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A Study of Immune-Regulation of Microbiota Supernatant on Macrophage Function

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A STUDY OF IMMUNE-REGULATION OF MICROBIOTA SUPERNATANT ON MACROPHAGE FUNCTION

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LIST OF ABBREVIATIONS:

#	numbers
%	percentage
(-)	negative
(+)	positive
<10KDa	less than 10 KDa
>10KDa	greater than 10KDa
Ab	antibody
AMPs	antimicrobial protein
B. breve	*Bacillus breve*
B. fragilis	*Bacillus fragilis*
BMDM	bone marrow derived macrophage cells
CD	cluster of differentiation
CLRs	C-type lectin receptors
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNAse	deoxyribonuclease
ELISA	enzyme-linked immunosorbent assay
EPS	exsopolysaccharides
FBS	fetal bovine serum
FitC-dextran	Fluorescein isothiocyanate–dextran
foxp3	forkhead box P3
GALT	gut associated lymphoid tissues
GF	germ free
GI	gastrointestinal
HCl	hydrochloric acid
i.p	intraperitoneal injection
IBS	irritable bowel syndrome
IEC	intestinal epithelium cell
IFN	interferron
Ig	Immunoglobulin
IL	interleukin
IL-1β	interleukin 1β
IL-6	interleukin 6
ILC	innate lymphoid cells
iNKT	invariant natural killer T cells

kDa	kilodalton
L. kefiranofaciens	*Lactobacillus kefiranofaciens*
LD	*Lactobacillus delbrueckii*
LF	*Lactobacillus fermentum*
LPS	lipopolysaccharide
LR	*Lactobacillus rhamnosus*
M cells	microfold cells
MAIT	mucosal-associated invariant T cells
MD-2	Lymphocyte antigen 96
mg	milligram
MHC	major histocomapbility complex
mL	millilitter
MR1	major histocomapbility complex, class I related
MSU	monosodium urate
Myd88	Myeloid differentiation primary response gene 88
NCLR4	Nod-like receptor family, CARD domain containing 4
NLRP3	Nod-like receptor family, pyrin domain containing 3
NLRP6	Nod-like receptor family, pyrin domain containing 6
NLRs	NOD-like receptors
PBS	phosphate buffer saline
PD-1	program cell death 1
pg	picogram
PMA	poly(methyl acrylate)
PPRs	pattern recognition receptors
PSA	polysaccharide A
PUFAs	poly unsatturated fatty acids
RLRs	RIG-like receptors
RNAse	Ribonuclease
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
s.e.m	standard error of the mean
SCFAs	short chain fatty acids
sd	strandard deviation
SO	*Streptococcus oralis*
TFF3	trefoil factor 3
TGF-β	transforming growth factor beta
Th1	T helper 1 cell
Th17	T helper 17 cell
Th2	T helper 2 cell
TLR	toll like receptor
$TLR2^{-/-}$	toll like receptor 2 knock out
TLR-2KO	toll like receptor 2 knock out

$TLR4^{-/-}$	toll like receptor 4 knock out
TLR-4KO	toll like receptor 4 knock out
Tregs	regulatory thymus (T) cells
Trif	toll- interleukin receptor -domain-containing adapter-inducing interferon-β
TS	tryptic soy bacteria culture medum
μg	microgram
μL	microlitter

ABSTRACT

The diverse human microbial ecosystem colonizes many different body sites. The gastrointestinal (GI) tract, especially, is a major region that that harbors a complex microbial community composed of trillions of commensal bacteria. Interactions between the commensal bacteria and the host immune system begin at birth and continue through the human lifespan. In the gut, commensal bacteria help the host to ensure the maintenance of intestinal homeostasis as well as the mucosal barrier structure through host stimulation of mucus and antimicrobial molecules. The secretion of these host factors functions to inhibit pathogenic invasion and maintain the population of commensal bacteria. The microbial communities also secrete their own metabolites to regulate host metabolism as well as host immunity. Together, this complex crosstalk allows a series of host-microbe interactions and host responses that are necessary to manipulate the host immunity, maintenanmucosal homeostasis and regulate the composition of the microbiota.

