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Date
Evaluating Systemic Effects of Synthetic Flaxseed Lignan LGM2605 in a Murine Model

by

Reagan Badger

A thesis
submitted in partial fulfillment
of the requirements for the degree of
Master of Science in the Department of Biological Sciences

Idaho State University

Spring 2021
Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of REAGAN BADGER find it satisfactory and recommend that it be accepted.

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Shannon Kobs Nawotniak, Ph.D.
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Animal Welfare Research Committee Approval

June 25, 2019

Kinta Serve
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Pocatello, ID 83209

RE: Your application dated 6/11/2019 regarding study number 782: Anti-oxidant Treatments for Immune and Lung Effects of Asbestos Fiber Exposure

Dear Dr. Serve:

Thank you for your response to requests from a prior review of your application for the new study listed above. Your study is eligible for Designated Member Review (DMR) under OLAW guidelines.

This is to confirm that your application is now fully approved. The protocol is approved through 6/25/22.

You are granted permission to conduct your study as most recently described effective immediately. The study is subject to annual review on or before 6/25/2020, unless closed before that date.

Please note that any changes to the study as approved must be promptly reported and approved. Some changes may be approved by administrative review; others require full board review. Contact Tom Bailey (208-282-2179; fax 208-282-4723; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Curt Anderson, PhD
IACUC Chair
Acknowledgements

I would like to thank my advisor Dr. Kinta Serve for her encouragement and guidance throughout every step of my project, and for helping to build me into the scientific researcher that I am today. Without Dr. Serve’s support, I would have had neither the confidence nor the means to pursue my master’s degree; I am profoundly grateful for the time I spent studying and working in her lab. I would also like to thank my committee member Dr. Ken Aho for his assistance with statistical analyses as well as the presentation and interpretation of my experimental data, which were undoubtedly crucial to the successful completion of this project. Furthermore, I would like to thank both Dr. Melpo Christofidou-Solomidou (University of Pennsylvania, Department of Medicine) and Dr. Jean Pfau (University of Montana, Department of Microbiology and Immunology) for both their mentorship and collaboration on these LGM2605 studies.

I would like to express my appreciation for the support of the Department of Biological Sciences at Idaho State University, which afforded me the opportunity to pursue my B.S./M.S. dual degree and allowed me to achieve my academic goals. Participating in research at the graduate level has greatly enriched my educational experience and has inspired me to continue my pursuit of research as a future physician. I would also like to express my appreciation for the Idaho State University MCRF for use of its equipment as well DNA amplification and sequencing. Finally, I would like to acknowledge the funding sources that made this work possible, including the Idaho State University Office for Research as well as funds allocated by the Boise State University Biomolecular Research Center via an NIH grant.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ARDs</td>
<td>asbestos-related diseases</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
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<tr>
<td>CBA</td>
<td>cytometric bead array</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>LA</td>
<td>Libby amphibole</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>NLRP3</td>
<td>nucleotide-binding domain (NOD)-like receptor protein 3</td>
</tr>
<tr>
<td>NMDS</td>
<td>non-metric multidimensional scaling</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor E2-related factor 2</td>
</tr>
<tr>
<td>OTUs</td>
<td>operational taxonomic units</td>
</tr>
<tr>
<td>PLF</td>
<td>peritoneal lavage fluid</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAID</td>
<td>systemic autoimmune disease</td>
</tr>
<tr>
<td>SCFAs</td>
<td>short chain fatty acids</td>
</tr>
<tr>
<td>SDG</td>
<td>secoisolarisiresinol diglucoside</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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Evaluating Systemic Effects of Synthetic Flaxseed Lignan LGM2605 in a Murine Model

Thesis Abstract—Idaho State University (2021)

Synthetic flaxseed lignan LGM2605 is noted for its antioxidant and anti-inflammatory properties. Previously, oral administration of LGM2605 was shown to reduce early (3-day) immune responses resulting from acute exposure to Libby amphibole (LA) asbestos; however, its effects on later (adaptive) immune responses have not yet been evaluated. Furthermore, no previous work has investigated the effects of orally administered LGM2605 on gut microbial composition. Through our research, we expanded upon the previous work by evaluating the effects of LGM2605 treatment within a 14-day murine model of asbestos exposure. We found that LGM2605 reduced inflammation as well as both innate and adaptive immune responses in LA-exposed mice at 14 days, supporting its possible use in the prevention of LA-induced diseases. We also considered the effects of 10-day LGM2605 treatment on gut microbiota of mice. We identified alterations in gut microbiota following LGM2605 treatment, in general shifting towards a more anti-inflammatory microbial composition.

Key words: asbestos; autoimmunity; dysbiosis; flaxseed; gut microbiota; LGM2605; Libby amphibole; secoisolarisiresinol diglucoside; systemic inflammation
Chapter I: Introduction

1. Research Overview

Flaxseeds are widely recognized for their extensive nutritional benefits, thanks in large part to their composition of omega-3 and omega-6 fatty acids, high quality protein and fiber, and rich lignan content\(^1\). Notably, secoisolarisiresinol diglucoside (SDG) is a bioactive lignan highly concentrated in flaxseed which has been shown to exhibit protective effects in a variety of systemic diseases, likely due to its antioxidant and free radical scavenging properties\(^2\). Recent studies have proposed the synthetic flaxseed lignan LGM2605 as a possible therapeutic alternative for SDG within inflammatory disease models\(^3\)–\(^5\). Similar to naturally occurring SDG, LGM2605 has been characterized as an antioxidant, free radical scavenger, and powerful chemopreventive agent. However, LGM2605 has significantly greater bioavailability compared to whole flaxseed, and it can also be readily produced in a laboratory setting\(^6\), making it an ideal candidate for potential therapeutic use.

LGM2605 has previously demonstrated protective effects in the context of radiation and asbestos exposure, which are known inducers of inflammation\(^5\)–\(^8\). Currently, we are investigating the potential for LGM2605 to reduce inflammation caused by exposure to Libby amphibole (LA) asbestos. LA has been linked to increased risk of asbestosis, mesothelioma, pulmonary disease, and systemic autoimmune disease. Our most recent work showed that oral administration of LGM2605 reduced hyper-acute immune responses following exposure to LA in a 3-day murine model\(^9\), demonstrating the possible use of LGM2605 as a treatment for LA-induced diseases. However, the effects of LGM2605 on adaptive (longer-term) immune responses following LA exposure are still unknown. Through our research, we examined the effects of LGM2605 treatment within a 14-day murine model of LA exposure (Chapter II),
thereby extending upon the previous work by considering a longer treatment period. We hypothesized that orally administered LGM2605 would reduce inflammation as well as both innate and adaptive immune responses at this time point.

In addition to evaluating the immune-modulating potential of LGM2605, we were also interested in any potential effects of LGM2605 on gut microbial composition. Dietary supplements, including flaxseed, may be utilized to promote gut health and reduce systemic inflammation via gut-modulating effects. Previous studies have demonstrated that dietary flaxseed supplementation beneficially alters the gut microbial composition of mice, supporting a reduction in systemic inflammation\(^\text{10, 11}\). However, the effects specifically of LGM2605 on gut microbiota have not yet been evaluated. We therefore examined the effects of 10-day LGM2605 treatment on the gut microbiota of mice (Chapter III). We hypothesized that orally administered LGM2605 would lead to beneficial changes in gut microbiota. Specifically, we predicted that LGM2605 treatment would increase the prevalence of anti-inflammatory bacteria, or bacteria that produce short chain fatty acids (SCFAs) and other metabolites associated with inhibition of inflammatory processes. We further predicted that LGM2605 treatment would decrease the prevalence of pro-inflammatory bacteria, or bacteria that produce lipopolysaccharides (LPS) and other toxic substances associated with increased pro-inflammatory cytokine production and increased risk of autoimmune/inflammatory disease.

Our studies demonstrate the beneficial systemic effects of LGM2605 treatment via oral administration in mice. We showed that LGM2605 treatment ameliorated inflammation and immune responses at 14 days post-LA exposure. Orally administered LGM2605 also significantly altered the gut microbial composition of mice. By investigating these effects, we were able to establish the usefulness of LGM2605 as a possible chemopreventive agent for LA-
induced diseases. Our findings may also be of interest to other investigators developing treatment plans for various other chronic, inflammatory conditions. Additionally, we established baseline changes in gut microbiota following oral LGM2605 administration, which will be an important reference point for future studies utilizing LGM2605 within gut dysbiosis models.

2. LGM2605 in Models of LA Exposure

“Asbestos” broadly refers to a group of naturally occurring mineral fibers used in various commercial applications. Asbestos fibers are long and thin, characterized by a length of at least 5μm and an aspect (length-to-width) ratio of 3:1 or longer. They are durable and heat resistant, making them ideal for use in construction—as building insulation and as a fire retardant—and in certain manufactured goods (e.g. ceiling and floor tiles, roofing shingles, and some paints). Inhalation of the fibers, however, can lead to diseases like lung cancer and fibrosis, which is why government-regulated asbestos fibers are no longer widely used for construction purposes, and significant efforts have been taken to remove them from existing structures. Asbestos fibers fall into two categories based on shape: chrysotile (serpentine) and amphibole (straight chain) fibers. While exposure to both chrysotile and amphibole fibers can cause disease, amphibole fibers are strongly associated with autoimmune responses. Libby amphibole (LA) asbestos, in particular, may pose significant health concerns and has been the subject of several recent studies.

LA is a mixture of amphibole and asbestiform fibers, including winchite, richterite, and tremolite. It was found as a contaminant of vermiculite ore mined outside of Libby, Montana, from the 1920s through 1990. During processing, vermiculite ore is “exfoliated,” or heated to high temperatures, causing it to expand/pop, thus forming a lightweight material useful for various commercial applications. In Libby, exfoliation of contaminated vermiculite released
LA fibers into the air, causing mine employees and townspeople to unknowingly inhale the toxic asbestos material. As a result, an estimated 400 Libby residents have died, and 3,000 more are currently suffering illnesses caused by asbestos exposure\textsuperscript{18}. After little national attention, Libby was finally placed on the Superfund list in 2002, initiating the largest, longest-running asbestos cleanup project in American history. The EPA later declared a Public Health Emergency in Libby, a first for the agency\textsuperscript{18}. Additionally, raw vermiculite ore was shipped to nearly 300 processing plants across the U.S., and Libby supplied in total roughly 80\% of the world’s vermiculite while its mines were operational, sending LA-containing products into businesses and residences across the globe\textsuperscript{16}. Thus, the true scope of LA contamination and risk of human exposure is likely much more widespread than previously considered.

Exposure to LA may contribute to the development of several asbestos-related diseases (ARDs), including mesothelioma, lung cancer, asbestosis, and pleural fibrosis. Asbestosis refers to lung disease resulting from the inhalation of asbestos fibers and is associated with severe fibrosis as well as an increased risk of mesothelioma\textsuperscript{19}. Pleural fibrosis is a thickening and stiffening of the pleura due to collagen deposition, which eventually contributes to loss of pulmonary function and severe chest pain. Compared to other asbestos types, exposure to LA causes pleural disease that is more severe and progresses more rapidly\textsuperscript{20}. LA exposure may also contribute to the development of systemic autoimmune disease (SAID), such as systemic scleroderma, lupus erythematosus, and rheumatoid arthritis\textsuperscript{19}. Of note, SAID and other ARDs have been reported in a much higher prevalence within the LA-exposed Libby population compared to the general public\textsuperscript{14}. 
LA-Induced Inflammation and Immune Responses

While there are many potential causes for LA-associated autoimmunity, recent studies have shown that unresolved inflammation may be a driving factor. LA initiates several inflammatory processes, including 1) cytokine release/immune cell trafficking; 2) oxidative stress/cytotoxicity; and 3) inflammasome activation, all of which contribute to immune dysfunction implicated in SAID.

Inhalation of asbestos deposits the fibers throughout the respiratory tract, leading to injury of the lung tissues. The fibers are then translocated from the lungs to the pleural cavity, where macrophages attempt (and fail) to remove them in what is known as frustrated phagocytosis. This process leads to cell damage and death, resulting in the release of cytokines as well as radical species. Local cytokines then recruit additional immune cells in the area (i.e. trafficking), propagating further injury and perpetuating the immune response.

Following interaction with asbestos fibers, macrophages and other immune cells release radical species (free radicals). Radical species include reactive oxygen species (ROS), reactive nitrogen species (RNS), and active chlorine species (ACS). The accumulation of radical species may lead to oxidative stress, which can in turn contribute to cytotoxicity, damaging the cell membrane and other cell components, such as proteins, lipids, lipoproteins, and DNA. Over time, oxidative stress may induce a variety of chronic, degenerative diseases, including SAID. Radical species, particularly ROS, also provide a stress signal prompting inflammasome activation, which elicits downstream inflammatory effects.

Inflammasomes are multi-protein intracellular complexes that detect cell stressors and respond to them by activating caspase proteins, which then proceed to activate various pro-inflammatory cytokines as part of the immune response. The NLRP3 inflammasome is an
important component of innate immunity, but inappropriate or prolonged activation of the inflammasome may lead to disease\textsuperscript{25}. NLRP3 is implicated in various autoimmune conditions and plays a key role in inflammation following asbestos exposure\textsuperscript{4, 22}. Stimulation of NLRP3 leads to activation of caspase-1, which then activates IL-1β, IL-6, IL-18, and TNF-α. Normally, these cytokines help fight off infection, but they can also induce severe inflammatory reactions associated with autoimmune disease\textsuperscript{26}. It is hypothesized that LA contributes to the development of SAID and other systemic diseases through these mechanisms of cell injury and chronic inflammation.

**Use of LGM2605 to Inhibit LA-Induced Inflammation**

SAID and other ARDs tend to be chronic and progressive, and they currently have few effective treatment options. Novel therapeutic intervention may provide a much-needed means of prevention/treatment for LA-induced diseases in exposed populations. In particular, a therapy that targets early immune responses may be effective in ameliorating inflammation that contributes to LA-associated autoimmunity as well as later disease progression.

