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Investigating the Role of Lipoglycan-Producing *Ruminococcus gnavus* in Autoimmune Flare Responses in Lupus-Prone Mice: A Research Protocol

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Abstract

Introduction: Systemic lupus erythematosus (SLE), more commonly known as lupus, is a chronic autoimmune condition that affects over 3 million adults worldwide. Its cause remains unknown, and there is currently no cure. Present-day treatment focuses on managing symptoms during periods of disease flare-ups. Previous literature has demonstrated a correlation between the increased presence of the gut bacterial strain *Ruminococcus (blautia) gnavus* and lupus nephritis flare-ups. However, the mechanism by which this bacterium may influence flare activity has not been determined, and causality has not been established.

Methods: This study proposes an animal model-based research protocol to evaluate whether the lipoglycan-producing strain of *R. gnavus* elicits a stronger autoimmune response than the regular strain. NZM2328 lupus-prone mice and wild-type controls will be divided into treatment and control groups. Treatment groups will receive oral administration of either regular or lipoglycan-producing *R. gnavus* strains. Following the treatment period, serum antinuclear antibody (ANA) levels will be measured, and glomerular kidney tissue will be extracted and analyzed via immunofluorescence and Western blotting to assess immune complex deposition.

Anticipated Results: It is hypothesized that the lipoglycan-producing *R. gnavus* strain will produce a more pronounced autoimmune response than the regular strain, particularly in lupus-prone mice. This will be reflected in elevated ANA levels and increased immune complex deposition in the kidneys.

Discussion: Data from this study will be analyzed to determine whether *R. gnavus* lipoglycan production and exhibition has a causal relationship with lupus flare activity. Statistical comparisons between groups will assess differences in immune markers and histological findings. Findings from this protocol could help clarify microbial contributions to lupus pathogenesis and guide future work in microbiome-targeted interventions.

Conclusion: This research protocol looks to explore a novel microbial trigger for lupus flare-ups. Understanding how lipoglycan-producing *R. gnavus* affects disease severity may inform future therapeutic strategies targeting the gut microbiome for improved management of lupus nephritis.

Keywords: antinuclear antibody; dysbiosis; gastrointestinal tract; lipoglycans; lupus nephritis; NZM2328 strain; *Ruminococcus (blautia) gnavus*; systemic lupus erythematosus; wild-type NZM strain

Introduction

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is the most common form of lupus, affecting 3.17 million adults worldwide, with a higher incidence occurring in female populations [1, 2]. SLE is a chronic autoimmune connective tissue disease that affects multiple organ systems simultaneously and can be uniquely marked by periods of flares (symptom worsening) and periods of remission (symptom relief) [3]. The initial symptoms of SLE flare-ups commonly include fever, fatigue, and inflammation, which can result in joint pain and stiffness. The severity of these symptoms often varies depending on the stage and progression of the disease. Another characteristic sign of SLE is the appearance of a butterfly-shaped rash that typically spans the cheeks and the bridge of the nose, although rashes may also develop on other areas of the body [2].

During disease flares, multiple organ systems may be affected, leading to widespread internal inflammation. If left unmanaged, this inflammation can cause significant damage to vital organs, potentially leading to dysfunction

or complete organ failure, becoming life-threatening. One of the most serious complications of SLE is lupus nephritis, a condition in which the immune system attacks the kidneys, causing inflammation [3]. This impairs the kidneys' vital ability to filter waste from the blood, regulate osmotic balance, and maintain blood pressure. Additionally, key markers of SLE include elevated antinuclear antibody (ANA) production and immune complex deposition in the kidneys [3, 4]. Up to 50% of SLE patients develop lupus nephritis [3].

