AWARD NUMBER: W81XWH-19-1-0626

TITLE: Gut Symbiotic Lipid A Family: Structures and Immunomodulation in IBD

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CONTRACTING ORGANIZATION: Brigham and Women's Hospital, Boston, MA

REPORT DATE: October 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2021	Annual report	30Sep2020-29Sep2021

4. TITLE AND SUBTITLE

Gut Symbiotic Lipid A Family: Structures and Immunomodulation in IBD

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0626

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

As essential molecules composing the outer membrane of gram-negative bacterial pathogens, glycolipids such as lipopolysaccharides (LPSs) and lipooligosaccharides (LOSs) have been extensively investigated, and their shared lipid A structure has been studied in depth. However, studies of the same family of molecules in commensal bacteria are disproportionately scarce. In fact, the LPS/LOS of commensals such as Bacteroides have been regarded as "inactive" in terms of their ability to stimulate host innate pattern recognition receptors (PRRs) and subsequent downstream responses. Recent studies have challenged this poorly documented view, revealing critical contributions of the LPS/LOS of mucosal commensals to host immune development and disease susceptibility. Indeed, commensal lipid A is synthesized with greater structural diversity and more extensive chemical modification than the lipid A of pathogens. However, only a few scattered reports have elucidated the structure–function relationships of these molecules.

We have identified and studied the immunomodulatory capsular polysaccharide A (PSA) of Bacteroides fragilis, a ubiquitous human gut symbiont that signals through Toll-like receptor 2 (TLR2). We have recently found that PSA is covalently attached to a glycolipid anchor (GLA), the structure of which is essentially identical to that of the lipid A of this microbe. Removal of the GLA abrogates the TLR2-mediated immunomodulatory activity of PSA, and much of the innate immune activity of PSA can be recapitulated with the GLA alone. These results led us to hypothesize that structurally diverse lipid A molecules of B. fragilis and related symbiotic bacteria can modulate host immune responses by distinct activation/regulation of PRR-mediated signaling pathways. To address this hypothesis, we propose a multidisciplinary investigation that takes advantage of high-sensitivity and LC-MS/MS platforms; genome-wide, high-throughput metabolomics screening technology; molecular microbiology; in vivo immunology; and gnotobiotic animal studies. All aspects of this work will be developed and expertized by two closely collaborating research groups.

15. SUBJECT TERMS

Gut commensal microbiota; Inflammatory bowel diseases; Metabolomics; immunomodulation by microbial symbionts; lipid A; lipooligosaccharide

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	11	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. Introduction

A diverse community of commensal microbes colonize the intestine and play a crucial role in immune homeostasis by producing a broad range of microbial factors that can modulate immune response of the host. Recent advances in microbiome research have uncovered a complex relationship between symbiotic microbes and the host immune system. The microbiome is indispensable for maintaining immune tolerance while eliciting a proper immune response against pathogen infection. However, there is still a fundamental lack of in-depth insights into the molecular structure of symbiotic molecules responsible for immunomodulatory activity. Among a myriad of commensal microbes inducing various immunomodulatory outcomes, we have studied a human gut symbiotic microbe, *Bacteroides fragilis*, and its outer membrane associated glycolipids, including polysaccharide A (PSA). We previously demonstrated that *B. fragilis* PSA can effectively inhibit the progression of colonic inflammation and autoimmune neuroinflammation by promoting IL-10 from $CD4^+$ T cells. Our detailed chemical analysis of PSA molecule discovered a glycolipid anchor moiety that is structurally similar, if not identical, to lipid A molecules found as part of the lipooligosaccharides (LOS) of this microorganism. Of significant interest, the removal by acid hydrolysis of this lipid A anchor from *B. fragilis* PSA significantly abolishes IL-10 activity suggesting a functional importance of this glycolipid moiety for PSA-mediated immunomodulation.

Our proposed research under support from DoD CDMRP is to investigate a functional correlation between the molecular structure of *B. fragilis* glycolipid and immunomodulatory activity. From our lipidomic analysis of *B. fragilis* PSA, we discovered that unlike conventional lipid A structures found in *Escherichia coli*, the glycolipid portion of individual molecules of PSA are comprised of structurally distinct lipid A variants with varying degrees of acylation and phosphorylation. During the first year of this grant, we conducted a groundwork of structure-activity investigation by using a series of chemically synthesized lipid A structural variants that recapitulate the molecular structure of variable lipid A molecules found in *B. fragilis* LOS. We examined the immunomodulatory activity of each synthetic lipid A molecular variants in dendritic cell (DC) culture *in vitro* and tested therapeutic potential of each lipid A variant using *in vivo* disease model. During our second year of work in this program, we continued our research to investigate structural diversity of gut symbiont-originated lipid A molecules as well as delineate underlying mechanisms of how these lipid A variants are recognized and how the immunological landscape in DCs are differentially modulated by lipid A structural variations.