In the gastrointestinal tract, the major immune cells that actively participate in the host defense mechanism are resident intestinal macrophages. These cells are primarily responsible for the first-line host defense that protects the host from an enormous number of potentially pathogenic bacteria and/or antigenic stimuli that are present in the intestinal lumen. Normally, during steady state, these cells are very anergic and tolerant towards the commensal bacteria. Yet, they still retain their phagocytic and bactericidal activity.

Although the mechanisms surrounding the anergy of the intestinal macrophages are not well understood, the abundance of commensal bacteria present in the gut lumen may suggest that the possible function of commensal bacteria is to influence the activity of gut macrophages.

Little is known about the commensal bacteria's ability to exert their function on the host intestinal macrophages through the production of commensal metabolites. It is well understood that commensal bacteria can secrete metabolites to regulate host metabolism as well as influence host immunity. We believe that the commensal bacteria might regulate intestinal macrophages in the same manner. In this study, we sought to understand the involvement of the secreted commensal bacterial factors/metabolites in response to the anergic function of intestinal macrophages. We hypothesized that the secreted bacteria factors/metabolites present in the bacterial supernatant can suppress macrophage activation upon the induction of TLR or NLRP3 agonists on macrophages.

To test our hypothesis, we will use four different strains of commensal bacteria that are normally present in the gastrointestinal tract. Those are *Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus rhamnosus,* and *Streptococcus oralis.* We will determine whether the secreted bacterial factors present in the commensal culture supernatants can actually suppress the activation of macrophages in the presence of LPS or MSU crystal stimuli *in vitro*. Furthermore, we will also test whether the bacterial factors present in the bacterial supernatant can suppress the recruitment of neutrophil in the peritoneal cavity in the presence of LPS stimulation *in vivo*. If the secreted commensal factors/metabolites can suppress the activation of macrophage or the recruitment of netrophils upon the induction of TLR or NLRP3 agonists on macrophages,

we will then seek to determine whether the secreted commensal factors can suppress macrophages activation in the transwell system, a system that mimics the gastrointestinal tract consisting of both epithelial cells and macrophages. By taking advantage of the transwell system, we will determine whether the secreted bacterial factors in the commensal supernatants can actually suppress macrophage activation during the induction of TLR or NLRP3 agonists on macrophages in the presence of epithelial layers. Together, these studies provide insights into how commensal bacteria can regulate macrophage responses in the gastrointestinal system.

We discovered that the secreted commensal factors, which have molecular weights of less than 10Kda, can suppress macrophage activation as well as neutrophil recruiment in both *in vivo* and *in vitro* as well as in the transwell system upon MSU crystal or LPS crystal stimuli. In contrast, the secreted factors present in the commensal supernatants which have molecular weight greater than 10KDa act as a stimulatory mediator and promote the activation of macrophages in TLR-2 dependent manner.

CHAPTER ONE

INTRODUCTION

I. ROLE OF COMMENSAL BACTERIA IN THE HOST IMMUNE SYSTEM.

Commensal bacteria are present in humans in massive numbers, and they colonize many different locations in the host. In humans, the commensals can be found easily on the human skin, the oral cavity, the respiratory tract, as well as the urogenital and gastrointestinal (GI) tract. The GI tract, especially, is a major region that houses a complex network of microbial communities [1,2,6]. The human GI tract has unique structures and functions that allow for multiple interactions between epithelial cells and other immune cells to recognize the external environment stimuli, including nutrients, pathogens and commensal bacteria [4]. Normally, in a healthy state of the GI tract, the host is immune-tolerant toward the commensal bacteria, in exchange for such a protection provided by microbial communities. Such protection provides a wide range of health aspects relating to host immune function, host metabolism, the process of food digestion, extraction as well as synthesis of nutrients, and lastly the protection against pathogenic infections [4]. However, in the presence of external factors that may disturb the microbial communities, the host immuno-tolerance toward the commensals may be altered. For example, the ability of the commensals to colonize in the host GI tract system may be reduced by antibiotic use or an altered diet. As a result, this may alter the

antimicrobial resistance in the host GI tract system and allow for pathogenic infection to happen [37-47]. This suggests that there is a relationship between commensals and chronic GI diseases in the host, such as irritable bowel syndrome (IBS), ulcerative colitis, and Crohn's disease [37-47,242]. Thus, it is important to understand how commensals impact the host GI tract immune system.This may provide a potential therapeutic to treat chronic GI diseases in humans by manipulating the functions of microbial communities in the host system.

