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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

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TABLE OF CONTENTS

Page

1. INTRODUCTION

During the first year of award, Kasper lab and Oh lab closely collaborated to execute four proposed specific aims.

Oh lab made progress in first three specific aims, 1) profiling lipid A structures of multiple symbiotic species (>50 gram negative commensals), 2) chemically characterizing and lipid A molecules from genetic knockout strains of B. fragilis and 3) preparing and purifying lipid A molecules with distinct structures. **Kasper lab** has carried out 1) *in vitro* experiments proposed in aim 3 with synthetic lipid A molecules showing immunomodulatory actions as well as 2) *in vivo* (DSS colitis) models proving host protective actions of lipid A molecules from gut symbionts.

2. KEYWORDS

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

3. ACCOMPLISHMENTS

A. **Scientific Goals-** These are listed by specific aims in the order they appeared in the application

Accomplishments of Oh lab

Specific Aim 1a

Subtask 1: Culturing 20 gut symbionts (month 1-6)

We have successfully cultured more than 100 gut symbionts in anaerobic monoculture system. Among them, we chose 67 gram-negative species for lipid A isolation and characterization.

Subtask 2: Chemical preparation of lipid A (month 4-6)

To isolate lipid A, we have optimized a chemical hydrolysis method which can directly cleave the acid-labile linkage of LOS from bacterial pellets.

Subtask 3: LC-MS/MS and IC-MS/MS profiling of symbiotic lipid A (month 7-24)

With the results of subtask 2, we have hydrolyzed and isolated lipid A species. Instead of IC-MS/MS which needs a harsh eluent condition, we have developed a HILIC-based LC-MS/MS method which can separate and characterize the structure of core oligosaccharides.

Specific Aim 1b

Subtask 1: Large scale purification of B. fragilis lipid A (month 1-12)

We have developed and optimized a HPLC method which can fractionate lipid As with diverse structures from Bacteroides fragilis. This method using semi-quantitative reverse phase column successfully separated six subclasses of lipid A with 90-99% purity. Milligram-level of LOS can be fractionated per single run, and up to 10ug of each molecular species and up to 50ug of each subclass of lipid A is estimated to be fractionated. This is enough amount to carry out further in vitro and in vivo studies to assess structure-activity relationship. We are also in the process

Subtask 2: Large scale purification of other symbiotic Lipid As (month 13-24)

We have tested and confirmed the above method can be applied to other Bacteroides and gram-negative species. We will choose 3-5 species which have characteristic lipid A species and isolate lipid A molecules for further in vitro and in vivo assay.

Specific Aim 2a

Subtask 1: Optimization of LC-MS/MS for high-throughput screening (month 1-6)

Subtask 2: Screen 7000 mutants (month 7-18)

We have optimized a LC-MS/MS (by adopting metal-free reverse phase (C8) column chromatography) method to separate and quantify symbiotic lipid A molecules from the transposon mutants. In parallel, to increase the throughput of the screening, we also have been developing a complementary MALDI-TOF/TOF methods which can rapidly and semi-quantitatively screen mutants.

Specific Aim 2b

Subtask 1: Knockout generation from LC-MS/MS results (month 13-24)

Prior to generate knockout strains from the transposon screening results, we also have generated knockouts of previously annotated lipid A biosynthesis genes of B. fragilis and other Bacteroides. By adopting a high-efficiency strategy, we were able to generate >10 knockout strains of B. fragilis.

Subtask 2: LC-MS/MS analysis of Lipid A of knockout strains (month 25-36)

By using workflow described in specific aim 1, we analyzed lipid A profiles of B. fragilis KO strains generated from subtask 1.

Specific Aim 3a

Subtask 1: In vitro assessment of *B. fragilis* lipid A and GLA (month 13-24)

We have developed an in vitro screening assay by using stably transfected TLR4 and TLR2 HEK293 cell lines. Synthetic lipid A molecules of which structure resemble B. fragilis lipid A were used for optimization. We have also developed a rapid, small-scale isolation method (proteinase K treatment followed by Trizol extraction) for GLA molecules from *B. fragilis* wild-type and knockout strains (specific aim 2b) and isolated GLA molecules has been assessed with above assay system.