Autoimmune Diseases

Autoimmune diseases profoundly affect multiple aspects of life including physical health, emotional well-being, and daily functioning. They are characterized by the body's immune response mistakenly attacking healthy tissue, via the production of autoantibodies, specifically through antinuclear antibodies (ANAs). A type of ANA called anti-dsDNA binds specifically to double-stranded DNA and histone complexes, while anti-Sm binds to the protein contained within the small nuclear ribonucleoprotein, snRNP. These two ANAs are hallmarks of the identification and diagnosis of SLE [4]. The presence of anti-dsDNA antibodies and ANAs is closely linked to the onset of lupus nephritis. An overabundance of these autoantibodies binding to their self-antigens forms immune complexes (ICs). In some cases, the ANAs will bind to glomerular antigens, leading to IC deposits in the glomeruli of the kidneys. The presence of these IC deposits in the kidneys, in turn, activates complement molecules, ultimately resulting in inflammation of the glomerular cells within the kidneys. This inflammation leads to the damage and injury of the kidneys experienced by patients with lupus nephritis [3]. Furthermore, a key biomarker of the periods of flares has been characterized by the increased presence of immunoglobulin G in serum, called anti-C3b IgG (IgG) [5]. This shows that as symptoms worsen, there is a corresponding increased immune response.

Lipoglycans

Lipoglycans are glycolipid molecules found on the surface of certain gut bacteria that can mediate host–microbe interactions, including adhesion and immune modulation. Stomach lining mucins are heavily glycosylated proteins that form a protective mucus layer in the gastrointestinal tract, serving as both a barrier and a nutrient source for mucin-degrading microbes, such as *Ruminococcus gnavus*, which is central to this study.

Ruminococcus gnavus

Ruminococcus (blautia) gnavus (*R. gnavus)* is a strictly anaerobic Gram-positive bacterium [6, 7]. First identified in human GI tract contents and feces by Moore et al. (1974) [7], this species is now recognized as a prevalent part of the gut microbiome in infants and adults, influencing digestion through its produced metabolites by Juge (2023) [8]. As a symbiont within the human gut, its adaptation to the GI environment is strain-dependent, associated with its ability to forage on the glycoprotein mucin, a major component of the GI extracellular mucus layer [9, 10]. *R. gnavus* produces a lipoglycan as well as a glucorhamnan polysaccharide, which are membrane-bound compounds associated with triggering an immune response and inflammation as well as influencing SLE pathogenesis though the exact mechanisms are not fully understood [11, 12]. As implied, the detection of an increased microbial bloom of *R. gnavus* in a region of the GI would indicate that the physical barrier lining the GI is being weakened. Consequently, studies have reported both positive and negative correlations between *R. gnavus* levels and various gastrointestinal and extraintestinal diseases, including neurological disorders. During Azzouz et al.'s (2023) [13] longitudinal gut microbiome analysis, *R. gnavus* microbial blooms occurred around the same times as lupus flare-ups were detected, particularly in patients with lupus nephritis. A particular *R. gnavus* lipoglycan was specifically observed in patients with lupus nephritis that was not observed in healthy patients [13]. However, the duration of these blooms could not be assessed accurately by the observational pilot study, and the co-occurrence was likely underestimated due to the only 16 SLE female patients being assessed, of which 5 exhibited such microbial blooms and 4 of whom had lupus nephritis [13]. Causality could not be established as the researchers were unable to determine whether the microbial bloom and microbiota instability began before or after the progression of lupus nephritis flare-ups, and whether the lipoglycan played a role in the cause of lupus nephritis flare-ups. Understanding the connection between the presence of lipoglycan-producing *R. gnavus* and the severity and presence of lupus nephritis markers could inform future research on treatment options involving the gut microbiota.

This study seeks to answer the following research question: Does lipoglycan-producing *R. gnavus* elicit a stronger autoimmune response than regular *R. gnavus* in lupus-prone mice?