LGM2605 is an antioxidant compound that has been shown to block early immune responses, especially inflammatory processes, observed following asbestos exposure; it may therefore be protective against SAID and other LA-induced diseases. LGM2605 reduces ROS generation by direct free radical scavenging\textsuperscript{6} and by upregulation of the Nrf2-ARE signaling pathway, which controls transcription of phase II antioxidant, cell protective enzymes\textsuperscript{3}. Previous studies have shown that LGM2605 reduces oxidative stress and cytotoxicity resulting from asbestos exposure\textsuperscript{3, 4}. LGM2605 also inhibits asbestos-induced activation of the NLRP3 inflammasome, presumably via involvement of ROS and Nrf2 pathways\textsuperscript{4}. Furthermore, LGM2605 has been shown to reduce the production of pro-inflammatory cytokines IL-1β, IL-6,
IL-18, and TNF-α, which are upregulated by asbestos exposure\(^3\). Taken together, the antioxidant, free radical scavenging, and anti-inflammatory properties of LGM2605 support its use as a possible chemopreventive agent in the context of LA-induced diseases. Further research is necessary to determine the usefulness of LGM2605 in controlling LA-induced immune responses and to establish its efficacy \textit{in vivo}.

The 2019 study by Christofidou-Solomidou et al. investigated for the first time the role of LGM2605 treatment within an \textit{in vivo} model of LA asbestos exposure. The study’s objective was to evaluate the effects of LGM2605 on early inflammatory processes previously demonstrated for LA in this model\(^{27, 28}\). Mice were given daily LGM2605 treatment (or saline control) for 3 days before and 3 days after a single intraperitoneal dose of LA asbestos (or saline control). Upon termination of treatment, peritoneal lavage was performed, and immune cell influx was measured via flow cytometry. LGM2605 was shown to reduce LA-induced hyperacute immune responses within the 3-day treatment period. LA exposure contributed to splenomegaly (spleen enlargement) indicative of immune activation, as well as increased trafficking of innate immune cells (i.e. PMN cells and macrophages) to the peritoneal cavity\(^9\). LGM2605 treatment countered these changes, contributing to a reduction of LA-induced splenomegaly and immune cell trafficking\(^9\). However, no significant changes in adaptive immune responses (i.e. recruitment/activation of B and T cells) were observed within this short time period, and no other inflammatory markers were considered.

Our current research (Chapter II) expands upon this previous work by lengthening the experimental duration to 14 days, thereby allowing sufficient time for evaluation of adaptive immune responses. We also measured several other parameters that were not considered in the 3-day trial in order to more fully evaluate the effects of LGM2605 treatment on LA-induced
inflammation and immune activation. Overall, the results of this trial, as detailed in Chapter II, suggest that LGM2605 treatment reduces inflammation as well as both innate and adaptive immune responses at 14 days post-LA exposure.

3. Gut Microbiome as a Potential LGM2605 Target

An emerging area of study is the connection between the gut microbiome and systemic inflammation. We are therefore interested in determining how LGM2605 affects gut microbial composition when administered as an oral supplement. In healthy individuals, the gut microbiome serves many functions: it aids in digestion, confers protection against pathogenic organisms, synthesizes essential vitamins and minerals, and plays an important role in the immune system. Dysbiosis of the gut microbiome can cause dysregulation of these functions and has been linked to autoimmune disease in both humans and animal models. It has been suggested that decreased bacterial diversity and/or overgrowth of more aggressive types of residential bacteria may contribute to dysbiosis as well as resulting inflammation. Dietary intervention has been shown to be an effective means of improving gut health and ameliorating inflammatory responses by altering the diversity of gut microbes in ways that benefit, rather than disturb, host functions.

While no previous studies have examined the effects specifically of LGM2605 on gut microbial composition, several have focused on dietary flaxseed supplementation in both mouse and human subjects, with promising results. Specifically, administration of dietary flaxseed oil in mice has been shown to attenuate gut dysbiosis, reduce pro-inflammatory cytokine concentrations, and improve the overall diversity of gut microbiota, supporting a reduction of systemic inflammation. Because LGM2605 is a synthetic derivative of flaxseed, we expected it to yield similar results when administered orally in mice. Our current research
(Chapter III) sought to investigate this effect. Utilizing a murine model, we administered daily LGM2605 treatment (or saline control) via oral gavage over a 10-day treatment period. We then identified changes in gut microbial composition by analysis of 16S rRNA genes of DNA extracted from mouse cecums and assessed community composition and diversity. LGM2605 treatment significantly altered the gut microbiota of mice, in general promoting a more anti-inflammatory microbial composition.

4. Summary

Overall, our research reveals beneficial systemic effects of orally administered LGM2605, supporting its utility as a chemopreventive agent. LGM2605 controls acute immune responses triggered by LA exposure; it may therefore hold significant therapeutic potential in treating various LA-induced diseases as well as other inflammatory conditions. We also demonstrated positive changes in gut microbiota following LGM2605 treatment, which may contribute to the drug’s previously reported anti-inflammatory and health protective effects. Our work lays the foundation for future studies investigating the use of longer-term LGM2605 treatment within models of chronic LA exposure, in order to evaluate the effects of LGM2605 on later disease development.
Chapter II

Synthetic Flaxseed Lignan LGM2605 Inhibits Libby Amphibole-Induced Inflammation in a 14-Day Murine Model

Abstract

Background: Libby amphibole (LA) asbestos-like fibers derived from Libby, Montana, contribute to inflammatory responses in both mice and humans. LA exposure is associated with increased risk of asbestosis, mesothelioma, pulmonary disease, and systemic autoimmune disease. The synthetic flaxseed derivative LGM2605 is a potent antioxidant and free radical scavenger, with demonstrated anti-inflammatory effects. LGM2605 was previously shown to decrease LA-induced acute inflammation and trafficking of innate immune cells at 3-days post-LA exposure in a murine model. The present study extends this work by evaluating immune responses at 14 days post-LA exposure.

Methods: Male and female C57BL/6 mice were administered daily LGM2605 treatment (100mg/kg) via Medigel cups for 3 days before and 14 days after a single intraperitoneal dose of LA fibers (200µg). Control mice were given unsupplemented Medigel cups and an equivalent dose of saline via i.p. injection. On day 14 post-LA treatment, peritoneal lavage was performed, and immune cell influx, cytokine concentrations, and immunoglobulin isotyping were analyzed via flow cytometry.

Results: LA exposure altered trafficking of both innate and adaptive immune cells. Specifically, LA exposure increased the percentages of peritoneal PMN cells, peritoneal macrophages, and both splenic and peritoneal B1a cells as well as decreased the percentage of total peritoneal B cells. In addition, LA exposure increased pro-inflammatory cytokine concentrations and induced
immunoglobulin isotype switching. These changes were countered by LGM2605 treatment, with similar trends among male and female mice.

**Conclusion:** LGM2605 ameliorated inflammation and altered immune responses at 14 days post-LA exposure, supporting its possible use in the prevention/treatment of LA-induced diseases.

**Summary:** Following exposure to Libby amphibole (LA) fibers via intraperitoneal (i.p.) injection, orally administered LGM2605 countered LA-induced trafficking of both innate and adaptive immune cells, reduced pro-inflammatory cytokine concentrations, and inhibited LA-induced immunoglobulin isotype switching among male and female mice. Our findings illustrate that LGM2605 has anti-inflammatory and immunomodulatory properties and thus may be an effective chemopreventive agent for various LA-induced diseases.

1. **Introduction**

   From the 1920s through 1990, approximately 80% of the world’s vermiculite was mined and shipped from Libby, Montana. Vermiculite is a naturally occurring mineral that expands when heated and is used for various applications, including home insulation. Unfortunately, the vermiculite mined from Libby was contaminated with amphibole asbestos and asbestiform fibers, including a mixture of winchite, richterite, and tremolite fibers. While Libby amphibole (LA) exposures were most significant among vermiculite mine and processing plant workers, they occurred anywhere the vermiculite was distributed, most notably as Zonolite Attic Insulation.

   Exposure to LA and similar asbestiform amphibole fibers has been linked to development of asbestos-related diseases (ARDs), including asbestosis and mesothelioma. A unique pulmonary disease is also described for LA-exposed populations, characterized by diffuse pleural thickening (i.e. pleural fibrosis) leading to progressive loss of lung function and increased
mortality rates\textsuperscript{20,42}. LA has also been linked to development of autoimmune responses, characterized by increased prevalence of anti-nuclear antibodies (ANAs) and cell-specific autoantibodies as well as increased incidence of systemic autoimmune diseases\textsuperscript{43-45}. While the etiology of amphibole-associated disease is undoubtedly complex and multifaceted, unresolved inflammation and oxidative damage have been linked to pathogenesis\textsuperscript{20,44,46}.

Following inhalation, LA fiber deposition initiates a complex set of inflammatory cascades beginning with activation of resident macrophages followed by recruitment of circulating macrophages and polymorphonuclear (PMN) leukocytes\textsuperscript{47-50}. These cells influx to the site of fiber deposition, where they release reactive oxygen species (ROS)\textsuperscript{4,23,51}, activate NLRP3 inflammasomes\textsuperscript{22,26}, and produce the pro-inflammatory cytokines IL-1β, IL-6, IL-18, and TNF-α\textsuperscript{52}, thus driving a feed-forward loop of inflammation and immune cell activation\textsuperscript{53,54}. Non-resolution of inflammation is thought to contribute to development of ARDs. Thus, we predicted that blocking early inflammatory steps would provide therapeutic benefits by reducing immune cell recruitment and activation at sites of LA fiber deposition.

Recent reports describe the usefulness of LGM2605, a synthetic secoisolariciresinol diglucoside or flaxseed lignan, in preventing amphibole asbestos-induced cytotoxicity, inflammation, and oxidative stress, both in vitro and in vivo\textsuperscript{4,5,55}. Mechanisms by which LGM2605 protects against asbestos-induced cytotoxicity include reduction of NLRP3 inflammasome activity\textsuperscript{4} and upregulation of the Nrf2/ARE antioxidant pathway\textsuperscript{56}. Additionally, LGM2605 has been shown to protect against oxidative stress in an Nrf2-independent manner by acting as a direct free radical scavenger\textsuperscript{3,6}.

Our most recent work examined the ability of LGM2605 to mitigate hyper-acute immune responses to LA exposure in a C57BL/6 mouse model\textsuperscript{9}. LGM2605 treatment (100mg/kg daily
via oral gavage) significantly reduced immune cell recruitment to the peritoneal cavity within 3 days of a single bolus intraperitoneal dose of LA (200µg). Specifically, LGM2605 significantly attenuated LA-induced splenomegaly and recruitment of macrophage and PMN cells. These data suggest that LGM2605 protects against very early innate immune events induced by LA exposure. However, due to the short experimental duration (3 days), we were unable to detect differences in adaptive immune responses to either LA alone or LA in combination with LGM2605. This current study expands upon our previous work by lengthening the experimental duration to 14 days, thereby allowing us to assess the ability of LGM2605 to ameliorate later effects of LA exposure. We hypothesized that LGM2605 treatment would reduce the recruitment of both innate and adaptive immune cells, limit the production of pro-inflammatory cytokines, and prevent LA-induced immunoglobin isotype switching at the site of fiber deposition.

2. Materials and Methods

**Animals** All experiments were approved by the Idaho State University Institutional Animal Care and Use Committee (IACUC). Ten-week-old female and male C57BL/6 mice (Jackson Laboratories) were housed three per cage in the Idaho State University Animal Care Facility with a 12-h light/dark cycle, constant temperature (22°C) and humidity (45%), and *ad libitum* access to standard rodent chow.

**LGM2605 Treatment** LGM2605 was prepared as previously described and provided as lyophilized aliquot by the University of Pennsylvania. Briefly, LGM2605 was synthesized from vanillin via secoisolariciresinol and glucosyl donor (perbenzoyl-protected trichloacetimidate under the influence of TMSOTf) through a concise route involving chromatographic separation of diastereomeric diglucoside derivatives (Chemveda Life Sciences, Inc., Hyderabad, India). Lyophilized samples of LGM2605 (100mg/vial) were reconstituted with sterile PBS to produce
stock solution of 33.3mg/mL. Mice were treated daily with approximately 100mg/kg LGM2605 (see Section 3.1) using Medigel Sucralose Clear H$_2$O cups (Thermo Fisher Scientific) as a delivery mechanism. Cups were prepared according to manufacturer recommendations. Briefly, reconstituted LGM2605 was added to room temperature cups by puncturing the foil lid. Lids were then taped shut and vigorously shaken for 30 seconds to disperse liquid LGM2605. A drop of food dye was added to the cup prior to mixing to visualize even distribution through the gel. Cups were changed daily so freshly prepared LGM2605 was administered. Medigel consumption was monitored daily by weighing cups at the start and end of each 24-hour period. Amount of LGM2605 added to each cup was calculated daily based on average Medigel consumption and weight of the mice in each cage for each day (see Section 3.1). Control mice were given Medigel cups supplemented with equal volume of sterile PBS. Figure 1 illustrates assignment of mice to treatments (A) as well as treatment schedule (B).

Mineral Fibers (LA) Libby amphibole (LA) asbestos was provided by the United States Environmental Protection Agency (EPA) as a composite sample of asbestos-rich rock samples collected from multiple sites in the W.R. Grace mine outside of Libby, Montana. The LA sample was previously characterized using a suite of methods including transmission electron microscopy (TEM) with selected area electron diffraction (SAED)$^{57}$, scanning electron microscopy (SEM) with energy dispersive spectroscopy (EDS), x-ray diffraction$^{58}$, and wavelength dispersive electron probe microanalysis (EPMA)$^{59}$. LA fibers were not elutriated.

LA Exposure LA fibers were prepared as previously described$^{57}$. Briefly, fibers were prepared as 1mg/ml suspensions in sterile phosphate buffered saline (PBS, pH 7.4) and sonicated (Branson Ultrasonics, Danbury, CT, USA) for 15 min prior to use to minimize aggregation. Fiber solutions were boiled for 1 hour to inactivate any bacterial contaminants$^{60}$ and then tested for endotoxin.
Endotoxin testing of fiber suspensions was performed prior to instillations using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Biotech Corp), following the manufacturer's protocol. Samples included phosphate-buffered saline (PBS) and suspended LA (1.0mg/ml) in sterile PBS; these were tested against a standard curve provided with the kit. Briefly, all samples were added to endotoxin-free tubes along with 100µl of LAL and incubated at 37°C for 6 min. Stop solution and color stabilizers were added to each tube, and then 200µl of each solution was moved to a 96-well plate. Absorbance was read in duplicate for each sample at 545nm. Endotoxin was detected at 0.071EU/mL, which is below the acceptable lower limit for animal exposures. Mice were subsequently exposed to LA fibers via intraperitoneal injection using a 25-gauge needle with 200µl, thus giving 200µg per mouse in a single bolus dose.

