr Ann Wozniak for providing THP-1 monocytes for this project.

Author Biography

Arianna Theleman is a graduating senior from the University of Kansas, earning her Bachelors of applied science in biotechnology. She plans to continue her education at KUMC in their IGPBS program. Arianna is passionate about discovery, family and her faith, and is motivated by the opportunity to participate in innovative research helping to impact and improve people's lives.

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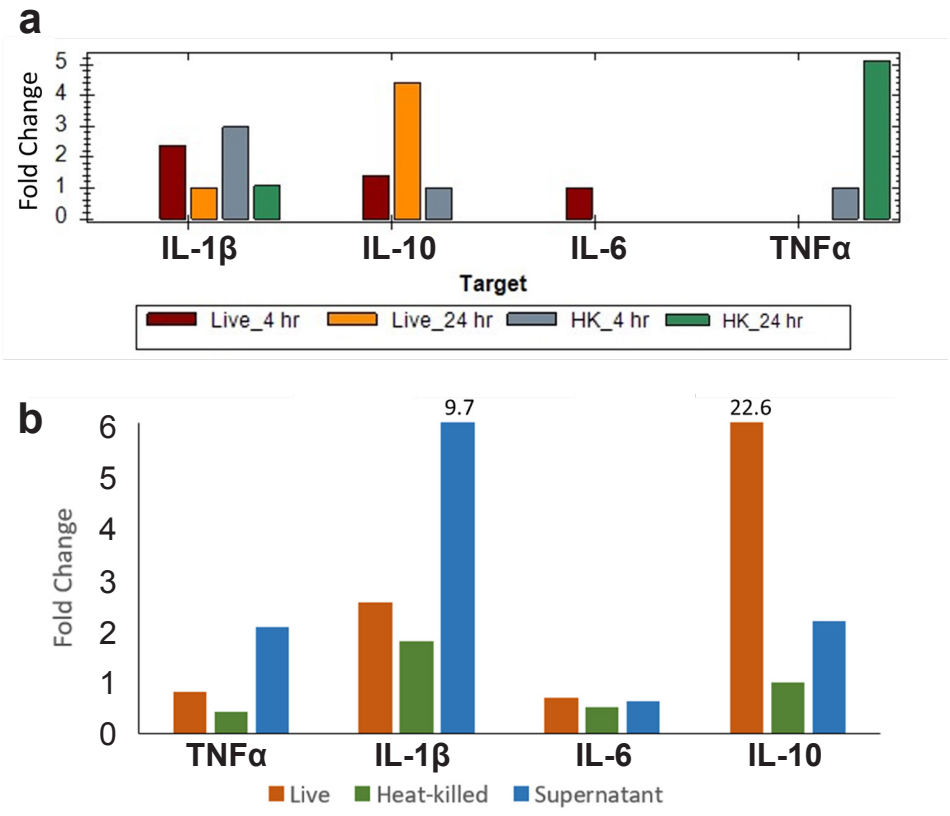


Figure 4 | RT-qPCR Fold Change Analysis. RT-qPCR was run on the M1 conditions containing probiotics and the mRNA expressions were compared against M1 + no probiotic added.
a, Fold change analysis of 10:1 [probiotic: macrophage] coculture at time points of 4 and 24 hours to compare the expression of cytokine mRNA template produced by the cells. **b**,Fold change analysis of mRNA expression at time point 24 hours, repeated to compare expression levels as seen in **a.**

Adipose Tissue-Derived Factors Promote Raji B-Cell Lymphoma Survival during Doxorubicin Treatment

Gia Luc Vo [1], JF Treml[1†], M. Daggett[1], B. Mattingly[1]., and S. Thomas[1]

Obesity is associated with increased risk and poorer outcomes in several cancers, but its effects on lymphoma responses to chemotherapy remain incompletely understood. This study examined whether soluble factors derived from adipose tissue influence the survival of Raji B-cell lymphoma cells during doxorubicin treatment. Raji cells were exposed to adipose tissue-derived medium with or without doxorubicin, and viability was assessed using LIVE/DEAD Fixable Red staining and flow cytometry. Addition of the adipose-derived preparation increased cell viability during doxorubicin exposure, with significant increases observed in the 20-, 30-, and 40-µL treatment groups relative to doxorubicin alone. Exploratory RT-qPCR analysis suggested treatment-associated changes in TNF-α and GLUT1 expression; however, these measurements were performed in a single experiment and require replication. These findings suggest that soluble adipose-derived factors may partially protect Raji lymphoma cells from doxorubicin-induced cytotoxicity and support further investigation of the relationship between adipose tissue and chemotherapy resistance.