Methods

In this study, the NZM2328 and Wild-type (WT) NZM mice strains [14] will be used to explore the effects of orally administrating the lipoglycan-producing *R. gnavus* strains into the subject male and female mice on the autoimmune response within their kidney cells, with strict adherence to National Research Council Animal Care Committees guidelines. Sample size (N) will be determined based on a power analysis using preliminary or pilot data, with a statistical power of 0.8 and an alpha level of 0.05 to ensure adequate sensitivity for detecting biologically meaningful differences between groups. The WT-NZM and NZM2328 negative control groups will be orally administered with saline. The WT-NZM and NZM2328 treatment groups will be orally administered with the bacterial treatment mixture

of the lipoglycan-producing *R. gnavus* strains RG2, S107-86, and S47-18 which have been shown to be reactive to immunoglobulin G (IgG) antibodies, above the baseline median abundance of 0.1% [6, 13]. The WT-NZM and NZM2328 positive control groups will be orally administered with the *R. gnavus* strain RG1 from healthy donors that do not contain IgG reactive lipoglycans [13], above the baseline median abundance of 0.1%. Each *R. gnavus* strain will be cultured in YCFA medium containing essential nutrients, mucin, and short-chain fatty acids physiologically relevant to the mouse gut microbiome [15]. The concentration of each strain in the bacterial mixture will be adjusted according to ratios calculated from the average abundances of each strain determined during pre-treatment gut microbiome analysis of the rodents. This oral administration will be performed for the number of weeks necessary to mimic gut colonization via oral gavage. The oral gavage will be used to introduce the bacterium strains into the gut microbiomes of the mice strains through the gastrointestinal tract. To determine the successful colonization of *R. gnavus* within each subject's microbiome, fecal samples will be taken regularly and cultured accordingly. Bacterial genome sequencing will be conducted on other identified bacterial species strains to assess the effects of their interactions with *R. gnavus* strains if known. Additionally, diagnostic serum ANA blood tests and IgG blood tests will be performed to validate the subject's SLE status and assess flare-ups regularly. After euthanasia via pentobarbital sodium overdose [16], kidney glomerular cells will be carefully harvested from each mouse using the isolation method described by Wang et al. (2019) [16]. Once isolated, the glomeruli will undergo in vivo Alcian Blue staining as described by Bankir & Hollenberg (1983) [16] to distinguish glomeruli from contaminants and monitor recovery efficiency during the isolation process. The total protein will be extracted from a sample of kidney glomeruli utilizing a radio-immunoprecipitation assay (RIPA) buffer [16] to prepare for western immunoblotting analysis, applying anti-dsDNA antibodies to identify the antigenic substance on glomeruli in lupus nephritis. In another sample of the isolated cells, thin slices of glomeruli are analyzed using immunofluorescent microscopy methods similar to those of Jain et al. (2021) who use IC targeting antibodies and appropriate microscopes [17].

To compare the level of *R. gnavus* bacterial presence alongside the presence of other prevalent bacterial species measured via fecal samples, ANA and IgG serum levels measured via blood tests, IC content volume measured via Western immunoblotting analysis, and immunofluorescence intensity measured via microscopy across the six subject groups, pre- and post-treatment. The two-tailed ANOVA tests will be conducted followed by post hoc tests.