**Tissues Harvest** At the experimental endpoint, animals were euthanized using CO2 asphyxiation followed by cardiac puncture for blood collection. Blood was allowed to clot and then centrifuged to collect serum. Serum was stored at -20°C until use. Peritoneal lavage fluid (PLF) was collected by first injecting four ml of sterile PBS with 5% fetal bovine serum (FBS) into the intact peritoneal cavity, gently agitating the peritoneal cavity, and then aspirating three ml using a 21-gauge needle. PLF was centrifuged, and the cells were prepared for flow cytometry. The resulting cell-free PLF was aliquoted and frozen for further analyses. Spleens were also harvested, weighed, and then minced and prepared as single cell suspensions as previously described, including a brief wash in Red Blood Cell Lysis solution (eBioscience/ThermoFisher Scientific, Waltham, MA, USA) to select for white blood cells. The splenocytes were counted using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, USA), and 1 million cells from each sample were placed in separate tubes containing 100µl PBS with 3% Bovine Serum
Albumin as blocking agent. Sets of cells were stained with BD Biosciences (San Jose, CA, USA) antibodies as follows:

- B cells: CD19 (PE) or IgM (PerCP Cy5.5)
- B1a B cells: IgM<sup>pos</sup> (PerCP Cy5.5), CD5<sup>pos</sup> (APC), CD23<sup>neg</sup> (PE)
- Polymorphonuclear cells (PMN): Ly-6G (APC)

Cells were stained for 30 min at 4°C and then washed with 1ml ice cold PBS, twice. Staining was analyzed on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA). Isotype control antibodies (BD Biosciences, San Jose, CA, USA) determined background staining, and less than 1% of these controls was allowed in the M1 gate for percent positive. Monocytes and lymphocytes were determined based on forward and side scatter, and then the major subsets within the lymphocyte gate were identified based on the antibodies listed above. Tight polygonal regions were used (instead of quadrants) to identify the subpopulations.

**Cytokine Detection** Cytokine levels in PLF and serum were measured using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose, CA, USA). The following cytokines were measured using CBA with flow cytometry: Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemotactic Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70). The CBA procedure was carried out according to the manufacturer’s instructions. Briefly, a standard curve was prepared by making a stock solution (5,000pg/mL) for each cytokine assayed followed by serial dilutions. Prior to cytokine assay, the PLF samples were concentrated three-fold using a CentriVap concentrator (Labconco, Kansas, MO, USA); serum samples were not concentrated. 50μl of concentrated PLF, serum, or prepared standard were then mixed with 50μl of capture beads resuspended in diluent. 50μl of PE detection reagent supplied with the kit was added to all samples and incubated in the dark for
2 hours at room temperature. 1.0ml of wash buffer from the kit was then added to all samples and centrifuged at 200g for 5 min. Supernatant was discarded, and pellets were resuspended in 300µl of wash buffer. All samples were analyzed on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA) following instrument set-up using the instrument set-up beads provided with the kit. Standard curve data was utilized to calculate cytokine concentrations in PLF and serum samples.

**Immunoglobulin Isotyping** Immunoglobulin isotypes in PLF were evaluated using the Cytometric Bead Array (CBA) Mouse Immunoglobulin Isotyping Kit (BD Biosciences, San Jose, CA, USA). The following isotypes were measured using CBA with flow cytometry: IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, and IgE. κ and λ light chains were also evaluated for each isotype. The CBA procedure was carried out according to the manufacturer’s instructions. Briefly, mouse Ig standards (2.5µg/mL) were reconstituted in buffer provided with the kit. 50µl of concentrated PLF or prepared standard was then mixed with 50µl of capture beads resuspended in diluent. 1.0ml of wash buffer from the kit was added to all samples and centrifuged at 1200rpm for 5 min. Supernatant was discarded, and pellets were resuspended in 100µl of wash buffer. 50µl of PE detection reagent supplied with the kit was then added to all samples and incubated in the dark for 15 min at room temperature. Next, 500µl of wash buffer from the kit was added to all samples and centrifuged at 1200rpm for 5 min. Supernatant was discarded, and pellets were resuspended in 500µl of wash buffer. All samples were analyzed on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA) following instrument set-up using the instrument set-up beads provided with the kit. Mouse Ig standards were utilized to determine immunoglobulin isotype concentrations in PLF samples.
Statistical Analysis

Two-way ANOVA was utilized to detect overall treatment and sex effects for all cell types and inflammatory markers that were measured. Tukey post-hoc tests were utilized to further evaluate effects of treatment by performing pairwise comparisons between individual treatment groups. For each parameter, effects of LA exposure were determined by comparing LA only v. control (significance represented by asterisks in Figures 2-8). Effects of LGM2605 treatment in LA-exposed mice were evaluated by comparing LGM2605+LA v. LA only (significance represented by pound symbols in Figures 2-8). One-way ANOVA was utilized to specifically examine sex effects within individual treatment groups and/or treatment effects subdivided by sex.

3. Results

3.1. Mice consumed approximately 100mg/kg LGM2605 daily.

Medigel cups were weighed at the start and end of each 24-hour period to measure mouse consumption. Average consumption of drug was calculated based on individual mouse body weights and was not altered by presence of food dye (used to ensure even distribution of drug throughout gel). The average daily amount of drug consumed was 104.3mg/kg for females (CI95% ±2.32) and 101.9mg/kg for males (CI95% ±3.23). There was no statistically significant difference between male and female consumption. Control mice were fed an equivalent dose of saline (vehicle). Overall, mouse weights remained constant over the course of the study, with an average daily weight change of +0.06g (CI95% ±0.02), suggesting adequate hydration and weight gain.

3.2. LA-induced increase in spleen weights is reduced by LGM2605 treatment.

Relative spleen weights were calculated by dividing the spleen weight by total mouse weight. Overall, relative spleen weights were significantly altered by treatment, $p<0.0001$ by two-way
ANOVA. LA treatment significantly increased relative spleen weights compared to control, $p=0.001$ by Tukey post-hoc test (Figure 2A). Treatment with LGM2605+LA reduced relative spleen weight back to control levels, $p<0.001$ by Tukey post-hoc test. Female mice demonstrated significantly higher relative spleen weights compared to males, $p<0.0001$ by two-way ANOVA (Figure 2B), but the overall pattern of LA-induced increases and LGM2605-induced decreases in splenomegaly were consistent within each sex.

3.3. LGM2605 treatment reduces peritoneal PMN and macrophage levels following LA exposure.

Overall, treatment effects were significant among peritoneal PMN cells, $p<0.0001$ by two-way ANOVA. LA treatment significantly increased the percentage of peritoneal PMN cells (as a percent of total cells) compared to saline control, $p<0.0001$ by Tukey post-hoc test (Figure 3A). Treatment with LGM2605+LA reduced the percentage of peritoneal PMN cells back to control levels, $p<0.001$ by Tukey post-hoc test. Treatment effects were also significant among peritoneal macrophages, $p<0.0001$ by two-way ANOVA. LA treatment significantly increased the percentage of peritoneal macrophages compared to saline control, $p<0.001$ by Tukey post-hoc test (Figure 3B). Treatment with LGM2605+LA reduced the percentage of peritoneal macrophages back to control levels, $p<0.0001$ by Tukey post-hoc test. No sex effect was noted for macrophages or PMN cells in PLF, and no differences in splenic macrophage or PMN cells were detected among any treatment groups (data not shown).

3.4. LGM2605 treatment alters peritoneal and splenic B1a B cell levels following LA exposure.

Overall, treatment effects were significant among peritoneal B1a B cells, $p<0.0001$ by two-way ANOVA. Treatment with LA alone significantly increased peritoneal B1a B cells (as a percent
of total lymphocytes) compared to saline control, \( p=0.017 \) by Tukey post-hoc test (Figure 4).

Interestingly, LGM2605 treatment, either alone or combined with LA, significantly reduced the percentage of peritoneal B1a B cells compared to both LA only \( (p<0.0001 \) by Tukey post-hoc test) and control \( (p<0.01 \) by Tukey post-hoc test). Treatment effects were also significant among splenic B1a B cells, \( p<0.0001 \) by two-way ANOVA. LA treatment significantly increased the percentage of splenic B1a cells compared to saline control, \( p=0.043 \) by Tukey post-hoc test (Figure 5A). LGM2605 treatment, either alone or combined with LA, significantly reduced the percentage of B1a cells back to control levels, \( p<0.01 \) by Tukey post-hoc test. No effect of sex was determined for peritoneal B1a B cell populations, though there was a significant sex effect for splenic B1a B cells, \( p<0.01 \) by two-way ANOVA (Figure 5B, 5C). Female mice had a significantly higher percentage of splenic B1a B cells compared to males in the control group \( (p<0.01 \) by one-way ANOVA) and in the LA only group \( (p=0.015 \) by one-way ANOVA). However, this difference was absent in the LGM2605 treatment groups.

### 3.5. LGM2605 treatment alters total peritoneal B cell but not T cell levels following LA exposure.

Overall, treatment effects were significant among total peritoneal B cells, \( p<0.0001 \) by two-way ANOVA. LA treatment significantly reduced total peritoneal B cells (as a percent of total lymphocytes) compared to saline control, \( p<0.001 \) by Tukey post-hoc test (Figure 6). Treatment with LGM2605+LA partially restored the percentage of total peritoneal B cells \( (p=0.017 \) by Tukey post-hoc test), though they were still significantly lower than saline control or LGM2605 alone \( (p<0.001 \) by Tukey post-hoc test). No sex effect was detected among any of the B cell populations examined. Interestingly, LA treatment did not alter the percentages of total
peritoneal T cells or Th cells (data not shown). In addition, no effects of LA were noted on the total splenic B or T cell populations or on Th cell populations (data not shown).

3.6. LGM2605 treatment significantly alters IL-6 and MCP-1 concentrations in PLF following LA exposure.

Table 1 reports overall treatment effects among cytokine concentrations in PLF. LA treatment significantly increased the concentration of IL-6 in PLF compared to saline control, $p<0.001$ by Tukey post-hoc test; treatment with LGM2605+LA significantly reduced the concentration of IL-6 in PLF back to control levels, $p<0.001$ by Tukey post-hoc test (Figure 7A). In addition, LA treatment significantly increased the concentration of MCP-1 in PLF compared to saline control, $p<0.01$ by Tukey post-hoc test; treatment with LGM2605+LA significantly reduced the concentration of MCP-1 in PLF back to control levels, $p<0.001$ by Tukey post-hoc test. PLF concentrations of IL-10, TNF, IFN-γ, and IL-12p70 were unaffected by either LA or LGM2605 treatment at this time point. No effect of sex was detected among any of the cytokines examined.

3.7. LGM2605 treatment significantly alters IL-6 and IL-10 concentrations in serum following LA exposure.

Table 2 reports overall treatment effects among cytokine concentrations in serum. LA treatment significantly increased IL-6 concentration in serum compared to saline control, $p=0.015$ by Tukey post-hoc test (Figure 7B). Treatment with LGM2605+LA reduced IL-6 concentration back to control levels, $p<0.01$ by Tukey post-hoc test. In addition, treatment with LGM2605+LA significantly increased IL-10 concentration in serum compared to LA only, $p=0.011$ by Tukey post-hoc test. However, neither LA nor LGM2605+LA led to significant alterations in IL-10 concentration relative to control. Serum concentrations of MCP-1, TNF, IFN-γ, and IL-12p70
were unaffected by either LA or LGM2605 treatment at this time point. No effect of sex was detected among any of the cytokines examined.

3.8. LGM2605 treatment significantly alters immunoglobulin isotypes in PLF following LA exposure.

Table 3 reports overall treatment effects among immunoglobulin isotypes in PLF. LA treatment significantly decreased the relative concentrations of IgG$_1$ ($p=0.032$ by Tukey post-hoc test) and IgG$_{2b}$ ($p<0.01$ by Tukey post-hoc test) in PLF compared to saline control (Figure 8). Treatment with LGM2605+LA significantly increased the concentrations of IgG$_1$ ($p<0.01$ by Tukey post-hoc test) and IgG$_{2b}$ ($p<0.01$ by Tukey post-hoc test) in PLF compared to LA treatment, thus returning concentrations back to control levels. In addition, LA treatment significantly increased the relative concentration of IgA in PLF compared to saline control, $p=0.037$ by Tukey post-hoc test; treatment with LGM2605+LA significantly decreased the relative concentration of IgA back to control levels, $p=0.025$ by Tukey post-hoc test. Interestingly, there was a significant sex effect for IgA ($p=0.020$ by two-way ANOVA), with control males demonstrating significantly higher IgA levels compared to control females ($p<0.01$ by one-way ANOVA). No other sex effects were noted. In general, LA treatment decreased the ratio of $\kappa$ antibody concentration/$\lambda$ antibody concentration compared to saline control (Table 3). Treatment with LGM2605+LA restored $\kappa/\lambda$ ratios back to control levels. Neither LA nor LGM2605+LA significantly altered the relative concentrations of IgG$_{2a}$, IgG$_3$, IgM, or IgE; however, trends in $\kappa/\lambda$ ratios were consistent across all isotypes.

4. Discussion

Libby amphibole (LA) asbestos is highly inflammatory and has been linked to the development of various asbestos-related diseases (ARDs), including autoimmune disease$^{44, 62, 63}$. Development
of ARDs and autoimmunity may be due to chronic, non-resolving inflammation and immune activation perpetuated by LA fiber deposition. Inhalation of LA fibers promotes increased trafficking of innate immune cells (i.e. PMN cells and macrophages) to the site of fiber deposition, where these cells contribute to oxidative stress and inflammation which drive further immune cell activation\textsuperscript{52,53}. A potent antioxidant and free radical scavenger, synthetic LGM2605 has been proposed as a possible chemopreventive agent for targeting many of these early immune responses in order to reduce progression to chronic, less treatable disease states\textsuperscript{64}. Together with the results of prior investigations, our findings suggest that LGM2605 inhibits acute inflammation induced by LA exposure and may therefore be protective against the development of various LA-induced diseases.