Cancer remains one of the leading causes of mortality worldwide, with chemotherapy serving as a cornerstone of treatment for many malignancies, including non-Hodgkin B cell lymphoma.[1,2] Among commonly used chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, is widely utilized due to its ability to induce cancer cell death through DNA intercalation, inhibition of topoisomerase II, and generation of reactive oxygen species.[3,4] Despite its effectiveness, resistance to doxorubicin remains a major clinical challenge, limiting treatment success and contributing to disease relapse. Emerging evidence suggests that the tumor microenvironment plays a critical role in modulating chemotherapy response.[5] Particularly, adipose tissue has gained attention as an active endocrine organ that can influence cancer progression and therapeutic outcomes. Adipocytes can secrete a wide range of cytokines, chemokines, and adipokines which can impact tumor cell behavior.[6] These secreted factors are increasingly recognized as key contributors to cancer cell survival, proliferation, and drug resistance.

Adipose tissue is now understood to be metabolically active and highly involved in systemic and local signaling. Adipocytes release signaling molecules such as tumor necrosis factor-alpha (TNF-α), which regulate inflammation, metabolism, and cellular survival pathways.[7,8] These adipocyte-derived factors can alter the tumor microenvironment and promote tumor progression. Adipocytes located near tumors, often referred to as cancer-associated adipocytes, undergo phenotypic changes that enhance their ability to support tumor growth.[9] These cells exhibit increased secretion of inflammatory mediators and metabolic substrates, such as free fatty acids, which can be utilized by cancer cells for energy production and survival. This metabolic crosstalk is a key mechanism contributing to therapy resistance.

In addition to secreting protective factors, adipocytes can also alter drug availability. Evidence suggests that adipocytes can sequester and metabolize chemotherapeutic agents, effectively reducing the concentration of active drug reaching cancer cells.[10] This phenomenon further contributes to decreased drug efficacy.

Cytokines play a central role in mediating communication between adipocytes and cancer cells. TNF- α, a pro-inflammatory cytokine produced by adipose tissue, has been implicated in cancer progression and treatment resistance.[11] While TNF-α can induce apoptosis under certain conditions, it can also activate survival pathways such as NF-kB signaling, promoting cell proliferation and resistance to chemotherapy.

In addition to inflammatory signaling, metabolic adaptations are critical for cancer cell survival. Glucose transporter 1 (GLUT1) is a key regulator of glucose uptake and is often upregulated in cancer cells to support increased metabolic demands.[12,13] Enhanced GLUT1 expression allows cancer cells to maintain energy production under stress conditions, including exposure to chemotherapy.

While previous studies suggest that adipocytes contribute to chemotherapy resistance, the specific role of adipocyte-derived cytokines in lymphoma cells during doxorubicin treatment remains unclear.[14] In particular, the effects of these cytokines on inflammatory signaling and metabolic adaptation require further investigation. This study aims to examine whether modelled adipocyte-conditioned media influences the survival of lymphoma cells treated with doxorubicin. In addition, the study evaluates changes in gene expression of TNF-alpha and GLUT1 to assess the impact of adipocyte-derived factors on inflammatory and metabolic pathways.

Understanding how adipocyte-derived cytokines influence cancer cell survival during chemotherapy is important for improving treatment outcomes. As obesity rates continue to rise, the interaction between adipose tissue and cancer cells becomes increasingly relevant in clinical settings. Insights from this study may help explain variability in chemotherapy response and contribute to the development of strategies targeting the tumor microenvironment.

[1]University of Kansas, Edwards Campus, Biotechnology

†Corresponding Author jtreml@ku.edu

Materials and Methods

Raji B-Cells Culture

Raji B-cell lymphoma cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Cytiva HyClone) and 1% penicillin-streptomycin (Gibco). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Cell concentration and viability were monitored every two days using a hemocytometer and trypan blue (Gibco) exclusion assay at a 1:1 ratio of cells to trypan blue.

Modelled Adipocyte Conditioned Media Preparation

To model adipocyte conditioned media, approximately 800 mg of porcine adipose tissue was trimmed and homogenized. A total of 3.5 mL of sterile PBS-Tween (0.1%) solution was added to the tissue sample. The mixture was vortexed for 2 minutes and centrifuged at 5000 rpm for 1 minute. Following centrifugation, the upper lipid layer was removed, and the adipocyte-conditioned supernatant was collected. The collected supernatant was filtered twice using a sterile 20 mm filter before use in cell culture experiments.