2. Keywords

Gut microbiota, Symbiotic microbes, Immunomodulation, Lipid A, Structure-activity relationship, Inflammatory bowel disease

3. Accomplishments

A. Scientific Goals

Oh lab

During year 2, Oh lab focused on the preparation and functional validation of saccharolipid species from various gut symbionts.

Specific Aim 1 (Aim 1a Subtask 3)

We have developed a generalizable method to prepare LPS and LOS from more than 40 strains symbiotic microbiota. Saccharolipids (SLs, which include both LPS and LOS). Purification of SLs from gut and oral symbiont species in the order Bacteroidales, which are reported to have similar lipid A structures[31], yields various types of polysaccharide structural patterns (Figure 1). Three major types have been characterized: (1) low-molecular-weight bands (lipid A–core oligosaccharides [“rough LPS”] similar to the *E. coli* Nissle strain) identified in *B. fragilis* and *B. thetaiotaomicron*, (2) short ladder-type bands identified in *B. dorei* and *B. vulgatus*, and (3) a medium- or high-molecular-weight ladder (similar to *E. coli* LPS but missing lipooligosaccharide [LOS] or lipid A) identified in the genus *Porphyromonas*. Of considerable interest, along with Bacteroidetes (many *Bacteroides* species and neighboring genera) and Proteobacteria (such as *E. coli*), we have determined

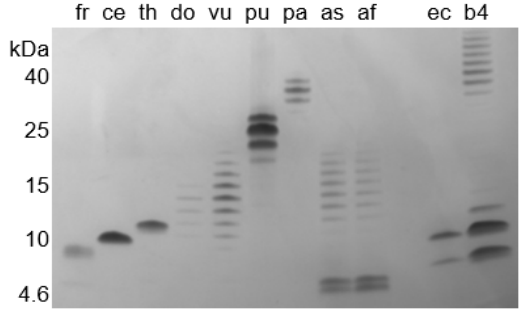


Figure 1. SL profiles of representative GI symbionts. SLs were purified from bacterial pellets and run in 10–20% Tris-tricine gel with zinc counterstaining. *fr, Bacteroides fragilis; ce, B. cellulolyticus; th, B. thetaiotaomicron; do, B. dorei; vu, B. vulgatus; pu, Porphyromonas ueonis; pa, P. asaccharolytica; as, Acidaminococcus intestini D21; af, Acidaminococcus fermentans canada; ec, E. coli* Nissle 1917; *b4, E. coli* serotype O111:B4.

that several species of Firmicutes (mostly classified as Negativicutes) produce LPS-type SLs consisting of lipid A and a short O-antigen ladder structure (similar to type 2 SLs).