Within the present study, LA exposure enhanced total immune cell influx to the site of fiber deposition and increased pro-inflammatory cytokine levels, both locally and systemically, at the 14-day time point. LGM2605 treatment reduced total immune cell influx in LA-exposed mice as well as decreased pro-inflammatory cytokine levels. We considered sex-specific responses to both LA exposure and LGM2605 treatment; in general, no differences between male and female mice were determined. Adaptive immune responses were noted in B but not T lymphocyte populations at 14 days post LA-exposure, and LGM2605 demonstrated protective effects against LA-induced inflammation which may be relevant for future long-term investigations.

Notably, mice were dosed with LA fibers via intraperitoneal (i.p.) injection; this method was also utilized in the previous 3-day trial. LA exposure in association with ARDs is typically by inhalation, affecting mainly the lungs and surrounding pleural cavity. However, LA fibers are known to access both the pleural and peritoneal cavities upon exposure\textsuperscript{65}. The peritoneal cavity
is utilized as a surrogate for the pleural cavity in LA exposure studies because collection of
pleural cavity tissue can be difficult, yielding few cells for subsequent analysis. In addition,
mice do not inhale asbestos fibers in the same way that humans do; this makes i.p. injection the
preferred method of LA exposure for inflammatory and immunological effects within murine
models. Despite this important distinction, we can still make useful parallels between the pleural
and peritoneal cavities due to the similarity of immune cells and inflammatory processes
characteristic of both sites.

Compared to the previous 3-day trial, the present study utilized a different method of
drug dosing in mice which may ultimately be more effective for future long-term investigations
into the therapeutic use of LGM2605. Previously, mice were dosed with LGM2605 by daily oral
gavage; although a precise method of drug delivery, this technique tends to be stressful for mice,
resulting in increased morbidity that may skew the observed physiological outcomes. Oral
gavage is also relatively time-consuming when utilized for daily treatments, making it
undesirable for long-term dietary intervention studies. The use of Medigel cups for oral
administration of LGM2605 offers a practical alternative. This method is much less stressful for
mice, as it reduces total handling time; it also requires less time for daily treatments. In order to
ensure dosing consistency, however, it is important that both mice and cups are weighed on a
daily basis, altering the dosage amount per cage as needed. Appropriate drug dosages are not
guaranteed by this strategy, as gel consumption may vary between individual mice. However, the
data presented here suggest that individual mouse consumption of LGM2605-containing gel was
sufficient for hydration and weight gain, and that drug consumption provided significant
protective effects against LA fiber exposure.
At 3 days post-LA exposure, LGM2605 treatment was previously shown to attenuate LA-induced splenomegaly, or enlargement of the spleen\(^9\). In the present study, a similar outcome was observed. Exposure to LA fibers led to an increase in the relative spleen weights of both male and female mice (Figure 2A), with female mice demonstrating larger relative spleen weights on average (Figure 2B). Increased spleen weights may serve as an indicator of immune activation since immune cells pass through the spleen when trafficking to the site of inflammation/infection\(^9\). Splenomegaly is a well-documented feature of autoimmune disease, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)\(^69\), which are commonly associated with LA exposure\(^14\). Treatment with LGM2605+LA significantly reduced spleen weights that were enlarged by LA exposure, suggesting a reduction in immune activation. These findings were substantiated by flow cytometry data, which in general showed an increase in immune cell trafficking among LA-exposed mice that was countered by LGM2605 treatment.

In the present study, we also reported similar trends in the recruitment/activation of PMN cells and macrophages compared to the previous 3-day trial. LA exposure increased the percentages of peritoneal PMN cells (Figure 3A) and macrophages (Figure 3B), demonstrating enhanced trafficking of these cells to the peritoneal cavity. Recruitment of PMN cells and macrophages occurs as part of the innate immune response; these cells are generally regarded as first responders in inflammation/infection and are important mediators of oxidative stress\(^70\). Therefore, alterations in innate immune responses were identified at 14 days post-LA exposure. Treatment with LGM2605+LA decreased the percentages of peritoneal PMN cells and macrophages back to control levels, demonstrating reduced trafficking of these cells to the site of fiber deposition. No differences between male and female mice were shown.
In order to further investigate the effects of LA exposure and LGM2605 treatment on innate immune responses, we also measured B1a cell populations. B1a cells are innate immune cells that may play a crucial role in LA-associated autoimmunity. Exposure to LA fibers led to a significant increase in the percentages of both peritoneal (Figure 4) and splenic (Figure 5A) B1a cells, while treatment with LGM2605+LA significantly reduced these populations back to control levels. B1a cells are long-lived, self-renewing lymphocytes that are found primarily in the peritoneal and pleural cavities. They are largely responsible for the spontaneous secretion of circulating IgM referred to as “natural antibodies,” allowing these cells to respond rapidly to inflammation/infection without the need for prior immunization. B1a cells are capable of recognizing self-antigens, which may serve in the clearance of apoptosis products. However, due to their autoreactivity, B1a cells have been linked to autoimmune disease in both human and animal models, playing a role in the development of such conditions as RA and SLE. B1a cells have been shown to respond to amphibole asbestos exposure by trafficking from the site of inflammation to the spleen and lymph nodes, where they may ultimately promote immune dysfunction. Therefore, the observed reduction of B1a cells following oral administration of LGM2605 suggests that early LGM2605 treatment may protect against later development of LA-associated autoimmunity. Interestingly, female mice had a significantly higher splenic B1a cell population compared to males within the control (Figure 5B) and LA treatment (Figure 5C) groups; further research may be necessary to explain this effect and account for the observed sex differences.

Previous in vivo studies have shown a decrease in both pleural and peritoneal B1a cells at 3 days post-LA exposure, indicative of B1a cell trafficking to secondary lymphatic organs. However, recovery of pleural B1a cells was reported at 6 days post LA-exposure, and trafficking
of these cells to the spleen and lymph nodes peaked at about 7 days post-LA exposure. In the present study, our results were consistent with these findings. The observed increase in peritoneal as well as splenic B1a cells suggests that B1a cells activated in the spleen may return to the site of local inflammation by 14 days post-LA exposure, allowing for a recovery of peritoneal B1a cell numbers. Follow-up studies are needed to more fully elucidate the effects of LA exposure on B1a cell populations over an extended treatment period. Future long-term investigations should also evaluate alpha-4 integrin, a surface marker that controls B cell retention in the peritoneum, in order to confirm active trafficking of B1a cells. Previously, alpha-4 integrin expression was shown to decrease on B1a cells following LA exposure, signifying a detachment of these cells from the peritoneal cavity and increased cell motility. Although we did not measure alpha-4 integrin expression within the present study, it is possible that alpha-4 integrin may be upregulated at 14 days post-LA exposure, contributing to an accumulation of these cells within the peritoneal cavity.

With respect to adaptive immune responses, our previous 3-day trial identified a significant decrease in Th cells following LA exposure that was countered by LGM2605 treatment. However, no other changes in B or T cell populations were observed within this short time period. In the present study, we predicted that changes in adaptive immune responses would be detectable within the 14-day treatment period; we therefore evaluated total B cell, total T cell, and Th cell populations in the peritoneal cavity and spleen. Interestingly, the percentages of total T cells and Th cells were unaffected by treatment at 14 days post-LA exposure. This outcome is consistent with that of a previous in vivo study reporting the effects of long-term (7-month) LA exposure, which found that LA did not alter total numbers of splenic or peritoneal T cells. Our findings suggest that Th cells suppressed at 3 days post-LA exposure experienced a recovery in
their numbers by 14 days, allowing for a return to control levels. Exposure to LA fibers also led to a significant decrease in the percentage of total peritoneal B cells over a 14-day period (Figure 6). Since B cells rely on activation by Th cells, it is possible that early Th cell suppression resulted in subsequent suppression of B cells, despite the recovery of Th cells to their pre-treatment levels by 14 days post-LA exposure. These results are supported by a previous in vitro study showing that asbestos exposure stimulates macrophages to release TNF-α and IL-6, and that these cytokines in turn suppress B-cell proliferation²⁷. Since both peritoneal macrophages and IL-6 production were significantly increased by LA in the present study, this effect may further explain the observed decrease in total peritoneal B cells following LA exposure.

Meanwhile, treatment with LGM2605+LA increased the percentage of total peritoneal B cells relative to LA only, with no effect of sex determined. Total splenic B cells were unaltered by either LA exposure or LGM2605 treatment, suggesting that B cells were not actively trafficking via the spleen at this time point.

Taken together, the alterations in B1a cells and total B cells suggest that both innate and adaptive immune responses were engaged at 14 days post-LA exposure, and that LGM2605 treatment ameliorated these responses. Interestingly, our data showed that LA exposure does not invoke a significant influx of lymphocytes to the site of fiber deposition, in agreement with the findings of previous studies⁹,⁴¹. Although not evaluated in the present study, it is possible that B and T cell maturation may alter in response to LA, despite limited changes in total cell numbers. In future investigations, flow cytometry should be utilized to differentiate between immature and mature lymphocytes following LA exposure and could provide additional insight into the effects of LA exposure on immune activation.
In order to further examine the effects of both LA exposure and LGM2605 treatment on inflammation, we measured cytokine levels in PLF and serum using a cytometric bead array (CBA). LA exposure significantly increased the concentrations of the pro-inflammatory cytokines IL-6 and MCP-1 in PLF (Figure 7A), indicative of localized inflammation within the peritoneal cavity. Notably, IL-6 promotes differentiation of monocytes to macrophages\(^\text{75}\), and MCP-1 is one of the major chemokines that regulates migration and infiltration of monocytes/macrophages as part of the inflammatory response\(^\text{76}\). These findings coincide with cellular flow cytometry data, which demonstrated an increase in peritoneal macrophages following LA exposure (Figure 3B). Treatment with LGM2605+LA significantly reduced PLF concentrations of IL-6 and MCP-1 back to control levels, reducing inflammation and attenuating LA-induced inflammatory responses within the peritoneal cavity. These results are supported by the fact that LGM2605 treatment contributed to a reduction in peritoneal macrophages within LA-exposed mice. In addition, LA exposure significantly increased the concentration of IL-6 in serum (Figure 7B), indicative of systemic inflammation. Elevated serum levels of IL-6 have been implicated in autoimmune disease (i.e. RA, SLE) as well as other chronic, inflammatory conditions\(^\text{76}\). Treatment with LGM2605+LA significantly reduced the serum concentration of IL-6 back to control levels, suggesting beneficial effects in the context of LA-induced inflammation and associated disease pathologies. Treatment with LGM2605+LA also increased the serum concentration of regulatory cytokine IL-10 compared to LA only (Figure 7B). Secreted by most cells of the immune system, IL-10 is regarded as both immunosuppressive and anti-inflammatory, as it suppresses the production of various pro-inflammatory cytokines and inhibits the antigen-presenting function of both macrophages and dendritic cells\(^\text{77,78}\). IL-10 may therefore combat chronic inflammation by mediating resolution of immune responses. Taken together,
these results suggest that LA promotes both local and systemic inflammation by increasing pro-inflammatory cytokine production; LGM2605 not only counters these effects but also demonstrates likely immunosuppressive properties.

Both LA exposure and LGM2605 treatment were found to significantly alter immunoglobulin isotypes in PLF. Specifically, LA exposure contributed to a significant decrease in the relative concentrations of IgG\(_1\) and IgG\(_{2b}\), while treatment with LGM2605+LA restored these concentrations back to control levels (Figure 8). Murine IgG\(_1\) is functionally similar to human IgG\(_4\)\(^{79}\), and we have previously noted low levels of IgG\(_4\) in sera of LA-exposed populations relative to other fiber exposures\(^{14}\). Both murine IgG\(_1\) and human IgG\(_4\) may possess immunosuppressive effects by inhibiting complement activation, thus protecting against inflammation and cell death\(^{14}\). Decreased levels of these antibodies may therefore suggest an increase in asbestos-induced cytotoxicity. LA exposure also contributed to a significant increase in the relative concentration of IgA (Figure 8). IgA is the most abundantly produced isotype in higher mammals and is commonly associated with mucosal surfaces, where it forms a first line of defense against inhaled or ingested pathogens. Notably, IL-6 has been shown to enhance IgA synthesis by promoting terminal differentiation of IgA-committed B cells\(^{80}\), and we demonstrated that IL-6 concentrations were significantly increased by LA exposure (Figure 7). Furthermore, murine B1 cells in the peritoneal cavity are distinguished from other B cells by their preferential class switching to IgA under minimal stimulatory conditions, via an IL-6-independent pathway\(^{81-83}\). Murine cell line models have shown that LA induces increased surface expression of IgA among peritoneal B1a cells\(^{27}\), indicative of isotype class switching. In the present study, we observed an increase in peritoneal B1a cells following LA exposure (Figure 4), suggesting a likely role of these cells in IgA production. Interestingly, a previous in vivo study
reported an increase in peritoneal IgM at 3 days post-LA exposure, followed by a decrease in IgM to control levels by days 6-7. Because we observed an increase in IgA but no changes in IgM, this result indicates isotype class switching from IgM to IgA at 14 days post-LA exposure. Treatment with LGM2605+LA reduced the relative concentration of IgA back to control levels, potentially by inhibiting LA-induced isotype class switching and effectively regulating immune responses.