Cell Seeding Assay

Raji cells were seeded into 24-well plates at different concentrations including 5×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 cells/ml. Each condition was prepared in triplicate. Cells were incubated for 24 hours before viability assessment.

Cell viability was measured using the LIVE/DEAD™ Fixable Red Dye assay (ThermoFisher, L23102). Cells were washed with sterile PBS and stained with 1 µl of LIVE/DEAD™ Fixable Red Dye for 30 minutes at room temperature in the dark. Samples were analyzed using flow cytometry. The optimal seeding density was selected based on the highest percentage of viable cells.

Lethal Dose 50 Determination

Raji cells were seeded at a concentration of 1×10^5 cells/ml in 24-well plates. Cells were treated with different concentrations of doxorubicin hydrochloride (MP Biomedicals, 25316-40-9), including 1, 2, 4, 8, 16, and 20 µg/ml, for 24 hours. Following treatment, cell viability was assessed using the LIVE/DEAD™ Fixable Red Dye assay and flow cytometry. The lethal dose 50 (LD_{50}) concentration was determined from the resulting viability curve.

Treatment with Modelled Adipocyte-Cytokine Conditioned Media and Doxorubicin

Raji cells were cultured with modelled adipocyte-conditioned media at concentrations of 10, 20, 30, and 40 µl. Cells were incubated with modelled adipocyte-conditioned media for 12 hours prior to treatment with the LD_{50} concentration of doxorubicin. Following doxorubicin addition, cells were incubated for an additional 24 hours.

Cell viability was assessed using the LIVE/DEAD™ Fixable Red Dye assay and analyzed by flow cytometry. All treatment conditions were performed in triplicate. Counts of live Raji cells and concentrations of drugs were controlled consistently to Raji cells with doxorubicin in adipocyte-cytokine condition media. Two-tailed, unpaired Student's t-tests assuming equal variances were used to determine statistical significance with a threshold of $p \leq 0.05$.

RT-qPCR

Total RNA was extracted from each treatment condition using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized in a 20 µL reaction containing 4 µL of 5X iScript Supermix, 500 ng RNA template, and nuclease-free water. Reverse transcription was performed using the following thermal conditions: priming at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, and RT inactivation at 95°C for 1 minute.

Quantitative PCR (qPCR) reactions were prepared in a total volume of 20 µL containing 10 µL SYBR Green qPCR Master Mix, 1 µL forward/reverse primer mixture, 1 µL cDNA template, and 8 µL nu-