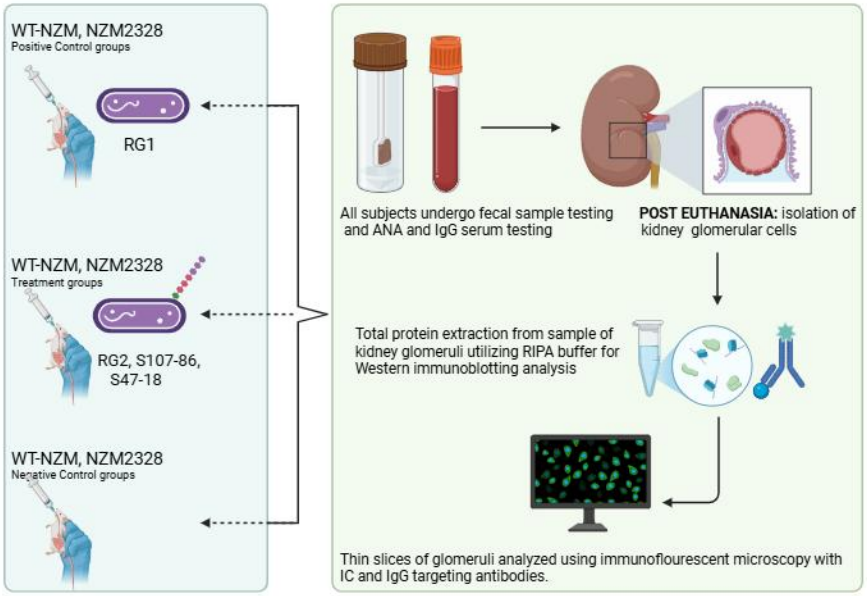

Figure 1. Graphical overview of methods (created with BioRender.com [18]) highlighting the treatment (administered with mixture of *R. gnavus* strains found in Lupus nephritis patients), positive control (administered with *R. gnavus* strain without immunoreactive lipoglycan), and negative control (saline administered) WT-NZM and NZM2328 groups. Subjects will undergo administration for several weeks while undergoing regular fecal sample, ANA serum, and IgG serum testing. Kidney glomerular cells will be isolated post euthanasia, and the total protein extract will be prepared for Western immunoblotting analysis using anti-dsDNA antibodies. Glomeruli slices will be analyzed using immunofluorescent microscopy methods with IC targeting antibodies.

Results

As this is a theoretical research protocol developed for a case competition, no data collection or experimental implementation has taken place. The proposed study has not been scheduled for execution, and results are not currently anticipated. This protocol was designed to explore a novel hypothesis and provide a potential framework for future experimental studies while adhering to ethical protocols.

Discussion

This study aims to elucidate the role of lipoglycan-producing *R. gnavus* in triggering lupus nephritis flare-ups by examining its effects on antinuclear antibody production and kidney immune complex deposition in lupus-prone NZM2328 mice. By comparing immune responses in mice exposed to lipoglycan-producing, IgG-reactive strains versus those exposed to strains lacking lipoglycans and IgG reactivity, the study seeks to determine the impact of these surface molecules on autoimmune activation. The experimental design utilizes the NZM2328 lupus-prone mouse model and controlled exposure to well-characterized *R. gnavus* strains, allowing precise comparisons between experimental and control groups while providing a targeted framework for studying systemic lupus erythematosus (SLE) through microbiome-host interactions.

If it is determined that these strains do not cause flares, the study would still offer insight into how lipoglycans and membrane-associated compounds affect gut composition and subsequent inflammatory responses. Data analysis will involve comparing serum ANA levels and IC deposition across experimental and control groups to assess whether lipoglycan-producing strains are associated with stronger autoimmune responses.

Upon considering limitations, such controlled bacterial exposure and mice handling does not entirely replicate natural microbiome dynamics. Inter-strain interactions between *R. gnavus* variants and interbacterial interactions with other gut microbiota could also influence disease flare-ups, impacting how a correlation, if found, will have to be evaluated.

To address the influence of sex differences on disease progression, future experiments will incorporate sex as a biological variable through the inclusion of both male and female cohorts and, where appropriate, hormonal controls. This approach will enable a more accurate assessment of sex-specific disease mechanisms and enhance the reliability and translational relevance of the findings, particularly in elucidating the biological and hormonal factors contributing to SLE flare-ups [19, 20]. This focus could, in turn, guide the development of targeted interventions aimed at controlling specific factors relevant to the onset and progression of SLE. One potential modification to incorporate this perspective would be the monitoring or even regulation of hormonal fluctuations, among other variables, which could help account for sex-based differences in gut microbiome composition and their role in disease dynamics. Incorporating microbiome composition controls would enhance the specificity and significance of the results by providing a clearer interpretation of their implications, even if lipoglycan-producing *R. gnavus* strain blooms are not found to be exclusively associated with SLE flare-ups. The complexity of the gut microbiome alongside the relationships between competing and non-competing bacterial populations exacerbate the importance of this consideration, highlighting that an increase in *R. gnavus* strains will likely influence the abundance and behaviors of other microbial organisms residing in the gut. This means that flare-ups could also be associated with the increased or decreased presence of other bacteria species, rather than being attributed solely to changes in the population of *R. gnavus.* Thus, this highlights other potential modifications to the proposed methodology, such as the use of germ-free mouse models, a thorough analysis of fluctuations in a defined set of bacterial species and strains found in the gut microbiome, alongside the initially proposed testing of IgG serum levels, ANA levels, and kidney IC deposition.