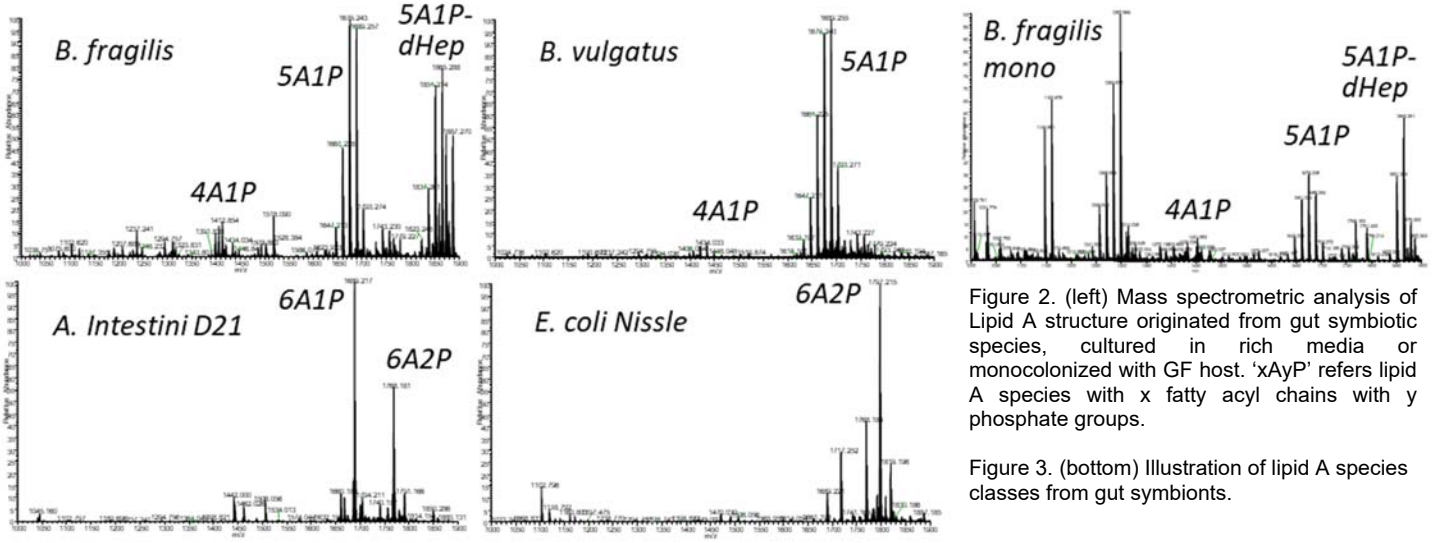
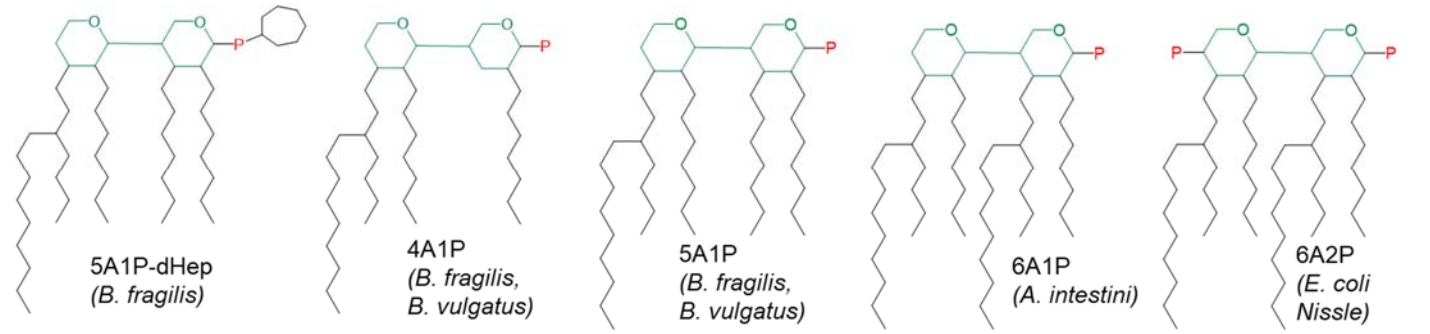


Figure 2. (left) Mass spectrometric analysis of Lipid A structure originated from gut symbiotic species, cultured in rich media or monocolonized with GF host. 'xAyP' refers lipid A species with x fatty acyl chains with y phosphate groups.

Figure 3. (bottom) Illustration of lipid A species classes from gut symbionts.



Further lipidomic analysis of the lipid A portion of SLs of gut and oral symbionts has confirmed shared structural components among gut symbiont–derived molecules, such as (1) longer acyl chains, (2) lower degrees of acylation, and (3) less phosphorylated diacylglucosamine (Figure 2-3) than that of *E.coli* lipid A. Many taxonomically related species share common structural components (such as 5A1P lipid A in *Bacteroides*), and there are also individual species–specific lipid A modifications, such as the deoxyheptose-conjugated lipid A (5A1P-dHep, assigned on the basis of a 176-Da difference in molecular mass) identified from *B. fragilis*. We also confirmed lipid A profiles of gut luminal content, originated from *B. fragilis* monocolonized mouse and SPF mice. As expected, lipid A profile of monocolonized mice resembles *B. fragilis* culture. SPF mice, whose major SL-producing species are Bacteroidetes, also has monophosphorylated lipid A as major species.