Accomplishments of Kasper lab

Specific Aim 3. To assess the immunomodulatory impacts of symbiotic lipid A *in vitro*

In our original proposal, we showed that *B. fragilis* LOS induces IL-10 production from $CD4^+$ T cells, and LOS oral administration reduces the susceptibility of DSS colitis. From our previous lipidomic analysis, we identified six representative lipid A variants, tri-, tetra-, and penta-acylated lipid A with/without monophosphorylation. However, *B. fragilis* LOS is a complex mixture of lipid A molecules in varying degree of acylation and phosphorylation. This could complicate our data to differentiate downstream immunomodulatory effects of each structural variant. While making an effort on the fractionation of lipid A variant from *B. fragilis* LOS, we conducted a groundwork of structure-activity investigation of lipid A by using synthetic lipid A molecules delineate the immunological activity of each individual lipid A structure. We used a series of synthetic lipid A molecules varying degree of acylation (tri-, tetra-, penta-, and hexa-acylated) and phosphorylation (mono- or di-phosphoryl). First, we cultured these synthetic lipid A variants with DCs. Cultured supernatants harvested from the lipid A-treated DCs were comprehensively examined for their cytokine production profile. We observed that the over-acylated

(penta- or hexa-acylated) monophosphoryl lipid A molecules (MPLAs) or diphosphoryl lipid A molecules (DPLAs) exhibit more potency to induce an array of proinflammatory cytokines, e.g. IL-1β, IL-6, IL-12 p70, and IFN-β, in comparison to the potency of under-acylated (tri- or tetra-acylated) MPLA (Figure 1, left). In striking contrast to this pattern, the under-acylated MPLAs prominently upregulate IL-18 production from DCs while the over-acylated MPLAs or DPLAs show no noticeable activity in IL-18 production. Of interest, IL-18 production from DCs in response to the underacylated MPLAs require TLR4 but not Myd88 or TRIF, which is very distinct usage of innate immune signaling found in the over-acylated lipid A molecules. We also found that NLRP3 signaling is essential for IL-18 induction by the under-acylated MPLAs.

Next, we examined the array of surface markers from DCs treated with various lipid A structural variants via flow cytometry. We found that the treatment of over-acylated MPLAs or DPLAs upregulate costimulatory molecules, e.g. CD40, CD80 and CD86, while the under-acylated MPLAs show a low capacity to induce these molecules on DCs. On the other hand, DCs treated with the under-acylated MPLAs induce a high expression of coinhibitory molecule PD-L1 comparing with the DCs treated with the over-acylated MPLAs or DPLAs. In addition, we found that ICOS (Inducible T cell co-stimulator) ligand (ICOSL) is highly expressed in the under-acylated MPLA-treated DC. However, the amount of ICOSL expression on the DC is drastically reduced as the degree of acylation and phosphorylation in the lipid A gets increased.

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

Along with the immunomodulatory effects observed *in vitro*, we next examined how these molecules affect host immune landscape in the steady-state condition. First, we orally administered 3 different lipid A acyl variants (tri-, tetra-, and penta-acylated) to B6 wild-type mice (15μg per day x 4 times, 60μg/mouse). Oral treatment of 3A- (tri-acylated) and 4A- (tetra-acylated) MPLA increased the proportion of RORγt-expressing peripheral Treg (pTreg, RORγt$^+$ Helios$^-$) in the colonic lamina propria (LP) while the proportion of thymic Treg (tTreg, RORγt$^-$ Helios$^+$) was reduced among colonic Treg population. Interestingly, the 5A- (penta-acylated) MPLA exerted a directly opposite effect to reduce colonic RORγt$^+$ Treg while increasing Helios$^+$ Treg in the colon LP. Next, we tested if these acylated MPLA variants have any translational potential for colitis disease model. To test this, we orally administered these lipid A molecules (D-3 to D2, 10μg x 6 times) to B6 WT mice and monitored their disease susceptibility to dextran sodium sulfate (DSS)-induced colitis model. We observed that 4A-MPLA-treated mice exerted the most protective effect on the weight loss compared with the control group while 3A-MPLA-treated or 5A-MPLA-treated mice showed a modest or minimal effect to DSS-induced colitis model (Figure 4, left). Associated with this data, the proportion of RORγt-expressing colon LP Treg was comparatively higher than other groups at D10 post-treatment of DSS.