Among all immunoglobulin isotypes examined in PLF, LA exposure consistently decreased the ratio of κ/λ light chains (Table 3), corresponding to increased λ light chain production. The normal κ/λ ratio in human sera is approximately 1.5, while the normal κ/λ ratio for mice is approximately 19, although a slightly different composition is expected in PLF. Any significant deviation from normal range may constitute an immune disturbance. Notably, disturbed κ/λ ratios have been described in various autoimmune conditions, including RA, and may serve as important prognostic factors. In addition, B1 cells in the peritoneal cavity are noted for their increased λ light chain production. These cells are responsible for the secretion of IgM autoantibodies, and their numbers have been shown to increase within autoimmune animals. The observed increase in λ light chain production at 14 days post-LA exposure—coupled with an increase in peritoneal B1a cells (Figure 4)—may therefore suggest increased autoimmune activity. Meanwhile, treatment with LGM2605+LA returned κ/λ ratios to control levels, suggesting protective effects in the context of LA-associated autoimmunity. However, more research is necessary to determine the physiological relevance of κ/λ ratios in LA exposure models and their implications for later disease development.
5. Conclusion

In summary, LA exposure via i.p. injection promoted inflammatory responses in both male and female mice over a 14-day period. Oral treatment with LGM2605 ameliorated these responses, as measured by changes in immune cell trafficking, cytokine production, and immunoglobulin isotype switching.

Author Contributions

R.B. and K.S. were responsible for the study design and manuscript preparation. Both authors carried out tissue harvest/cell collection and cellular flow cytometry. R.B. performed cytokine detection, immunoglobulin isotyping, and statistical analyses; R.B. also generated tables and figures included in the manuscript. K.S. managed the in vivo experiment, administering LA and LGM2605 treatments.

Conflicts of Interest

The authors report no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements and Funding

This research was supported by funds allocated by the Idaho State University Office for Research. We would like to thank Dr. Melpo Christofidou-Solomidou (University of Pennsylvania, Department of Medicine) and Dr. Jean Pfau (University of Montana, Department of Microbiology and Immunology) for their mentorship as well as critical review of the manuscript. We would also like to thank Dr. Melpo Christofidou-Solomidou for provision of LGM2605 and Medigel cups utilized in this study. We further acknowledge Zach Ditzig of the Serve lab for his assistance with tissue harvest/cell collection.
Figure 1. Assignment of treatment groups and schedule of treatment. (A) Mice were randomly divided into one of 4 treatment groups, with 6 males and 6 females per group. Each treatment is designated by diet/i.p. injection: control (saline/saline), LGM2605 only (LGM2605/saline), LA only (saline/LA), and LGM2605+LA (LGM2605/LA). (B) For 3 days prior to i.p. injection, mice received daily treatment of LGM2605 or saline control via Medigel cups. LA fibers or saline control were then delivered by i.p. injection. Mice received daily treatment of LGM2605 or saline control for an additional 14 days following LA exposure. On day 14, mice were euthanized, and tissues were collected for subsequent analysis.
Figure 2. Spleen weight analysis. Relative spleen weights were determined by dividing raw spleen weights (g) by individual mouse body weights (g). (A) LA treatment significantly increased relative spleen weights compared to saline control (*** \( p=0.001 \)); LGM2605+LA treatment reduced relative spleen weights back to control levels, (### \( p<0.001 \)). Following two-way ANOVA, data were analyzed via Tukey post-hoc test. The observed patterns were consistent with sex; however, female mice demonstrated significantly higher relative spleen weights compared to males (B), \( p<0.0001 \) by two-way ANOVA.
Figure 3. Detection of peritoneal PMN cells and macrophages by flow cytometry. (A) LA treatment significantly increased the percentage of peritoneal PMNs compared to saline control (** p<0.0001); LGM2605+LA treatment reduced the percentage of peritoneal PMNs back to control levels (### p<0.001). (B) LA treatment significantly increased the percentage of peritoneal macrophages compared to saline control (*** p<0.001); LGM2605+LA treatment reduced the percentage of peritoneal macrophages back to control levels (#### p<0.0001). Following two-way ANOVA, data were analyzed via Tukey post-hoc test.
Figure 4. Detection of peritoneal B1a B cells by flow cytometry. LA treatment significantly increased the percentage of peritoneal B1a B cells compared to saline control (* p=0.017). LGM2605 treatment, either alone or combined with LA, significantly reduced the percentage of peritoneal B1a B cells compared to both LA only (#### p<0.0001) and control (p<0.01). Following two-way ANOVA, data were analyzed via Tukey post-hoc test. No sex effects were observed.
Figure 5. Detection of splenic B1a B cells by flow cytometry. (A) LA treatment significantly increased the percentage of splenic B1a B cells compared to saline control (* p=0.043); LGM2605+LA treatment reduced the percentage of splenic B1a B cells back to control levels (## p<0.01). Following two-way ANOVA, data were analyzed via Tukey post-hoc test. Female mice had a significantly higher percentage of splenic B1a B cells compared to males in both the control group (B), p<0.01 by one-way ANOVA, and the LA only group (C), p=0.015 by one-way ANOVA.
Figure 6. Detection of total peritoneal B cells by flow cytometry. LA treatment significantly reduced the percentage of total peritoneal B cells compared to saline control (*** p<0.001). LGM2605+LA treatment partially restored the percentage of total peritoneal B cells (# p=0.017), though they were still significantly lower than saline control or LGM2605 only (p<0.001). Following two-way ANOVA, data were analyzed via Tukey post-hoc test.
Figure 7. Detection of cytokines in PLF and serum by cytometric bead array. (A) In PLF, LA treatment significantly increased the concentrations of IL-6 (*** $p<0.001$) and MCP-1 (**) $p<0.01$) compared to saline control; LGM2605+LA treatment reduced the concentrations of IL-6 (### $p<0.001$) and MCP-1 (#### $p<0.001$) back to control levels. (B) In serum, LA treatment significantly increased IL-6 concentration compared to saline control (*) $p=0.015$); LGM2605+LA treatment reduced IL-6 concentration back to control levels (## $p<0.01$). LGM2605+LA treatment also significantly increased IL-10 concentration in serum compared to LA only (# $p=0.011$); however, neither LA nor LGM2605+LA led to significant alterations in IL-10 concentration compared to saline control. Following two-way ANOVA, data were analyzed via Tukey post-hoc test.
Figure 8. Detection of immunoglobulin isotypes in PLF via cytometry bead array. LA treatment significantly decreased the relative concentrations of IgG1 (* $p=0.032$) and IgG2b (** $p<0.01$) compared to saline control; LGM2605+LA treatment significantly increased the concentrations of IgG1 (## $p<0.01$) and IgG2b (## $p<0.01$) back to control levels. LA exposure also significantly increased IgA concentration compared to saline control (* $p=0.037$); LGM2605+LA treatment reduced IgA concentration back to control levels (# $p=0.025$). Following two-way ANOVA, data were analyzed via Tukey post-hoc test.
Table 1. PLF cytokines by cytometric bead array. Data are presented as mean concentration in pg/ml ± std error. Data were analyzed by two-way ANOVA (for overall treatment effects) and by one-way ANOVA (for treatment effects subdivided by sex).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LGM2605 only</th>
<th>LA only</th>
<th>LGM2605+LA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.36 ± 0.17</td>
<td>0.89 ± 0.42</td>
<td>16.84 ± 4.16</td>
<td>0.22 ± 0.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male</td>
<td>0.26 ± 0.12</td>
<td>0.41 ± 0.14</td>
<td>12.86 ± 1.77</td>
<td>0.11 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.46 ± 0.22</td>
<td>1.62 ± 0.59</td>
<td>18.03 ± 5.89</td>
<td>0.33 ± 0.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>1.11 ± 0.75</td>
<td>0.71 ± 0.30</td>
<td>0.29 ± 0.21</td>
<td>0.00 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.77 ± 0.53</td>
<td>0.59 ± 0.27</td>
<td>0.88 ± 0.58</td>
<td>0.00 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>0.60 ± 0.07</td>
<td>0.59 ± 0.30</td>
<td>1.25 ± 0.74</td>
<td>0.00 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>IFN-gamma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.10 ± 0.07</td>
<td>0.59 ± 0.30</td>
<td>1.25 ± 0.74</td>
<td>0.00 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>1.11 ± 0.75</td>
<td>0.71 ± 0.30</td>
<td>0.29 ± 0.21</td>
<td>0.00 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.16 ± 0.08</td>
<td>0.19 ± 0.07</td>
<td>0.22 ± 0.08</td>
<td>0.05 ± 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>0.15 ± 0.08</td>
<td>0.10 ± 0.05</td>
<td>0.32 ± 0.09</td>
<td>0.01 ± 0.00</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>IL-12p70</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.52 ± 0.32</td>
<td>0.21 ± 0.07</td>
<td>0.78 ± 0.26</td>
<td>0.34 ± 0.24</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>1.22 ± 0.69</td>
<td>1.84 ± 0.39</td>
<td>0.78 ± 0.26</td>
<td>0.34 ± 0.24</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Table 2. Serum cytokines by cytometric bead array. Data are presented as mean concentration in pg/ml ± std error. Data were analyzed by two-way ANOVA (for overall treatment effects) and by one-way ANOVA (for treatment effects subdivided by sex).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LGM2605 only</th>
<th>LA only</th>
<th>LGM2605+LA</th>
<th>p-value treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6</strong></td>
<td>5.47 ± 0.82</td>
<td>4.25 ± 0.38</td>
<td>7.87 ± 1.06</td>
<td>4.38 ± 0.50</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Female</td>
<td>5.28 ± 1.42</td>
<td>3.67 ± 0.37</td>
<td>7.04 ± 1.30</td>
<td>5.26 ± 0.81</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>5.66 ± 0.97</td>
<td>4.83 ± 0.65</td>
<td>8.71 ± 1.73</td>
<td>3.49 ± 0.31</td>
<td>0.036</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>15.83 ± 1.35</td>
<td>18.46 ± 1.65</td>
<td>13.09 ± 1.33</td>
<td>21.31 ± 2.59</td>
<td>0.016</td>
</tr>
<tr>
<td>Female</td>
<td>17.69 ± 2.02</td>
<td>17.03 ± 1.94</td>
<td>11.86 ± 2.19</td>
<td>25.29 ± 3.85</td>
<td>0.014</td>
</tr>
<tr>
<td>Male</td>
<td>13.97 ± 1.59</td>
<td>19.89 ± 2.71</td>
<td>14.31 ± 1.57</td>
<td>16.54 ± 2.00</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td>9.30 ± 1.37</td>
<td>6.50 ± 0.46</td>
<td>5.41 ± 0.42</td>
<td>6.47 ± 0.47</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Female</td>
<td>9.19 ± 2.16</td>
<td>7.35 ± 0.54</td>
<td>5.18 ± 0.59</td>
<td>6.60 ± 0.54</td>
<td>0.045</td>
</tr>
<tr>
<td>Male</td>
<td>9.41 ± 1.89</td>
<td>6.47 ± 0.47</td>
<td>5.64 ± 0.63</td>
<td>6.31 ± 0.88</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>IFN-gamma</strong></td>
<td>3.28 ± 0.17</td>
<td>3.86 ± 0.26</td>
<td>3.34 ± 0.21</td>
<td>3.45 ± 0.14</td>
<td>n.s.</td>
</tr>
<tr>
<td>Female</td>
<td>3.33 ± 0.13</td>
<td>4.06 ± 0.45</td>
<td>3.10 ± 0.37</td>
<td>3.60 ± 0.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>3.22 ± 0.33</td>
<td>3.67 ± 0.27</td>
<td>3.58 ± 0.18</td>
<td>3.29 ± 0.17</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td>15.84 ± 0.78</td>
<td>15.47 ± 1.00</td>
<td>15.09 ± 1.32</td>
<td>15.14 ± 0.92</td>
<td>n.s.</td>
</tr>
<tr>
<td>Female</td>
<td>17.07 ± 0.91</td>
<td>14.96 ± 1.84</td>
<td>13.77 ± 1.71</td>
<td>16.08 ± 1.39</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>14.62 ± 1.12</td>
<td>15.97 ± 0.95</td>
<td>16.42 ± 2.02</td>
<td>14.01 ± 1.06</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>IL-12p70</strong></td>
<td>3.34 ± 0.63</td>
<td>3.28 ± 0.67</td>
<td>2.23 ± 0.62</td>
<td>3.81 ± 0.89</td>
<td>n.s.</td>
</tr>
<tr>
<td>Female</td>
<td>3.73 ± 0.85</td>
<td>2.19 ± 1.10</td>
<td>1.81 ± 0.82</td>
<td>5.91 ± 1.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>2.95 ± 0.97</td>
<td>4.38 ± 0.53</td>
<td>2.66 ± 0.96</td>
<td>1.71 ± 0.60</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Table 3. PLF immunoglobulin isotypes by cytometric bead array. Immunoglobulin concentrations are presented as relative concentration ± std error, using saline control as standard. κ/λ ratios are presented as mean concentration κ antibody/mean concentration λ antibody for each isotype. In general, LA treatment decreased κ/λ ratios; treatment with LGM2605+LA restored κ/λ ratios to control levels. Data were analyzed via two-way ANOVA to analyze overall treatment effects (sex differences not shown).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LGM2605 only</th>
<th>LA only</th>
<th>LGM2605+LA</th>
<th>p-value treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 conc.</td>
<td>1.00 ± 0.10</td>
<td>1.19 ± 0.09</td>
<td>0.53 ± 0.08</td>
<td>1.13 ± 0.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>93.18</td>
<td>91.58</td>
<td>32.44</td>
<td>81.19</td>
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<tr>
<td>IgG2a conc.</td>
<td>1.00 ± 0.08</td>
<td>1.16 ± 0.10</td>
<td>0.72 ± 0.11</td>
<td>1.05 ± 0.10</td>
<td>0.023</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>63.00</td>
<td>89.61</td>
<td>24.76</td>
<td>32.95</td>
<td></td>
</tr>
<tr>
<td>IgG2b conc.</td>
<td>1.00 ± 0.08</td>
<td>1.06 ± 0.07</td>
<td>0.53 ± 0.04</td>
<td>1.13 ± 0.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>89.32</td>
<td>114.34</td>
<td>16.33</td>
<td>93.87</td>
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<tr>
<td>IgG3 conc.</td>
<td>1.00 ± 0.09</td>
<td>1.27 ± 0.06</td>
<td>0.85 ± 0.09</td>
<td>1.21 ± 0.15</td>
<td>0.036</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>12.42</td>
<td>16.77</td>
<td>7.68</td>
<td>17.19</td>
<td></td>
</tr>
<tr>
<td>IgA conc.</td>
<td>1.00 ± 0.14</td>
<td>0.86 ± 0.03</td>
<td>1.68 ± 0.26</td>
<td>0.94 ± 0.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>7.24</td>
<td>6.97</td>
<td>0.98</td>
<td>5.38</td>
<td></td>
</tr>
<tr>
<td>IgM conc.</td>
<td>1.00 ± 0.06</td>
<td>1.29 ± 0.14</td>
<td>1.14 ± 0.07</td>
<td>1.16 ± 0.09</td>
<td>n.s.</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>32.93</td>
<td>64.81</td>
<td>8.27</td>
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<tr>
<td>IgE conc.</td>
<td>1.00 ± 0.10</td>
<td>1.83 ± 0.48</td>
<td>1.45 ± 0.18</td>
<td>1.20 ± 0.24</td>
<td>0.032</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>2.37</td>
<td>6.92</td>
<td>0.37</td>
<td>2.67</td>
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</tbody>
</table>
Chapter III

Short-Term Exposure to Synthetic Flaxseed Lignan LGM2605 Alters Gut Microbiota in Mice

Abstract

Scope: LGM2605 is a synthetic version of the naturally occurring flaxseed lignan secoisolariciresinol diglucoside (SDG), with known anti-inflammatory and antioxidant properties; however, its effects on gut microbial composition have not previously been evaluated. In the present study, we sought to determine how 10-day oral administration of LGM2605 alters gut microbiota of mice.