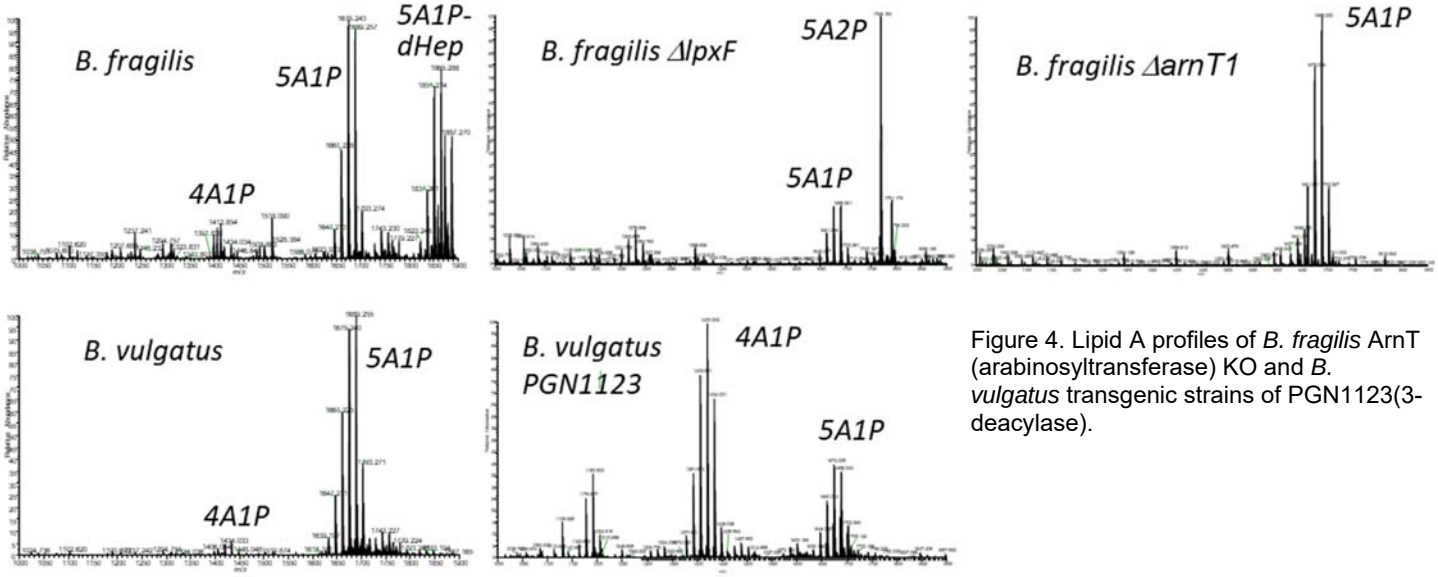


Figure 4. Lipid A profiles of *B. fragilis* ArnT (arabinosyltransferase) KO and *B. vulgatus* transgenic strains of PGN1123(3-deacylase).

Specific aim 2 (Aim 2B subtask 2): Structural modification of lipid A by genetic approaches

We have identified and genetically characterized several genes responsible for lipid A structural modification, such as an *arnT* (arabinosyl transferase) and an *lpxF* (1-phosphate phosphatase) homologs (BF3175 and BF3167, respectively). SLs isolated from individual knockout strains shows higher induction of IL-8 than SLs from wild-type strain, suggesting these genes are critical specific structural moiety (Figure 4). Further mass spectrometric analysis identified clear molecular phenotypes, such as loss of monosaccharide capping in the ΔarnT strain or retention of 4'-phosphate in the ΔlpxF strain (Figure 4). We also have generated and characterized a transgenic strain of *B. vulgatus,* heterologously expressing 3-deacylase of *Porphyromonas gingivalis* (PGN1123); this addition shifts the major lipid A species from pentaacylated to tetraacylated diglucosamine. These results from chemical and genetic studies illustrate the technical feasibility of manipulating target genes of interest in SL biosynthesis and purifying molecules with structural relevance for assessment.