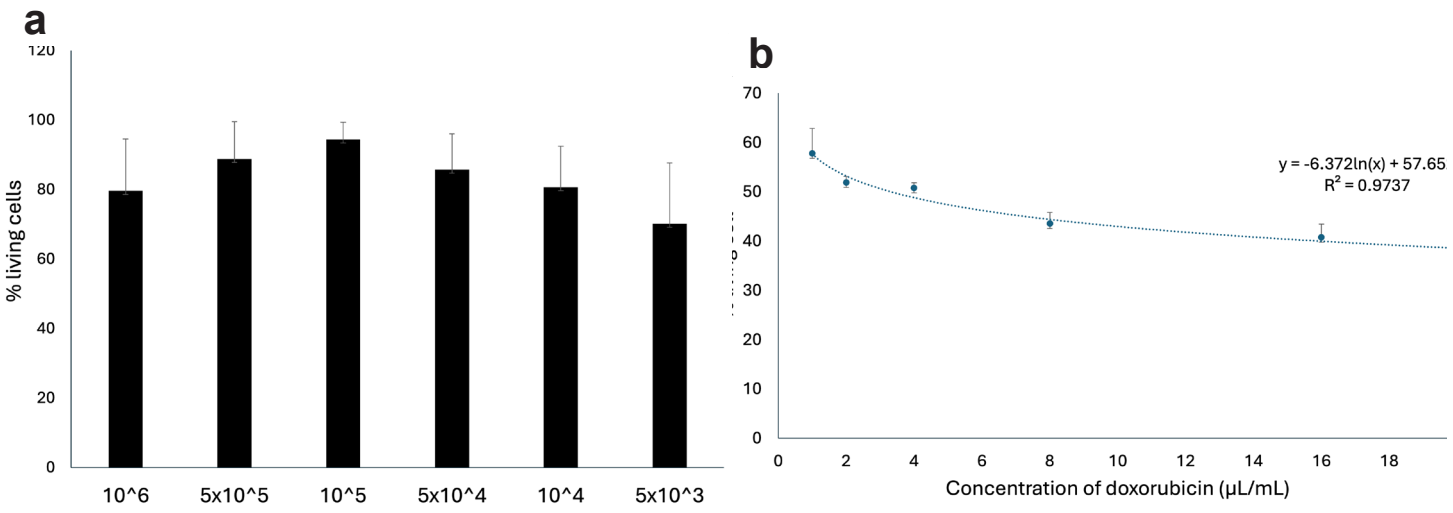


Figure 1 | Optimization of RAJI cell culture conditions and determination of doxorubicin LD_{50}. a, RAJI cell viability at different seeding densities after 24 hours. **b,** Dose-response curve of RAJI cells treated with increasing concentrations of doxorubicin. Cell viability decreased as doxorubicin concentration increased. The regression curve used to estimate the LD_{50} concentration is shown. Data are presented as mean ± SD (n=3).

clease-free water. qPCR amplification was performed on a real-time PCR system under the following cycling conditions: initial denaturation at 95°C for 4 minutes, followed by repeated cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. Relative gene expression of TNF-α and GLUT1 was analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene, GADPH.

Results

Cell Seeding Optimization

Different Raji cell densities, including 5×10^3, 1×10^4, 1×10^5, 5×10^5, and 1×10^6 cells/mL, were evaluated to determine the optimal concentration for viability assays. Among the tested conditions, 1×10^5 cells/mL demonstrated the highest percentage of viable cells after 24 hours and was selected for all subsequent experiments (**Fig. 1a**).

Determination of Doxorubicin LD_{50}

Treatment with increasing concentrations of doxorubicin resulted in a progressive decrease in Raji cell viability. The dose-response curve demonstrated a nonlinear relationship between drug concentration and survival, where higher concentrations produced greater cytotoxic effects. he fitted dose–response curve yielded an estimated LD_{50} of approximately 3.3 µg/mL. (**Fig. 1b**). This concentration was selected for downstream experiments involving modelled adipocyte-conditioned media because it produces substantial but incomplete cell death, allowing potential protective effects of adipocyte-derived cytokines to be observed.

Effect of Modelled adipocyte-conditioned media on Raji Cell Viability

Raji cells treated with modelled adipocyte-conditioned media alone maintained high viability across all tested concentrations. No substantial decrease in cell survival was observed, indicating that adipocyte-derived cytokines were not directly toxic to the cells (**Fig. 2**).

In contrast, Raji cells treated with doxorubicin alone demonstrated a marked reduction in viability. However, the addition of modelled adipocyte-conditioned media increased cell survival compared to doxorubicin treatment alone (**Fig. 3**). This protective effect became more pronounced at higher concentrations of modelled adipocyte-conditioned media, suggesting that adipocyte-derived factors may reduce the cytotoxic effects of doxorubicin.

Statistical analysis demonstrated significant increases in viability in several modelled adipocyte-conditioned media treatment groups compared to the doxorubicin-only condition, supporting the observation that adipocyte-derived factors promote lymphoma cell survival during chemotherapy exposure.

Statistical analysis showed significant increases in cell viability in the 20 µL, 30 µL, and 40 µL modelled adipocyte-conditioned media groups compared to doxorubicin treatment alone ($p < 0.05$), while the 10 µL group was not statistically significant ($p > 0.05$).