Methods and Results: 8-week-old female C57BL/6 mice were treated with either LGM2605 or saline, administered daily via oral gavage over a 10-day treatment period. Upon termination of treatment, mouse cecums (n=31 total) were collected, and cecal DNA was isolated. 16S rRNA genes were sequenced and analyzed in Mothur in order to identify changes in gut microbial composition induced by LGM2605 treatment (v. saline control). We then assessed community composition, performed indicator taxa analysis, and measured alpha and beta diversity. Overall, LGM2605 significantly altered the gut microbiota of mice; we reported alterations in 3 bacterial phyla and 22 genera as a result of treatment.

Conclusions: The study here identifies for the first time significant alterations in gut microbiota of mice following oral administration of LGM2605, in general shifting towards a more anti-inflammatory composition, similar to the results of previous studies utilizing dietary flaxseed oil. These findings lay the foundation for future investigations utilizing LGM2605 to control gut dysbiosis and, by extension, systemic inflammation.
1. Introduction

In healthy individuals, the gut microbiome serves many vital functions: it aids in digestion, confers protection against pathogenic organisms, synthesizes essential vitamins and minerals, and plays an important role in the immune system\textsuperscript{29}. Dysbiosis can cause dysregulation of these functions and has been linked to local and systemic inflammation as well as autoimmune disease in both humans and animal models\textsuperscript{29}. Factors contributing to dysbiosis and resulting inflammation include decreased overall bacterial diversity and overgrowth of more aggressive types of residential bacteria\textsuperscript{30}. For instance, a higher ratio of \textit{Firmicutes}/\textit{Bacteroidetes} in the gut is associated with increased body weight and increased systemic inflammation, while a lower \textit{Firmicutes}/\textit{Bacteroidetes} ratio supports growth of beneficial bacteria and inhibits growth of potential pathogens\textsuperscript{88}. Furthermore, dysbiosis may increase intestinal permeability or “leaky gut,” allowing for the passage of bacteria and toxins from the gut to the bloodstream or peritoneal cavity, where they promote inflammation and disease progression\textsuperscript{89}.

Dietary intervention has been shown to be an effective means of improving gut health and reducing inflammatory responses by altering the diversity of gut microbes in ways that benefit, rather than disturb, host functions\textsuperscript{29}. Several previous studies have focused on dietary flaxseed supplementation in both human and animal subjects as a means of controlling inflammation and promoting healthy gut microbiota. Zhang et al. demonstrated in a 2017 study that administration of dietary flaxseed oil in mice ameliorated alcoholic liver disease by attenuating gut dysbiosis (decreasing the relative abundance of \textit{Proteobacteria} and \textit{Porphyromonadacea}), decreasing intestinal permeability, and reducing pro-inflammatory cytokine levels (i.e. TNF-\textit{\alpha}, IL-1\textit{\beta}, and IL-6) in plasma\textsuperscript{10}. Millman et al. further demonstrated in a 2019 study that dietary flaxseed oil administration in mice improved the overall gut microbial
diversity and decreased the relative abundance of Firmicutes, outcomes that are typically associated with a reduction in systemic inflammation\textsuperscript{11, 88}.

Despite the beneficial impacts of flaxseed consumption on gut microbiota, the utility of whole flaxseed as a dietary supplement is limited by its low bioavailability\textsuperscript{21}. Thus, researchers have turned to more readily metabolized flaxseed compound derivatives. Secoisolariciresinol diglucoside (SDG), a bioactive lignan highly concentrated in flaxseed, exhibits protective effects in a variety of systemic diseases, likely due to SDG’s antioxidant properties\textsuperscript{2}. Recently, the synthetic flaxseed lignan LGM2605 has been evaluated as a therapeutic alternative to naturally occurring SDG; it has significantly greater bioavailability compared to whole flaxseed and can be readily produced in a laboratory setting\textsuperscript{6}. Previous studies have demonstrated the usefulness of LGM2605 in controlling inflammation and oxidative stress responses in models of radiation and asbestos exposure, likely resulting from the ability of LGM2605 to scavenge radical species and reduce inflammatory cytokine production\textsuperscript{5, 7, 8, 90}. Our most recent study showed that oral LGM2605 administration reduced hyper-acute immune responses in mice exposed intraperitoneally to amphibole asbestos\textsuperscript{9}. Much like that of SDG, the biological activity of synthetic LGM2605 depends largely on metabolism by residential gut bacteria\textsuperscript{88, 91}. However, the influence of LGM2605 on gut microbial composition has not yet been investigated.

In the present study, we sought to determine the effects of orally administered LGM2605 on the gut microbiota of mice. Identifying changes in gut microbiota will allow us to better understand the mechanism of action through which LGM2605 exerts its anti-inflammatory effects, as the gut and immune system are inextricably linked. Utilizing a murine model, we administered daily LGM2605 treatment (or saline control) via oral gavage. Previous studies have reported alterations in the gut microbiota of mice as a result of dietary intervention in as little as
The human gut is generally less responsive to dietary intervention\textsuperscript{93}, but relatively minor compositional changes have been observed within just 10 days of treatment\textsuperscript{94}. Therefore, we selected a time frame of 10 days for the current study. Changes in gut microbial composition were identified by analysis of 16S rRNA genes of DNA extracted from mouse cecums. The cecum has been utilized in previous studies of gut microbiota in animals due to its high microbial diversity, which greatly exceeds that achieved by fecal sampling\textsuperscript{93}. We predicted that oral administration of LGM2605 would lead to beneficial changes in gut microbial composition relative to control, increasing the prevalence of bacteria characterized as anti-inflammatory while decreasing the prevalence of bacteria characterized as pro-inflammatory.

2. Materials and Methods

**Animals and Diet** All experiments were approved by the Idaho State University Institutional Animal Care and Use Committee (IACUC). 48 female C57BL/6 mice (8 weeks old) were obtained from Jackson Laboratories. The animals were housed four per cage in the Idaho State University Animal Care Facility, with a 12-h light/dark cycle, constant temperature (22°C), and constant humidity (45%). Over the course of the study, mice were given *ad libitum* access to standard rodent chow and filtered water. Mice were allowed to acclimate for 3 days preceding administration of treatment.

**LGM2605 Treatment** Synthetic SDG (referred to as LGM2605 in the literature) was independently generated as previously described\textsuperscript{6}. Briefly, LGM2605 was synthesized from vanillin via secoisolariciresinol and glucosyl donor (perbenzoyl-protected trichloacetimidate under the influence of TMSOTf) through a concise route involving chromatographic separation of diastereomeric diglucoside derivatives (Chemveda Life Sciences, Inc., Hyderabad, India). Lyophilized samples of LGM2605 (100mg/vial) were reconstituted with sterile, endotoxin-free
water to produce stock solution of 50mg/mL. Endotoxin testing of water was performed prior to preparation of stock solution using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Biotech Corp), following the manufacturer’s protocol. Water samples were tested against a standard curve provided with the kit. Briefly, the samples were mixed with 100µL of LAL, added to endotoxin-free tubes, and incubated at 37°C for 6 min. Stop solutions and color stabilizers were added to each tube, and then 200µL of each solution was analyzed by measuring absorbance at 545 nm in triplicate for each sample. Endotoxin was detected at 0.59EU/mL, which is below the acceptable lower limit for oral administration in mice. LGM2605 (100mg/kg body weight) was administered daily using curved 2-inch 18-gauge stainless steel feeding tubes (sterilized before use). Individual mouse body weights were measured daily to calculate appropriate dosage volumes. Control animals received an equivalent volume of saline via oral gavage. Mice were randomly assigned to either the experimental group (LGM2605) or the control group (saline); treatments were administered for a total of 10 days.

**Cecal DNA Extraction and 16S rRNA Sequencing** Following the 10-day treatment period, mice were euthanized by CO₂ asphyxiation. A total of 31 cecal samples were collected into sterile microfuge tubes and frozen at -20°C until needed for analysis. Cecums were then thawed at room temperature, and cecal DNA was extracted using the Qiagen QIAamp™ PowerFecal™ DNA Kit. Briefly, each cecum was sectioned into two pieces of approximately equal size, and one section was transferred to a Dry Bead Tube provided in the kit (up to 0.25g biosolid/tube). The second section was re-frozen for later use. Subsequent steps were performed according to the manufacturer’s instructions. The bead-beating step was performed using the Mini-Beadbeater-8 (BioSpec, OK, USA). DNA was eluted in 100µL of C6 elution buffer solution. Recovered DNA quantity and quality were assessed using the NanoDrop ND-1000 spectrophotometer (Marshall
Scientific, NH, USA). Cecal DNA was then amplified and sequenced by the Molecular Core Research Facility (MRCF) of Idaho State University. Briefly, variable region 4 (V4) of bacterial 16S ribosomal RNA genes was amplified by PCR, and subsequent cleanup was performed. Library integrity was assessed by running a portion of the samples through AATI Fragment Analysis and qPCR for quality control. Samples were then quantified with the Qubit 2.0 Fluorometer and pooled in equimolar amounts. The sample pool was sequenced on an Illumina MiSeq platform using a 2x250-bp Miseq Reagent Kit v3 (Illumina, CA, USA). Sequencing of the 31 samples yielded a total of 6,256,091 reads, with a mean read count of 201,809 and a range of 37,544-428,248 reads. Amplicon sequence data was processed in Mothur. Sequences were clustered into operational taxonomic units (OTUs) at 97% identity using SILVA Taxonomy; a total of 9,914 unique OTUs were identified.

Statistical Analyses The effects of LGM2605 treatment on gut microbial composition were investigated primarily using analysis methods in Phyloseq (R Bioconductor package). PERMANOVA was used to test for significant differences in the composition of bacterial communities resulting from LGM2605 treatment, relative to control. Compositional patterns of communities were visually depicted using non-metric multidimensional scaling (NMDS). The indicator taxa analysis of Dufrene-Legendre was used to determine which groups of bacteria exhibited significant differences in abundance as a result of treatment, assessing both phylum- and genus-level comparisons. We used the nonparametric `indval` function from the package `labdsv` to derive indicator values and to determine statistical significance. Measures of alpha and beta diversity were also calculated for each treatment group. Shannon’s and Simpson’s diversity indices were used to calculate alpha diversity; the Bray-Curtis dissimilarity index was used to calculate beta diversity. Beta diversity was partitioned into balanced variation and
abundance gradients using the `beta.pair.abund` function from the package betapart\textsuperscript{101}.

Differences in beta diversity with respect to treatment were assessed using Anderson’s PERMDISP\textsuperscript{2} procedure\textsuperscript{102}, which provides an analysis of multivariate homogeneity of group variances.

3. Results

3.1. LGM2605 treatment was shown to significantly alter gut microbial composition in mice.

Oral administration of LGM2605 significantly altered gut microbial composition as compared to saline only ($p<0.001$ by PERMANOVA). The NMDS plot (Figure 9) illustrates the level of similarity between constituent bacterial communities in cecal samples, comparing cecums of LGM2605-treated v. saline-treated mice. Stacked bar plots illustrate bacterial phylogenetic distribution by phylum (Figure 10A) and by genus (Figure 10B), again comparing cecums of LGM2605-treated v. saline-treated mice.

3.2. LGM2605 treatment altered the total abundance of 3 bacterial phyla and 22 genera compared to saline only.

Indicator taxa analysis revealed significant differences in bacterial abundance across 3 phyla as a result of LGM2605 treatment (Table 4). Notably, LGM2605 treatment decreased the *Firmicutes*/*Bacteroidetes* ratio relative to saline only. In addition, LGM2605 treatment significantly altered 22 bacterial genera compared to saline (Table 5). Of those identified, 7 genera were clearly defined in scientific literature as having pro- or anti-inflammatory activity, as described in the discussion.

3.3. LGM2605 treatment did not impact alpha diversity of gut microbiota.
Alpha diversity was calculated using both Shannon’s diversity index (H) and Simpson’s diversity index (D). These indices reflect the richness and evenness of bacterial communities (OTUs) in individual cecal samples. For LGM2605-treated mice, H=3.766 and D=0.953; for saline-treated mice, H=3.564 and D=0.929. Box plots illustrate alpha diversity of gut microbiota by treatment group (Figure 11). Both LGM2605-treated and saline-treated mice exhibited high alpha diversity in cecal samples, but no significant difference in alpha diversity by either Shannon’s or Simpson’s method was observed as a result of LGM2605 treatment.

3.4. LGM2605 treatment did not impact beta diversity of gut microbiota.

Beta diversity was calculated using the Bray-Curtis dissimilarity index, characterizing abundance-based dissimilarity between cecal samples. For LGM2605-treated mice, total beta diversity=0.657; for saline-treated mice, total beta diversity=0.670. Among both treatment groups, beta diversity was primarily attributed to balanced variation in bacterial abundance rather than unidirectional abundance gradients (data not shown). No significant difference in beta diversity was observed as a result of LGM2605 treatment using the PERMDISP2 procedure.