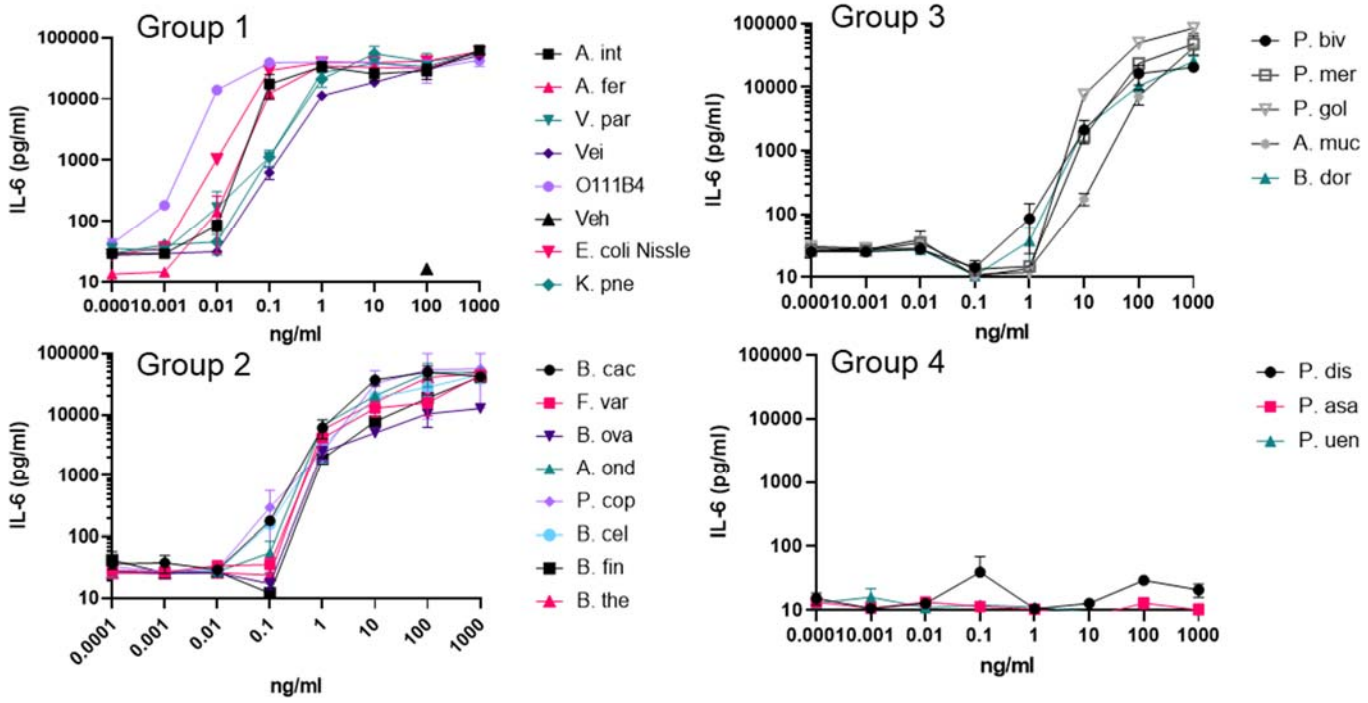


Figure 5. Classification of saccharolipid activity of gut and oral symbionts, shown as induction of IL-6 from human monocytes. Based on efficacy (EC50 value), SLs can be clustered in four types.

Specific aim 3 (Aim 3a subtask 1 and 2) : In vitro immunostimulatory actions of gut commensal LOS

Immunostimulatory activities of purified LOS and lipid A has been assessed with human monocytes. Human peripheral blood monocytic cells (PBMCs) was isolated by Ficoll-gradient centrifugation. Adherent monocytes were isolated and treated with wide range of LOS. Results clearly show that gut symbiont-derived LOS can be classified in four groups by their efficacy (Figure 5): 1) high (EC_{50}<1ng/mL), 2) intermediate (EC_{50} 1~10ng/mL), 3) low (EC50>10ng) and 4) inactive. LOS structures largely correlate with efficacy: Most of group 1 LOS resembles typical *E. coli* lipid A (step-ladder pattern with LOS), group 2 shows prominent lipid A and/or LOS, group 3 has short o-antigen ladder pattern and group has 4 long o-antigen ladder pattern. Quantitative analysis of lipid A content, as well as lipid A pattern by MALDI-TOF/TOF analysis is under way.

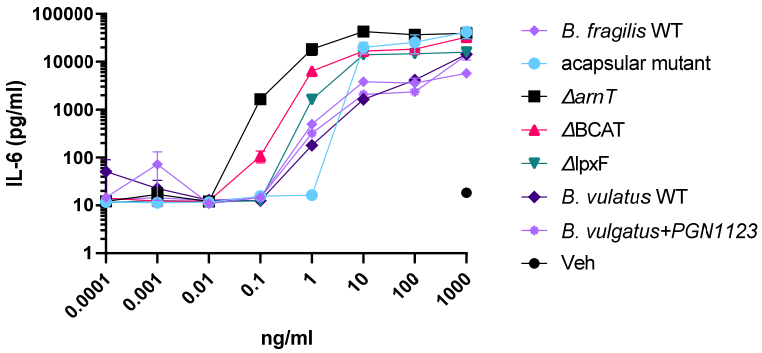


Figure 6. Activities of LOS purified from genetically manipulated *B. fragilis* strains and a *B. vulgatus* strain with *P. gingivalis* deacylase *PGN1123*.

We also have generated several B. fragilis mutants of lipid A biosynthesis. Several mutants such as ΔArnT (phosphate capping enzyme), ΔlpxF (4'-phosphatase) and ΔBCAT (involved in acyl chain branching) showed higher responses from monocytes (Figure 6).

Kasper lab

Among the proposed aims in our proposal, Kasper lab has mainly focused on the tasks described in the Specific Aim 3 and Aim 4.