Conclusions

The intent of publishing this research protocol is to provide a novel, testable framework for investigating whether lipoglycan-producing *Ruminococcus blautia gnavus* has a causal or associative role in the development and progression of lupus nephritis flare-ups. By offering a structured methodology that compares the immune response in lupus-prone mice to different *R. gnavus* strains and examines the autoimmune damage to the kidneys that is associated with lupus nephritis, this protocol contributes to the growing body of literature examining the gut microbiome's role in autoimmune diseases.

Understanding the mechanisms through which gut bacteria may exacerbate or trigger systemic lupus erythematosus (SLE) is critical to developing new therapeutic strategies. If an enlarged population of lipoglycan-producing *R. gnavus* species is found to elevate antinuclear antibody levels and immune complex deposition, this strain may serve as both a biomarker and a potential target for microbiome modulation therapies.

There are also novel questions which may be raised by the design of this study, for example: To what extent do sex-based hormonal differences influence bacterial interactions with the immune system? Could additional membrane-bound microbial products beyond lipoglycans play a synergistic role in triggering immune activation? How might germ-free models or microbiome-controlled environments refine these findings?

Future research could build upon these findings through more focused investigations into sex-specific immune responses, long-term monitoring of microbiome dynamics, and the application of advanced and genetically relevant animal models. Such approaches may yield deeper insights into the complex interplay between host

immunity and microbial communities. This research protocol is designed to foster interdisciplinary collaboration between microbiology and immunology, ultimately contributing to the development of more personalized and effective interventions. The overarching goal is to enhance the quality of life for individuals affected by lupus by informing better diagnostics, treatments, and preventive strategies.

List of Abbreviations
ANA: antinuclear antibody
dsDNA: double-stranded DNA
GI: gastrointestinal
IC: immune complex
IgG: immunoglobulin G
NZM: New Zealand mixed (mouse strain)
RIPA: radio-immunoprecipitation assay
SLE: systemic lupus erythematosus
snRNP: small nuclear ribonucleoprotein
WT: wild-type

Conflicts of Interest
The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent
This manuscript describes a theoretical research protocol developed for an undergraduate case competition. No experiments involving animal or human subjects have been conducted, and therefore research ethics board (REB) approval and participant consent were not required at this stage.

Authors' Contributions
AA: contributed to the conception and design of the study, participated in the literature review, co-drafted the manuscript, and approved the final version to be published.
IM: contributed to the conception and design of the study, participated in the literature review, co-drafted the manuscript, and approved the final version to be published.
SP: contributed to the conception and design of the study, participated in the literature review, co-drafted the manuscript, and approved the final version to be published.

Acknowledgements
The authors would like to thank the SciNapse Undergraduate Case Competition organizers for providing the opportunity to develop and share this proposal, and acknowledge the helpful feedback received during the poster preparation process, which informed aspects of this protocol.

Funding
This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Mikayla Hall-Bruce, Sara Sara
Article Dates: Received May 06 25; Accepted Dec 25 25; Published Mar 04 26

Citation
Please cite this article as follows:
Mansoor I, Ammeer A, Puvindran S. Investigating the role of lipoglycan-producing Ruminococcus gnavus in autoimmune flare responses in lupus-prone mice: A research protocol. URNCST Journal. 2026 Mar 04: 10(3).
https://urncst.com/index.php/urncst/article/view/913
DOI Link: https://doi.org/10.26685/urncst.913