4. Discussion

The gut microbiome and systemic inflammation are inextricably linked, as emerging research has demonstrated. Dietary intervention may help to beneficially alter gut microbial composition, producing anti-inflammatory effects. In the present study, we utilized the synthetic flaxseed lignan LGM2605 as a form of dietary intervention in mice. Previous studies have shown that LGM2605 acts as an antioxidant and free radical scavenger, reducing inflammation associated with radiation and asbestos exposure; however, its effects on gut microbiota have not previously been evaluated. We therefore sought to determine how a 10-day period of oral LGM2605 administration affects the gut microbial composition of mice. We analyzed 16S rRNA
genes of DNA extracted from mouse cecums. The cecum is proportionately larger in mice as compared to humans due to its importance in the digestion of plant compounds within the mouse gut; thus, it is not a perfect homologue for humans\textsuperscript{103}. However, the mouse cecum is still a valid model for human health associations because major bacterial community composition is conserved among mammals\textsuperscript{93}. Additionally, the cecum is a preferred site of DNA sequencing and more representative than fecal sampling based on the high microbial diversity in this intestinal region\textsuperscript{93}; it was therefore selected for analysis in this study.

LGM2605 treatment led to significant alterations in gut microbial composition compared to saline only ($p<0.001$ by PERMANOVA). Subsequent analyses were performed to evaluate the implications of these changes for systemic inflammation and human health. In general, we predicted that the gut-modulating effects of synthetic LGM2605 would mirror those of orally administered flaxseed oil, as observed in previous studies\textsuperscript{10, 11}. In the gut, dietary SDG is converted to the enterolignans enterodiol (ED) and enterolactone (EL), which greatly improves the compound’s bioavailability and biological activity\textsuperscript{104-106}. This process supports the growth of various beneficial bacteria that are responsible for lignan metabolism; in turn, the metabolites that these bacteria produce exert further downstream effects on community composition\textsuperscript{91, 107}. LGM2605 is thought to be metabolized in a similar way. We hypothesized that oral administration of LGM2605 would increase the relative abundance of bacteria characterized as anti-inflammatory, while decreasing the relative abundance of bacteria linked to inflammation and autoimmunity.

At the phylum level, LGM2605 treatment led to a decreased \textit{Firmicutes}/\textit{Bacteroidetes} ratio (Table 4), which is generally associated with lower intestinal and systemic inflammation, lower risk of obesity, and higher overall bacterial diversity\textsuperscript{108}. However, LGM2605 treatment
also led to an increased prevalence of *Proteobacteria*, in contrast to the 2017 study by Zhang et al., which reported a decrease in *Proteobacteria* as a result of dietary flaxseed oil supplementation\(^\text{10}\). Because *Proteobacteria* is often a marker of intestinal dysbiosis\(^\text{109}\), further research is needed to understand what factors may have contributed to this effect in LGM2605-treated mice and the potential health implications.

At the genus level, LGM2605 treatment contributed to an increase in several genera associated with anti-inflammatory effects, including *Alistipes, Anaeroplasma,* and *Faecalibaculum* (Table 5). Both *Alistipes* and *Anaeroplasma* are associated with an increased production of the anti-inflammatory cytokine TGF-β, which upregulates mucosal IgA expression, thereby strengthening the intestinal barrier and reducing gut permeability\(^\text{110,111}\). *Alistipes* is also associated with an increased production of the anti-inflammatory cytokine IL-10, which may help to suppress overactive immune responses\(^\text{110}\). During digestion, both *Alistipes* and *Faecalibaculum* produce short-chain fatty acids (SCFAs), bacterial metabolites that have been shown to improve intestinal barrier function\(^\text{110,112}\). SCFAs help suppress Th-17 cells and support differentiation of T-regulatory cells (T-regs), which is crucial in maintaining gut homeostasis and combatting inflammation\(^\text{113}\). The *Faecalibaculum* genus includes only one species, *Faecalibaculum rodentium*, which is found exclusively in mice; its human homologue is *Holdemanella biformis*. Studies of intestinal tumorigenesis have shown that *F. rodentium* and *H. biformis* may possess both anti-inflammatory and anti-tumorigenic effects through the production of SCFAs\(^\text{114}\).

LGM2605 treatment also contributed to a decrease in several genera that are associated with pro-inflammatory effects, including *Turicibacter* and *Streptococcus* (Table 5). Both of these genera are implicated in various forms of autoimmune disease, including inflammatory bowel
disease and rheumatoid arthritis\textsuperscript{115, 116}. \textit{Streptococcus} induces expression of the pro-inflammatory cytokines TNF-\(\alpha\), IL-6, and IFN-\(\gamma\), which are associated with autoimmunity\textsuperscript{117}. The role of \textit{Turicibacter} in disease pathology remains largely uncharacterized, but some studies report that its presence may be related to TNF expression\textsuperscript{115}.

Interestingly, LGM2605 treatment led to an increased abundance of \textit{Desulfovibrio}, which is generally regarded as pro-inflammatory (Table 5). \textit{Desulfovibrio} is a genus of gram-negative bacteria that produces lipid A, the toxic subunit of lipopolysaccharides (LPS)\textsuperscript{118}. In the case of “leaky gut,” LPS translocates from the intestines to the blood stream, where it stimulates pro-inflammatory cytokine release (e.g. TNF-\(\alpha\), IL-1, IL-6) in macrophages, contributing to systemic inflammation\textsuperscript{30}. Zhang et al. reported decreased plasma LPS following flaxseed oil supplementation; while not measured within the current study, plasma LPS may serve as a more accurate representation of LPS translocation and resulting inflammation\textsuperscript{10}. Taken together, our findings suggest that the increased \textit{Desulfovibrio} abundance following LGM2605 treatment was either insufficient to affect the gut lumen or was overridden by other microbial alterations, such as the increased prevalence of anti-inflammatory bacteria. More research is needed in order to tease apart this complicated relationship and to more fully understand the effects of \textit{Desulfovibrio} on systemic inflammation.

LGM2605 treatment also led to a decreased abundance of \textit{Bifidobacterium}, which is considered to be both anti-inflammatory and probiotic (Table 5). As a probiotic, \textit{Bifidobacterium} alters gut microbial composition by stimulating growth of residential bacteria while reducing growth of bacterial pathogens\textsuperscript{119}. Furthermore, \textit{Bifidobacterium} may help to regulate the balance of T-helper 1 (Th1)/T-helper 2 (Th2) cells\textsuperscript{120}. Th1-biased immune responses are often implicated in autoimmune disease and lead to increased production of the pro-inflammatory cytokines IFN-
\( \gamma \) and TNF-\( \alpha \). Restoring the balance of Th1/Th2 cells may therefore be beneficial in disease prevention. *Bifidobacterium* has been shown to suppress pro-inflammatory cytokine production as well as improve intestinal barrier function via this mechanism\textsuperscript{119,120}. The effects of LGM2605 on *Bifidobacterium* abundance and their associated health implications may be another consideration for future research.

Effects of dietary intervention on gut microbial composition are conventionally measured not only through the response of individual bacterial communities but also through alterations in the overall biodiversity of gut microbiota, i.e. alpha diversity. Decreased alpha diversity has been reported in various forms of autoimmune disease, including inflammatory bowel disease, rheumatoid arthritis\textsuperscript{115}, and systemic lupus erythematosus\textsuperscript{121}. Although LGM2605 treatment significantly altered the bacterial abundance of multiple phyla/genera within cecal samples (Tables 4-5), it did not impact alpha diversity measures by either Shannon’s or Simpson’s method (Figure 11). The lack of change in overall gut biodiversity may be partially explained by the short study duration of 10 days, as opposed to 6 or more weeks, which is the experimental length of other similar dietary studies. However, LGM2605 treatment still contributed to significant changes in gut microbial composition within just 10 days, providing compelling evidence for future studies investigating the effects of longer-term treatment.

Additionally, we found that LGM2605 treatment did not impact beta diversity measures, which describe the heterogeneity in microbial composition between cecums, i.e. inter-sample variation. Among both treatment groups, beta diversity was driven primarily by balanced variation, or variation in the prevalence of specific bacterial communities from one sample to the next, as opposed to variation in the number of individual organisms detected\textsuperscript{101}. Future studies may seek to determine the inflammatory effects of individual genera and/or species by
colonizing the GI tracts of germ-free mice with microorganisms of interest. This approach would allow for the differentiation of specific bacterial communities and their respective roles in disease, independent of other physiologic influences. However, differences observed should also be studied collectively in consideration of their combined effects on inflammation and disease pathology.

Since this study focused on the effects of short-term LGM2605 treatment, we were unable to fully evaluate the compound’s ability to modulate gut microbiota or systemic inflammation. However, the alterations in community composition that we observed—including changes in the relative abundance of 3 bacterial phyla as well as 22 genera—are encouraging and evoke the need for future studies investigating the effects of longer-term LGM2605 treatment. Future studies should also consider application of LGM2605 in gut dysbiosis models, in order to further establish the ability of LGM2605 to beneficially alter gut microbial composition. LGM2605 has previously demonstrated protective effects in the context of radiation and asbestos exposure, which are known inducers of inflammation. While these effects have been largely attributed to the compound’s direct antioxidant and free radical scavenging abilities, its gut-modulating effects may also play a role in reducing systemic inflammation within these models. Finally, the present study was limited by its exclusive use of 16S rRNA gene sequencing; future studies should also utilize transcriptomics and/or metabolomics in order to more fully elucidate the functional responses of gut microbes to LGM2605.

Overall, this study provides evidence that short-term dietary treatment with the synthetic flaxseed derivative LGM2605 significantly alters the gut microbiota of mice, in general shifting towards a more anti-inflammatory composition. Despite the short experimental duration, the findings presented here suggest that LGM2605 treatment positively impacts the gut microbiota.
of mice, which may contribute to the previously reported anti-inflammatory, antioxidant, and chemoprotective effects of LGM2605\textsuperscript{4, 5, 8, 90}. This study also establishes baseline changes in gut microbiota following oral LGM2605 administration, which will be an important reference point for future studies.

**Author Contributions**

R.B., K.A., and K.S. collaborated on the study design and manuscript preparation. R.B. and K.S. performed the *in vivo* experiments and prepared the final manuscript. R.B. carried out cecal DNA extraction. R.B. and K.A. performed statistical analyses. All authors critically reviewed the manuscript and approved the final version for publication.

**Conflicts of Interest**

The authors report no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Acknowledgements and Funding**

This research was supported by funds allocated by the BSU Biomolecular Research Center as part of an Institutional Development Award (IDeA) received from the National Institute of General Medical Sciences of the National Institute of Health (P20GM103408), (P20GM109095). We acknowledge the Molecular Research Core Facility (MRCF) of Idaho State University for amplification and sequencing of cecal DNA. We would like to thank Dr. Melpo Christofidou-Solomidou (University of Pennsylvania, Department of Medicine) for her mentorship, as well as the provision of LGM2605 utilized in this study. We further acknowledge several student researchers from the Serve lab: Zach Ditzig, for assistance with tissue collection and administration of treatments; Jake Shields, for assistance with DNA extraction; and Magdalena Alba, for assistance with animal care.
Figure 9. NMDS plot. Ordination is based on Bray-Curtis dissimilarity matrix generated from operational taxonomic unit (OTU) table of sequencing data. Points represent individual cecal samples (n=31); distance between points represents the level of similarity between them. The plot demonstrates that bacterial communities differ between cecums of mice treated with LGM2605 v. cecums of mice treated with saline only ($p < 0.001$ by PERMANOVA).
Figure 10. Stacked bar plots of bacterial phylogenetic distribution, by phylum (A) and by genus (B). Classifications were assigned using SILVA Taxonomy. OTUs were averaged among individual cecal samples (n=31) so that group differences in relative bacterial abundance could be compared. The plots demonstrate that gut microbial composition differs at both the phylum and genus levels between cecums of mice treated with LGM2605 v. cecums of mice treated with saline only.
Figure 11. Box plots of Shannon’s diversity (A) and Simpson’s diversity (B), quantifying overall biodiversity within individual cecal samples (n=31). Shannon’s diversity is reported as H; Simpson’s diversity is reported as D. Greater richness and evenness of bacterial communities correspond with higher diversity measures. For LGM2605-treated mice, H=3.766363 and D=0.9531014; for saline-treated mice, H=3.564396 and D=0.9287959. Based on these measures, we report no significant difference in the level of alpha diversity observed in cecums of LGM2605-treated v. saline-treated mice.
Table 4. Results of indicator taxa analysis, phylum level. Indicator values account for fidelity and relative abundance and range from 0 to 1, with higher values for stronger indicators. Significant differences in bacterial abundance as a result of LGM2605 treatment (relative to saline only) were observed across 3 phyla within cecal samples (n=31). Table reports increased/decreased relative abundance in response to LGM2605 treatment. Phyla that were not significantly altered by LGM2605 treatment are not reported.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Median # Reads ± IQR</th>
<th>Indicator Value</th>
<th>Effects of LGM2605 on Bacterial Abundance</th>
<th>p-Value</th>
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</thead>
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<td></td>
<td>Saline</td>
<td>LGM2605</td>
<td>Saline</td>
<td>LGM2605</td>
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<tr>
<td>Proteobacteria</td>
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<td>17.314 ± 18.320</td>
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Table 5. Results of indicator taxa analysis, genus level. Indicator values account for fidelity and relative abundance and range from 0 to 1, with higher values for stronger indicators. Significant differences in bacterial abundance as a result of LGM2605 treatment (relative to saline only) were observed across 22 genera within cecal samples (n=31). Of these, 7 genera (indicated by asterisks) were identified in literature as playing important roles in inflammation and/or autoimmune disease. Table reports increased/decreased relative abundance in response to LGM2605 treatment. Genera that were not significantly altered by LGM2605 treatment are not reported.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Median # Reads ± IQR</th>
<th>Indicator Value</th>
<th>Effects of LGM2605 on Bacterial Abundance</th>
<th>p-Value</th>
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<td>Saline</td>
<td>LGM2605</td>
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<td>Alistipes*</td>
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Chapter IV: Discussion

1. Summary of Results

Synthetic flaxseed lignan LGM2605 is a potent antioxidant, free radical scavenger, and anti-inflammatory compound. Through our research, we demonstrated that orally administered LGM2605 exerts beneficial systemic effects in mice, supporting its utility as a chemopreventive agent. LGM2605 controls acute immune responses triggered by LA exposure (Chapter II). We also demonstrated positive changes in gut microbiota following LGM2605 treatment (Chapter III). These findings are summarized in greater detail below.