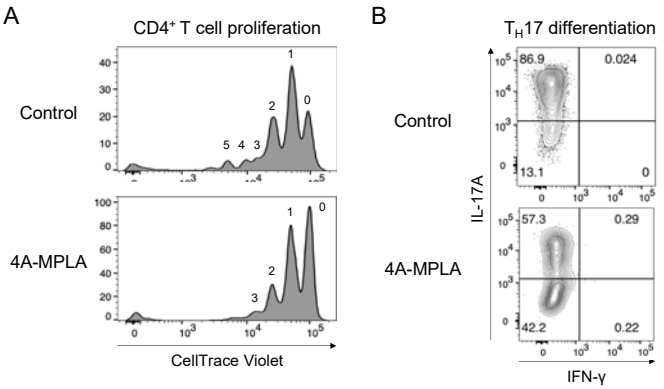


Figure 7. Immunomodulatory effect of 4A-MPLA on CD4⁺ T cell proliferation (A) and T_H17 differentiation (B)
* Numbers shown on histogram indicate the number of cell division starting from 0 (undivided) to 1 (less divided) through 5 (more divided)

<u>Specific Aim 3. To assess the immunomodulatory impact of symbiotic lipid A *in vitro*</u>

Our previous findings revealed a graded pattern of cytokine production that is correlated with chemical structure of lipid A molecular variants with different degrees of acylation (tri- to hexa-acylated) and phosphorylation (mono- or di-phosphorylated). We found that the under-acylated monophosphoryl lipid A (MPLA) weakly induced proinflammatory cytokines (e.g. IL-1β, IL-6, IL-8, IL-12, and TNF-α) in DCs, while highly inducing expression of co-inhibitory molecules such as PD-L1 and ICOSL. We further tested the immunomodulatory activity of the under-acylated MPLAs, especially 4A (tetra-acylated)-MPLA, which induced the highest PD-L1 expression in DCs compared to other synthetic lipid A variants. We expanded our investigation of the immunomodulatory activity of this particlar structural variant in DC-CD4⁺ T cell co-cultures. First, we labeled OT-II TCR-transgenic T cells with a cell proliferation dye, CellTrace Violet, and then stimulated them with $OVA_{323-339}$ peptide (restricted to MHC class II)-pulsed DC with or without 4A-MPLA. Consistent to our previous findings, 4A-MPLA treatment markedly impaired antigen-induced OT-II T cell proliferation (Figure 7A). We also examined the effect of 4A-MPLA on T helper (T_H) differentiation. For this experiment, we isolated CD4⁺ T cells from the spleen and co-cultured them with splenic DCs under various T_H-polarizing conditions (T_H1, T_H2, T_H17, and T_{FH}) in combination with various cytokines and neutralizing antibodies. Interestingly, 4A-MPLA exhibited a prominent inhibitory effect on T_H17 differentiation while showing no noticeable effect on other T_H lineage differentiations (Figure 7B). This result corresponds to our first-year findings from DSS-induced colitis, which shows a therapeutic potential of under- acylated MPLA in preventing colonic inflammation.

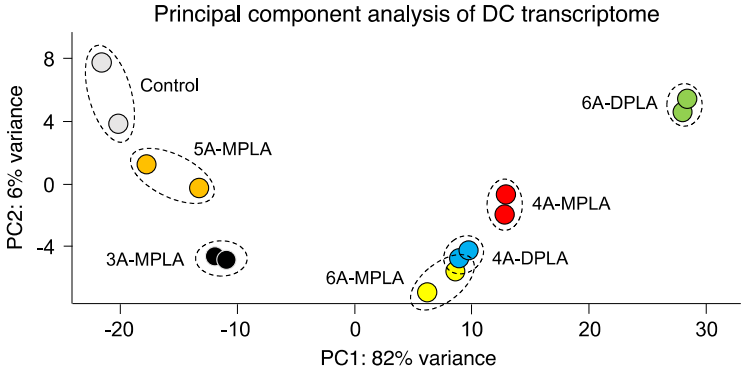


Figure 8. Principal component analysis of DC transcriptome data
* 3A- (tri-acylated); 4A- (tetra-acylated); 5A- (penta-acylated); 6A- (hexa-acylated)
* MPLA (mono-phosphoryl lipid A); DPLA (di-phosphoryl lipid A)

To have a better understanding of the immunological impact of lipid A structure on DCs, we performed a comprehensive transcriptomic analysis via RNA-Seq. First, we analyzed the entire transcriptomic data of DC treated with different lipid A structural variants. Of significant interest, principal component analysis of RNA-Seq data reveals that each individual lipid A variant modulates DC to exhibit unique transcriptomic signatures that are mutually distinguishable (Figure 8). Among various changes in immune transcriptome in DCs, we noticed a unique pattern of transcriptional activation that exclusively responds to the under-acylated MPLA variants. As shown on the heatmap in Figure 9, we found that 3A- and 4A-MPLA significantly activates interferon (IFN)-stimulated genes (ISG), such as *Oasl1*, *Oas2*, *Ifit1*, *Mx2*, and *Irf7*, all functionally associated with type I IFN-mediated immune signature. These results indicate that the molecular variation in lipid A structure influences the immunomodulatory activity to regulate T cell activation in DC-T cell co-cultures and induces transcriptomic changes in type I IFN-related signature in DCs.