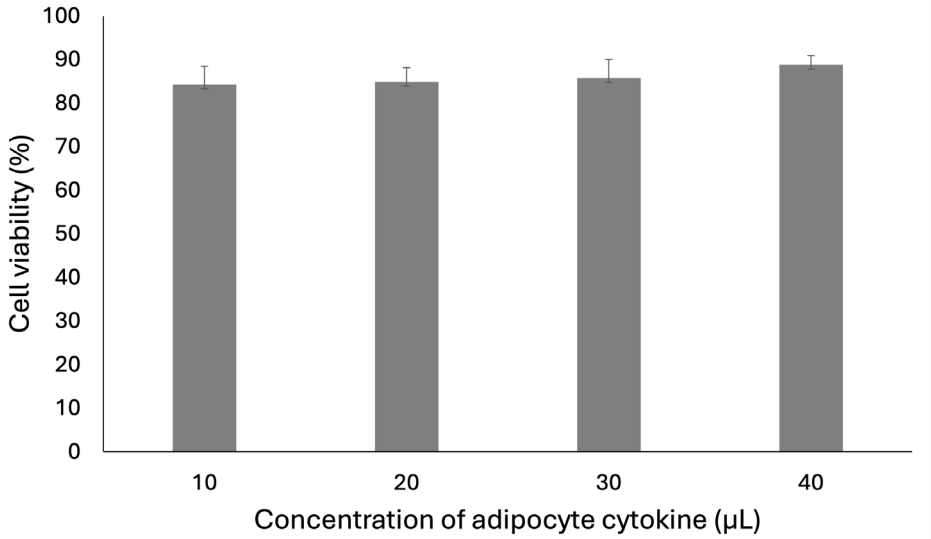


Figure 2 | Effects of adipocyte-conditioned media on Raji cell viability. Rajicells were treated with increasing volumes of adipocyte-conditioned media, and cell viability was measured after 24 hours using the LIVE/DEAD assay. Data are presented as mean ± SD (n=3).

TNF-α Gene Expression

Relative TNF-α expression was evaluated using RT-qPCR. Doxorubicin treatment resulted in a strong decrease in TNF-α expression compared to untreated Raji cells. Unexpectedly, Raji cells treated with modelled adipocyte-conditioned media alone also showed reduced TNF-α expression relative to the control condition (**Fig. 3a**). In the combined modelled adipocyte-conditioned media and doxorubicin treatment group, TNF-α expression remained low.

These findings suggest that modelled adipocyte-conditioned media did not increase TNF-α expression in Raji cells under these experimental conditions. However, because this experiment was performed only once, additional replicates are required to validate these observations.

GLUT1 Gene Expression

GLUT1 expression was analyzed to evaluate potential metabolic changes associated with modelled adipocyte-conditioned media treatment. Raji cells exposed to modelled adipocyte-conditioned media demonstrated increased GLUT1 expression compared to untreated control cells, suggesting enhanced metabolic activity and glucose uptake (**Fig. 3b**).

Doxorubicin treatment alone caused a substantial reduction in GLUT1 expression. However, cells treated with both modelled adipocyte-conditioned media and doxorubicin exhibited higher GLUT1 expression compared to the doxorubicin-only condition. Although expression levels remained below untreated controls, this partial restoration suggests that adipocyte-derived factors may support metabolic adaptation and survival during chemotherapy stress.

Discussion

This study investigated whether adipocyte-derived factors influence lymphoma cell survival during doxorubicin treatment. The results demonstrated that modelled adipocyte-conditioned media increased Raji cell viability during chemotherapy exposure, supporting the hypothesis that soluble

adipocyte-derived factors may contribute to chemotherapy resistance.

Modelled adipocyte-conditioned media alone did not reduce cell viability, indicating that the adipose-derived preparation was not detectably toxic to Raji cells under the tested conditions. However, when combined with doxorubicin treatment, modelled adipocyte-conditioned media partially protected lymphoma cells from drug-induced cytotoxicity. This protective effect became more pronounced with increasing concentrations of modelled adipocyte-conditioned media, suggesting that the apparent protective effect increased with the volume of preparation added.

The observed increase in GLUT1 expression following modelled adipocyte-conditioned media treatment suggests that adipocyte-derived factors may promote metabolic adaptation in lymphoma cells. GLUT1 is responsible for glucose uptake and is commonly upregulated in cancer cells to support increased metabolic demands. The exploratory GLUT1 result is consistent with a possible effect on metabolic regulation, but replication and functional metabolic assays are needed.

Unexpectedly, TNF-α expression decreased rather than increased following modelled adipocyte-conditioned media treatment. Since adipocytes are known to produce inflammatory cytokines, an increase in TNF-α expression was initially expected. This result may indicate that adipocyte-derived factors do not directly induce TNF-α transcription in Raji cells, or that Raji cells respond differently to adipocyte signaling under these conditions. Additional experiments are required to further investigate this observation.

Overall, these findings support previous evidence suggesting that adipose tissue contributes to cancer cell survival and chemotherapy resistance through interactions within the tumor microenvironment.

Limitations

Several limitations were present in this study. First, RT-qPCR experiments were performed only once, limiting the ability to interpret these TNF-α and GLUT1 gene expression results as anything more than suggestive and exploratory. Additional biological replicates are required to validate these findings. Second, the sample size for viability experiments was relatively small consisting of three technical wells from one experiment, which may reduce statistical power. Third, modelled adipocyte-conditioned media contains a mixture of cytokines and signaling molecules, making it difficult to identify which specific factors contributed to the observed protective effects. Finally, gene expression was evaluated only at the mRNA level and was not confirmed at the protein level using methods such as Western blotting or ELISA.