Chapter II

Mice were given daily LGM2605 treatment (or saline control) for 3 days before and 14 days after a single intraperitoneal dose of LA (or saline control). Upon termination of treatment, peritoneal lavage was performed; immune cell influx, cytokine concentrations, and immunoglobulin isotyping were then analyzed via flow cytometry. In agreement with the findings of the previous 3-day trial\textsuperscript{9}, LGM2605 attenuated LA-induced splenomegaly (spleen enlargement) and reduced trafficking of PMN cells and macrophages to the peritoneal cavity.

In the present study, we also noted additional changes that were not observed within the previous 3-day trial. LA exposure increased the percentages of both peritoneal and splenic B1a cells and decreased the percentage of total peritoneal B cells; treatment with LGM2605+LA restored these populations back to control levels. LA exposure also contributed to an increase in pro-inflammatory cytokine concentrations, including MCP-1 in PLF and IL-6 in both PLF and serum. Treatment with LGM2605+LA reduced pro-inflammatory cytokine concentrations back to control levels. Additionally, LGM2605 treatment increased the serum concentration of IL-10, which is regarded as both anti-inflammatory and immunosuppressive. Finally, LA exposure
induced immunoglobin isotype switching in PLF, decreasing the relative concentrations of IgG$_1$ and IgG$_{2b}$, while increasing the relative concentration of IgA. These changes were countered by LGM2605 treatment. Overall, we showed that LGM2605 treatment ameliorated inflammation and altered both innate and adaptive immune responses at 14 days post-LA exposure.

Chapter III

Mice were given daily LGM2605 treatment (or saline control) via oral gavage for a total of 10 days. Upon termination of treatment, mouse cecums were collected, and cecal DNA was isolated. 16S rRNA genes were then sequenced and analyzed in order to detect changes in gut microbiota induced by LGM2605 treatment; we assessed community composition, performed indicator taxa analysis, and measured alpha and beta diversity. Orally administered LGM2605 significantly altered the community composition of gut microbiota compared to saline control. We reported alterations in the relative abundances of 3 bacterial phyla and 22 genera as a result of treatment, several of which have important implications for inflammation and autoimmunity. However, LGM2605 treatment did not impact either alpha or beta diversity. Overall, we showed that LGM2605 significantly altered the gut microbiota of mice, in general shifting towards a more anti-inflammatory composition.

2. Implications/Future Directions

Chapter II

Upon initial exposure, LA contributes to early immune responses, i.e. acute inflammation, including 1) cytokine release/immune cell trafficking; 2) oxidative stress/cytotoxicity; and 3) inflammasome activation$^{21}$. A non-resolution of these early immune responses may cause chronic inflammation$^9$ contributing to LA-induced autoantibody formation and fibrosis. Notably, LA exposure may contribute to various forms of systemic autoimmune
disease (SAID), including systemic scleroderma, lupus erythematosus, and rheumatoid arthritis. Due to their chronic nature, these conditions currently have few effective treatment options, particularly within later disease stages. Early therapeutic intervention is therefore necessary to slow or stop the development of SAID before it progresses to a refractory state. In particular, targeting acute inflammatory processes may help to prevent chronic inflammation and later disease development. Through our research, we utilized LGM2605, which is a known anti-inflammatory and antioxidant agent, in order to block early immune responses triggered by LA exposure. Our findings suggest that LGM2605 may be an effective chemopreventive agent against SAID and other asbestos-related diseases (ARDs).

First, we showed that LGM2605 ameliorates LA-induced inflammation via reduction of pro-inflammatory cytokines in both PLF and serum (i.e. MCP-1 and IL-6). When asbestos is inhaled, the fibers are translocated from the lungs to the surrounding pleural cavity. Resident macrophages attempting to remove the fibers undergo frustrated phagocytosis, resulting in cell damage and death. This process prompts inflammatory cytokine production as one of the early features of LA-induced inflammation. Furthermore, LA exposure contributes to activation of NLRP3 inflammasomes, resulting in additional release of pro-inflammatory cytokines (e.g. IL-6). Locally released cytokines then cause additional immune cells to traffic into the site of LA exposure, propagating further injury and perpetuating the immune response. Although we did not investigate these mechanisms directly, our results show that LGM2605 may prompt a resolution of early inflammatory processes that are implicated in LA-induced diseases. We also noted changes in immune cell trafficking that were consistent with these findings.

We showed that LGM2605 may protect against LA-associated autoimmunity via reduction of both splenic and peritoneal B1a cells. B1a cells are innate immune cells that are
largely responsible for the production of IgM autoantibodies\textsuperscript{14,21}. They have been implicated in such conditions as rheumatoid arthritis\textsuperscript{73} and lupus erythematosus\textsuperscript{74}, and their numbers have been shown to increase within autoimmune animals\textsuperscript{87}. Notably, B1a cells respond to LA exposure by trafficking from the site of inflammation to the spleen and lymph nodes, where they may ultimately promote immune dysfunction\textsuperscript{66}. We noted an increase in both splenic and peritoneal B1a cells following LA exposure, indicative of increased autoimmune activity. By reducing these B1a cell populations in LA-exposed mice, LGM2605 demonstrates a likely chemopreventive role against immune dysfunction implicated in SAID.

We also showed that LGM2605 may regulate immune responses via inhibition of LA-induced isotype switching. IgA is commonly associated with mucosal surfaces and forms a first line of defense against inhaled or ingested pathogens. The observed increase in IgA levels following LA exposure suggests isotype class switching from IgM to IgA within the 14-day time point. Notably, IL-6 has been shown to enhance IgA synthesis by promoting terminal differentiation of IgA-committed B cells\textsuperscript{80}, and we demonstrated an increase in IL-6 levels following LA exposure. Furthermore, murine B1 cells in the peritoneal cavity display preferential class switching to IgA without stimulation by IL-6\textsuperscript{81-83}, and LA exposure has previously been shown to upregulate IgA surface expression among peritoneal B1a cells\textsuperscript{27}. We observed an increase in peritoneal B1a cells following LA exposure; these cells are likely responsible for the increased IgA production. By reducing IgA production in LA-exposed mice, LGM2605 demonstrates immunomodulatory properties which may contribute to its therapeutic effects.

Finally, we showed for the first time that LGM2605 alters adaptive immune responses induced by LA exposure, specifically by increasing total peritoneal B cells. We did not, however,
note any changes in total splenic B cells, total T cells, or Th cells. Results of a previous *in vitro* study showed that asbestos stimulates macrophages to release pro-inflammatory cytokines, which in turn suppress B cell proliferation\textsuperscript{27}. Both peritoneal macrophages and IL-6 production were significantly increased by LA at the 14-day time point, supporting a reduction in total peritoneal B cells following LA exposure. Previous long-term investigations of LA exposure have not noted any significant alterations in total T cells or Th cells\textsuperscript{41}, in agreement with the findings of the present study. Therefore, LA exposure does not seem to contribute to a significant influx of lymphocytes to the site of fiber deposition. The response may instead be limited to the localized action of B1a cells and other innate immune cells within the peritoneal cavity. Despite this, chronic inflammation and later disease development likely result from an interaction of both adaptive and innate immune cells. By increasing total peritoneal B cell populations in LA-exposed mice, LGM2605 demonstrates its ability to regulate later (adaptive) immune responses to LA, which may be useful in the prevention of SAID and other ARDs.

Our findings show that LGM2605 treatment reduces both inflammation and immune activation caused by acute LA exposure. The next step in our research would be to investigate whether treatment and resolution of inflammation can prevent LA-induced autoantibody formation and fibrosis. Future studies should therefore consider the effects of LGM2605 treatment on long-term LA exposure and later disease development. Furthermore, in the present study we considered how LA exposure alters the total levels of peritoneal and splenic B and T cells; we showed that LA exposure does not invoke a significant influx of lymphocytes to the site of fiber deposition. However, based on the results summarized above, it is possible that B and T cell maturation may alter in response to LA, despite limited changes in total cell numbers. Flow
cytometry may be utilized to differentiate between immature and mature lymphocytes following LA exposure and could be another potential direction for further research.

Future studies may also evaluate the role of tertiary lymphoid structures (TLS) in LA exposure models. TLS are discrete, structured organizations of lymphocytes that develop within nonlymphoid tissues. They are not present under normal conditions but are induced at sites of chronic inflammation in response to pro-inflammatory cytokine production. Lymphocytes are recruited from the bloodstream to the TLS; there, B cells are activated by Th cells to produce antibodies. Compared to secondary lymphoid organs (e.g. spleen and lymph nodes), TLS may lack the proper microenvironment to allow for screening of autoreactive B cells, thereby promoting autoantibody production. In the case of autoimmune disorders, including rheumatoid arthritis, TLS are usually associated with increased persistence and severity of disease. However, their role in LA-induced inflammation and associated autoimmunity has not yet been evaluated. It is possible that, in the case of LA exposure, lymphocytes circumvent the spleen/lymph nodes by aggregating within TLS. This may partly explain the lack of alteration in total splenic B cells at 14 days post-LA exposure and would suggest a more localized immune response to LA. Further consideration of TLS may also offer some insight into the role of T cells and Th cells in LA exposure models. Future studies may utilize techniques such as H&E staining and immunohistochemistry in order to detect and quantify TLS both locally within peritoneal cavity tissue and within kidneys, a classic site of TLS development. Identification of these structures may help to interpret the effects of LA on B and T cell populations as well as tease apart local v. systemic responses, thereby addressing some of the knowledge gaps within the present study.
Chapter III

Recent studies have investigated the potential for prevention/treatment of chronic inflammation and resulting autoimmunity through dietary intervention. Certain dietary components may produce anti-inflammatory effects, either directly (following absorption) or indirectly (by influencing gut microbial composition). Previous *in vitro* studies have attributed the anti-inflammatory effects of LGM2605 to its antioxidant and free radical scavenging properties\(^3,4,21,125\). However, despite their numerous health benefits, lignans such as LGM2605 are poorly absorbed; residential gut bacteria are essential for their activation from plant substrates\(^32,104,105\). Food-associated SDG, in particular, reaches the colon undigested; it is then transformed into enterolignans, which greatly improves the compound’s bioavailability and biological activity\(^106\). Furthermore, enterolignan production has been associated with increased bacterial diversity and altered gut microbial composition\(^126\). Digestion of synthetic LGM2605 presumably occurs via a similar pathway; however, the effects of LGM2605 on gut microbiota have not previously been investigated.

Our findings demonstrate for the first time that LGM2605 alters gut microbial composition, increasing the prevalence of bacteria characterized as anti-inflammatory while decreasing the prevalence of bacteria characterized as pro-inflammatory. These results suggest a new potential pathway for chemoprevention via LGM2605 treatment. In addition to its direct antioxidant and free radical scavenging properties, LGM2605 may demonstrate indirect anti-inflammatory effects via modulation of gut microbiota. Although our present study was limited in duration, the changes that we observed after just 10 days of treatment with LGM2605 are highly encouraging. LGM2605 may be an effective means of dietary intervention in the context of gut dysbiosis and other disease models, helping to improve gut health and reduce systemic
inflammation. The relationship between the immune system and the gut microbiome is undoubtedly complex, but the therapeutic potential of LGM2605 may be at least partly attributed to the cross talk between these two systems.

Through our research, we establish baseline changes in gut microbial composition following LGM2605 treatment, which will be an important reference point for future studies. However, changes in gut microbiota (especially alpha/beta diversity) were limited by the short study duration; the experimental length of other similar dietary studies is typically ≥6 weeks. Future studies should therefore investigate the effects of longer-term LGM2605 treatment. Furthermore, we consulted the literature to determine how changes in gut microbiota resulting from LGM2605 treatment might influence inflammation and/or autoimmunity. However, in order to further explore these effects, we would need to employ a controlled study of gut microbiota. Future investigations should therefore colonize the GI tracts of germ-free mice with bacteria of interest, such as those identified above. This approach would allow for the differentiation of specific bacterial communities and their respective roles in inflammation/autoimmune processes, independent of other physiologic influences. Future studies should also consider application of LGM2605 in gut dysbiosis models, in order to further establish the ability of LGM2605 to beneficially alter gut microbial composition.

Another limitation of the present study was the use of only one method for studying changes in gut microbiota. We utilized 16S rRNA gene sequencing, which provides a useful measure of gut microbial composition but does not assess functionality of the gut microbiota. Future studies may implement RNA sequencing methods (i.e. transcriptomics) in order to determine how LGM2605 treatment alters gene expression within the gut, and to evaluate the effects of these transcriptomic changes with respect to inflammation/autoimmune processes.
Future studies may also implement metabolic assessment or measures of certain gut-derived metabolites, such as SCFAs, which are produced by beneficial gut bacteria and have demonstrated anti-inflammatory effects. These alternative methods would help to assess the physiological outcomes of LGM2605 treatment via modulation of gut microbiota and may support its efficacy as a dietary supplement.

3. Overall Conclusions

Based on our findings, LGM2605 demonstrates significant therapeutic potential in the context of LA-induced inflammation and associated disease outcomes, including SAID and other ARDs. In addition, orally administered LGM2605 positively impacts gut microbial composition, supporting a reduction of systemic inflammation. LGM2605 may provide an effective means of prevention/treatment for a variety of autoimmune or otherwise chronic, degenerative diseases by blocking the underlying inflammation that contributes to disease progression. Future LA exposure models should focus specifically on chronic inflammation through the use of repeated, low-level LA dosing and longer-term LGM2605 treatment. Furthermore, while we evaluated the immune- and gut-modulating properties of LGM2605 separately, future studies may seek to examine the interplay between gut microbiota and LA-induced inflammation by integrating these distinct approaches.
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