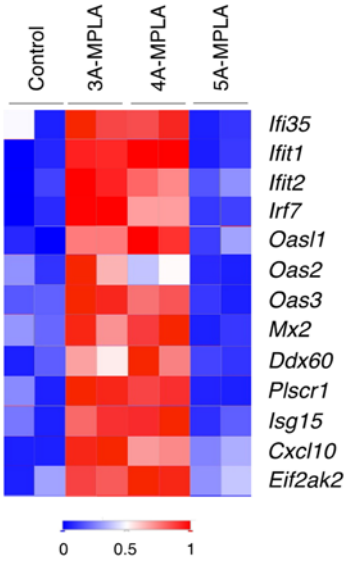


Figure 9. DC transcriptomic signature of genes highly upregulated upon treatment of the under-acylated MPLA structural variants

Specific Aim 4. To determine the immunomodulatory function of symbiotic lipid A in gnotobiotic animal models

In our prior annual report, we examined the immunomodulatory activity of synthetic lipid A structural variants in DSS-induced colitis model. We demonstrated that among the MPLA variants we tested, oral administration of 4A-MPLA induces the best protective response against colonic inflammation. To elucidate underlying molecular mechanism of such response, we used transgenic mouse model system (*Itgax-cre* x *Tlr4*$^{flox/flox}$) to ablate TLR4 signaling specifically in DCs and examined the immunomodulatory response induced by 4A-MPLA treatment in DSS colitis model (Figure 10). This result demonstrates that DC-mediated TLR4 signaling is indispensable for the immunomodulatory response driven by lipid A variants. Based on this result, we are now testing the immunomodulatory activity of these lipid A variants in gnotobiotic conditions to more clearly examine differential activity driven by lipid A structural variation with no interference from the lipid A molecules produced by existing gut microbiota.

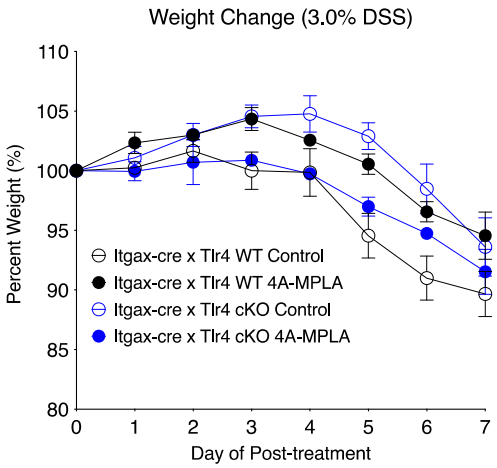


Figure 10. DC-specific ablation of TLR4 signaling abrogates lipid A-driven immune-protective response to DSS-induced colitis
* Tlr4 WT (Itgax-cre x Tlr4$^{+/+}$)
* Tlr4 cKO (Itgax-cre x Tlr4$^{flox/flox}$)

B. Training and Professional Development

Ji-Sun Yoo, Ph.D., postdoctoral fellow at Oh lab, has been investigating lipid A biosynthesis pathways and functions of gut symbiotic bacteria. Dr. Yoo has been awarded a postdoctoral research fellowship from National Research Foundation of Korea, on “Human antiviral immune responses induced by symbionts”.

A postdoctoral trainee at Kasper lab, **Hyoung-Soo Cho, Ph.D**., has been working on the investigation of chemical structure-immunologic activity relationship of lipid A molecules since the beginning of the project. He submitted an abstract to Keystone eSymposia, Harnessing the Microbiome for Disease Prevention and Therapy 2021 Conference, and the abstract was selected for Trainee Scholarship awarded by the conference committee. Also, he was recently invited to present our current work in Korean-American Biomedical Society at University of Massachusetts Medical School and Crohn’s and Colitis Foundation Investigators Research Symposium. For our transcriptome data analysis, he has been collaborating with the Biostatistician (Alos Diallo) in our department and learning how to process raw RNA-Seq data and visualize data by creating a heatmap or performing pathway analysis using various software (Morpheus and Cytoscape).

4. Impact

Our findings suggest that molecular variation in glycolipid structure can dictate an immunomodulatory potential of commensal microbes. We strongly believe that this research will provide the molecular insight to predict the immunomodulatory activity of commensal microbes based on lipid A structural composition. This research will provide a key information for the design and development of new therapeutics for inflammatory bowel disease based on symbiotic glycolipids.