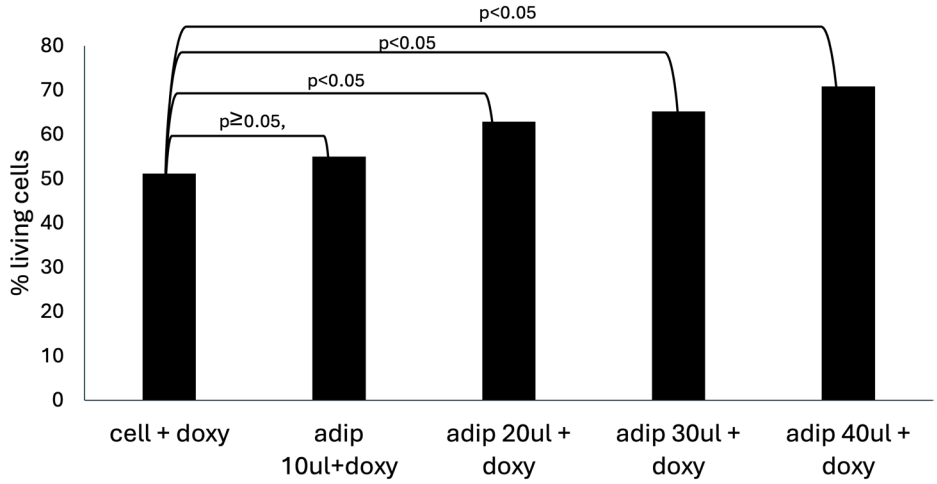


Figure 3 | Effects of adipocyte-conditioned media on RAJI cell viability during doxorubicin treatment. RAJI cells were treated with doxorubicin at the LD50 concentration in the presence of increasing volumes of adipocyte-conditioned media. Cells were cultured with adipocyte-cytokine condition media for 12 hours before add doxorubicin to LD50 concentration. Cell viability was measured after 24 hours using the LIVE/DEAD assay. Statistical significance was determined by Student's t-test compared with the doxorubicin-treated control group. Data are presented as mean ± SD (n=3).

Future studies should repeat the RT-qPCR experiments with more biological replicates to confirm the trends observed in TNF-α and GLUT1 expression. Since this study only examined gene expression at the mRNA level, additional experiments such as Western blotting or ELISA could be used to determine whether these changes are also seen at the protein level.

Future Direction

It would also be important to identify which specific adipocyte-derived cytokines are responsible for increasing Raji cell survival during doxorubicin treatment. Testing additional lymphoma cell lines could help determine whether this protective effect is unique to Raji cells or occurs more broadly in lymphoma.

Future work could further investigate survival pathways involved in chemotherapy resistance, such as NF-κB and PI3K/AKT signaling. In addition, comparing modelled adipocyte-conditioned media from lean and obese adipose tissue may provide more insight into how obesity contributes to lymphoma progression and drug resistance.

Author Biography

Gia Luc Vo is a graduate of the KU Biotechnology program. He completed an internship at the University of Kansas Medical Center, where he conducted research on Autosomal Dominant Polycystic Kidney Disease. Following graduation, he plans to pursue a career in biomedical research and continue studies in cancer biology and biotechnology.