B. Training and professional development

In Oh lab, one postdoctoral fellow (Dr. Jisun Yoo) and one research technician (Dr. Eungyo Choi) contributed to the research activities. Working on this project and the work has afforded significant training in solving the

chemical, immunologic, and microbiologic aims of this grant.Teaching has come from several sources, including direction and advice from PI's and senior lab members, conferences, and regular lab meetings.

4. IMPACT

The techniques we have developed provide important tools that will enable research at the interface of host microbe interactions during disease. An understanding of these interactions provides key insights into potential avenues for therapeutic intervention.

Among various immunomodulatory molecules that we examined, IL-18 has recently emerged as a key module for the host-microbe interaction in the intestine by promoting intestinal epithelial integrity and protection from colonic inflammation. Current studies indicate gut microbiota is closely related to the level of IL-18, but the investigation of structure-activity relationship behind this host-microbe interaction remain largely elusive (Elinav et al, *Cell*, 2011; Levy et al, *Cell*, 2015; Nowarski et al, *Cell*, 2015). Our study delves into how lipid A structural modification, e.g. the degree of acylation, differentially affects IL-18, which is closely related to intestinal homeostasis and etiology of inflammatory bowel disease. Furthermore, recent studies have shown that RORγt^{+} Treg is important for regulation of T_H1/T_H17 colonic inflammation and also is highly responsive to microbial antigens (Sefik et al, *Science*, 2015). Combining these findings with our *in vivo* results, we hypothesize that RORγt^{+} Treg residing in the colon are highly sensitive and also adaptable to the immunomodulatory cues derived from lipid A structural variations. Functional change in this Treg subset may play an essential role in intestinal tolerance responding to the lipid A structure of symbiotic microbes.

5. CHANGES/PROBLEMS

Due to global COVID-19 pandemic and related shutdown of research facility, there was a minor change in the order of executing specific aims. Majority of work has been done as scheduled and no significant delay took place or is expected in following years.

6. PRODUCTS

We published 2 research articles to date which this award partially funded.

1. Defined A molecular mechanism of *B. fragilis* PSA-mediated immunomodulation via TLR1/2 in cooperation with Dectin-1 receptor and its downstream signaling pathway.

Deniz Erturk-Hasdemir, Sungwhan F. Oh, Nihal A. Okan, Giuseppe Stefanetti, Francesca S. Gazzaniga, Peter H. Seeberger, Scott E. Plevy, and Dennis L. Kasper, Symbionts exploit complex signaling to educate the immune system, *Proc. Nat. Acad. Sci.*, 2019, 116 (52), 26157-166

2. Discovered the molecule and mechanism by which a specific commensal microbe induces IFNβ. The outer membrane-associated LOS of commensal microbes of the Bacteroidetes phylum induce expression of IFNβ. Using Bacteroides fragilis as the paradigm, we determined that IFNβ expression was induced via the TLR4-TRIF signaling pathway. In an in vivo model of VSV infection, we discovered that commensal-induced IFNβ regulates

natural resistance to virus infection.

Kailyn L Stefan, Myoungjoo V Kim, Akiko Iwasaki, Dennis L. Kasper. Commensal microbiota modulate natural resistance to virus infection. *Cell, in press*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

Name:	*Sungwhan Oh*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0002-0280-7903
Nearest person month worked:	*1.5*
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:	*12.0*
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:	-

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 (Award ID: 649279)

8. SPECIAL REPORTING REQUIREMENTS

Collaboratory award with partnering PI options

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

9. APPENDICES

Deniz Erturk-Hasdemir, Sungwhan F. Oh, Nihal A. Okan, Giuseppe Stefanetti, Francesca S. Gazzaniga, Peter H. Seeberger, Scott E. Plevy, and Dennis L. Kasper, Symbionts exploit complex signaling to educate the immune system, Proc. Nat. Acad. Sci., 2019, 116 (52), 26157-166

Kailyn L Stefan, Myoungjoo V Kim, Akiko Iwasaki, Dennis L. Kasper. Commensal microbiota modulate natural resistance to virus infection. Cell, in press