5. Changes/Problems

1. During our DC transcriptomic analyses, we found transcriptomic activation of ISG signature in DCs responding to the under-acylated MPLA structural variants. These genes are functionally associated with anti-viral immune function mediated by IFN-β, which has a dichotomous function based on various immunological contexts. We anticipate that these lipid A structural variants may enhance anti-viral immunity through the activation of type I IFN-associated genes while preventing an excessive immune response of the host. As a proof-of-concept, we will also try to test whether these lipid A variants in a lethal influenza A/PR8 challenge model.

2. Because of pandemic-related facility shutdown and relocation (Mar 2020-Jun 2021), there has been adjustments in the timeline of subaims using MALDI-TOF/TOF instrument. We have mitigated delay in two ways: 1) While preparing for the transposon-based metabolomic screening method set up, we have carried out targeted genetic study and lipid A profiling of KO and transgenic strains. Among various genetically manipulated strains generated, several strains producing one form of lipid A as dominant chemical structure (Figure 4) have been grown in larger scale and LOS prepared for functional validation as well as further purification of lipid A. Examples

are: a) *B. fragilis* ArnT KO with pentaacylated-monophosphorylated form and b) *B. vulgatus* transgenic strain with heterologously expressed *P. gingivalis* PGN_1123.
2) We have adjusted timelines, and changed order of several experiments. We have prepared LOS from up to 25 species and 10 genetically modified strains. *In vitro* assays using purified LOS and lipid A were carried out, while MALDI-TOF/TOF methodology is being established.

6. Products

During year 2, we published one research articles to date which this award partially funded.
Oh SF†*, Praveena T*, Song HB, **Yoo JS**, Jung DJ, Erturk-Hasdemir D, Hwang YS, Lee CC, Le Nours J, Kim HS, Lee J, Blumberg RS, Rossjohn J†, Park SB†, **Kasper DL†**. Host immunomodulatory lipids created by symbionts from dietary amino acids. *Nature. In press.* († Co-corresponding authors, * co-first authors)

7. Participants & Other Collaborating Organizations

Name:	Sungwhan Oh
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0002-0280-7903
Nearest person month worked:	*3.0*
Contribution to Project:	*Dr. Oh did three major tasks for the project.* *1) Structural analysis of purified lipid A molecules from B. fragilis* 2) *supervised genetic and chemical characterization of B. fragilis and other commensal lipid A / LOS structures.* 3) *Worked closely with Dr. Kasper planning overall directions.*
Funding Support:	-
Name:	Jisun Yoo
Project Role:	*Research fellow*
Researcher Identifier (e.g. ORCID ID):	0000-0003-3100-0794
Nearest person month worked:	*6.0*
Contribution to Project:	*Dr. Yoo carried out three major tasks.* *1) Genetic characterization of gut commensal lipid A molecules, generating multiple knockout strains of lipid A biosynthesis in B. fragilis and other Bacteroides.* *2)* Optimization of in vitro assay system for TLR2/TLR4 response to commensal Lipid A, using transfected cell lines. *3) Developed a method to isolate LOS molecules from multiple gut commensal strains.*
Funding Support:	National Research Foundation of Korea (Sep 2021~)
Name:	Byoungsook Goh
Project Role:	Research fellow

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.0
Contribution to Project:	Dr. Goh carried out three major tasks. *1)* MALDI-TOF/TOF analysis methodology optimization for lipid As *2)* Establishing high-throughput lipidomic screening platform of bacterial transposon library
Funding Support:	
Name:	Eungyo Choi
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6.0*
Contribution to Project:	*Dr. Choi contributed to the preparation and isolation of B. fragilis lipid A molecules for further chemical and biological characterizations.*
Funding Support:	-

Name:	Dennis Kasper
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Kasper supervised this project, organized and planned the execution of specific aims and worked with Dr. Oh on planning chemical and microbiological studies.*
Funding Support:	-

Name:	Hyoung-Soo Cho
Project Role:	*Research Fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-0899-8541*
Nearest person month worked:	*12.0*
Contribution to Project:	*1) Structure-activity analysis of individual lipid A structural variants in vitro* *2) Examination of immunomodulatory activity of lipid A structural variants in the steady-state condition and DSS-induced colitis model in vivo*
Funding Support:	*Crohn's and Colitis Foundation Research Fellows Award*

8. SPECIAL REPORTING REQUIREMENTS

Collaboratory award with partnering PI options

- Accomplishments during the reporting period were specified under specific PI.

9. APPENDICES

Oh SF†*, Praveena T*, Song HB, **Yoo JS**, Jung DJ, Erturk-Hasdemir D, Hwang YS, Lee CC, Le Nours J, Kim HS, Lee J, Blumberg RS, Rossjohn J†, Park SB†, **Kasper DL†**. Host immunomodulatory lipids created by symbionts from dietary amino acids. *Nature. In press.* († Co-corresponding authors, * co-first authors)