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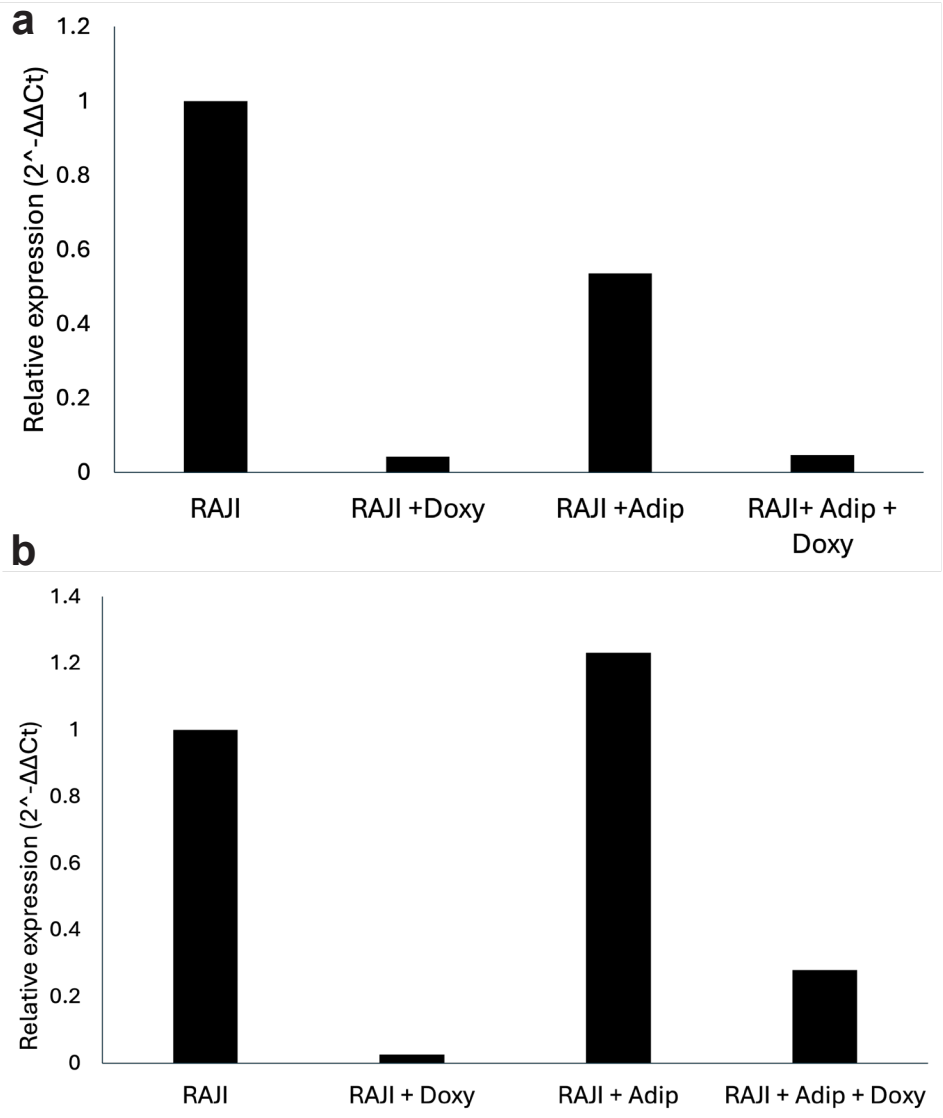
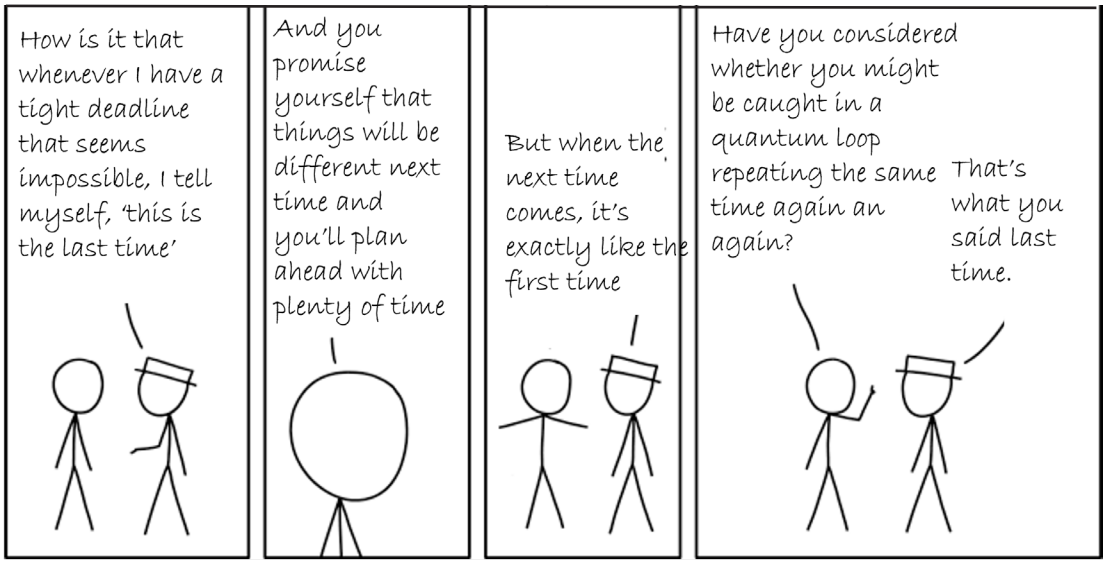


Figure 4. Relative expression of TNF-α and GLUT1 in RAJI cells under different treatment conditions. **a,** Relative TNF-α expression in untreated RAJI cells, doxorubicin-treated cells, adipocyte-conditioned media-treated cells, and combined adipocyte-conditioned media plus doxorubicin treatment. **b,**Relative GLUT1 expression under the same treatment conditions. Gene expression levels were normalized to a housekeeping gene and analyzed using the 2^-ΔΔCt method.

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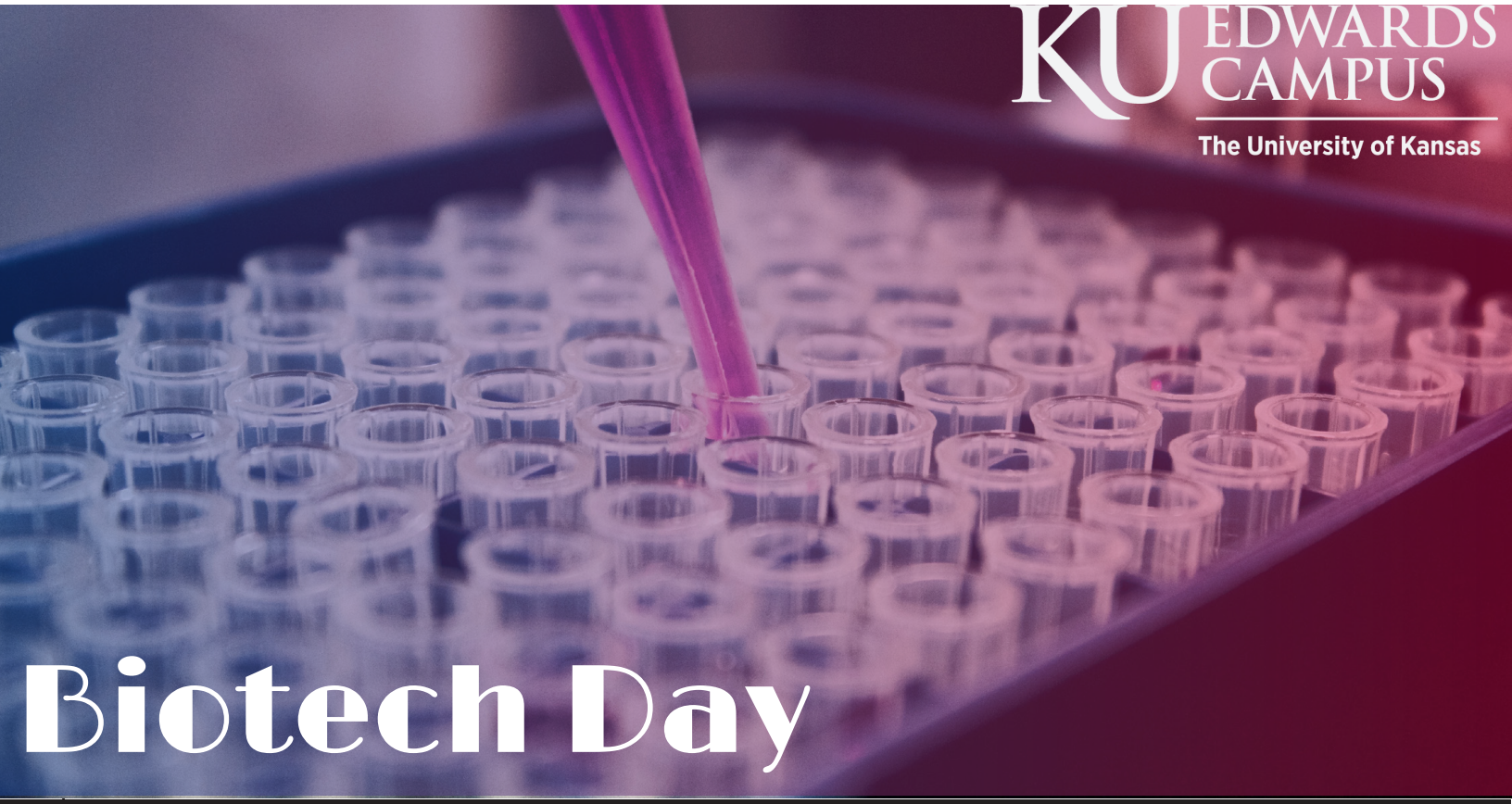
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