Such complex interactions between commensals and host immunity start at birth [7]. For instance, the commensals shape the development of host immunity as the host immunity, in turn, shapes the composition of the commensal communities. This communication between host immune responses and commensals requires multiple signaling pathways from many different microbial products together with host sensors that regulate the development and function of many host immune cells in both innate and adaptive immunities.

1.1 Host sensing of microbes by Toll-like receptors.

The GI tract is the largest defense barrier in the host immune system. There are at least 60% of the host's immune cells within the GI tract that are ready to recognize and defend from potential pathogenic infections and prevent uncontrolled inflammation within host GI mucosal region [48, 49]. Notably, the GI tract is also home to a vast array of microbial communities including commensal bacteria, yeasts, and lastly bacteriophages [50]. With abundant commensal species together with potential pathogenic threats present in the GI tract, it is important for the host immunity to survey

and monitor carefully so that the host immunity can distinguish commensal species from pathogenic potentials to prevent pathogenic infection. The host immune system must balance detection and elimintation of pathogens with tolerance towards commensal communities residing in the GI tract.

In the host immune system, a large family of pattern recognition receptors (PPRs) are responsible for host recognition and initiating the innate immune responses upon their interaction with pathogen-associated molecular patterns (PAMPs) present on microbial pathogens. Also, they interact with the damage-associated molecular patterns (DAMPs) present on host cell components released during cellular stress. In humans, both immune and non-immune cells can express PRRs. However, these PRRs are mainly found in antigen-presenting cells, such as macrophages, or dendritic cells. There are four main classes of PRRs that are recognized including the Toll-like receptor (TLRs), NOD-like receptor (NLRs), RIG-like receptor (RLR), and C-type lectin receptor (CLR).

A. Toll-like receptors. Among PRRs, Toll-like receptors (TLRs) are major ones that can elicit the innate immune response. TLRs can be found in many innate immune and non-immune cells. However, these receptors are mainly found in innate immune cells, such as macrophages and dendritic cells. Interestingly, the activation of TLRs requires their engagement with a defined set of conserved molecular motifs present on microbes [51] that leads to several intracellular signaling cascades mediated by Myd88 adaptors [53] or Trif domains [54] to produce cytokines, chemokines, and transcription factors. In turn, these molecules have a function to maintain gut homeostasis and to prevent pathogenic infections [52]. Among these TLRs, TLR-2 and TLR-4 are two types

of receptors that can be found on the surface of innate cells. Here, they recognize products secreted from Gram (+) and Gram (-) bacterial cell walls, respectively [55-57]. Under the homeostatic condition, TLR-2s and TLR-4s normally have low expressions in intestinal epithelial cells (IECs), and they do not respond to TLR stimuli [59-61]. However, when host immunity experiences chronic inflammation or pathogenic infection, both TLR-2s and -4s are increased on IECs and trigger an intracellular signaling cascade that promotes a pro-inflammatory state [62-66].

TLR-2s frequently involve the recognition of Gram (-) and Gram (+) bacteria along with fungal wall components [56-57, 67-68]. Also, TLR-2s play an important role in oral tolerance as well as gastrointestinal inflammation during pathogenic infection, and these receptors have an ability to elicit both pro- and anti-inflammatory reactions by their dimerization with other TLRs such as TLR-2 itself or with TLR-1, TLR-6, and TLR-10 [69].

Oral tolerance is defined as a state of host immunity being unresponsive upon oral antigenic exposure [70, 71]. The induction of oral tolerance requires the activation of TLR-2s in which TLR-2s exert their effects on Tregs and B cells to promote both pro-and anti- inflammatory reactions. The adaptive immune Treg cells are primarily responsible for the immune tolerance toward food antigens [72-75] while B cells have a function to produce IgA against allergic responses to food [77-86]. During homeostatic condition in the presence of microbiota, activation of TLR-2s on dendritic cells (DCs) directs Treg cell homing to the lamina propria but they do not drive Treg cell differentiation [73-76]. The homing of Treg cells promotes anti-inflammatory immunity of the host to prevent

allergic responses to food antigens. On the other hand, upon their activation, TLR-2s can also elicit pro-inflammatory responses on resting B cells. When TLR-2s become activated on resting B cells, they promote resting B cells to activate, and later differentiate into plasma cells and promote IgA class switching and recombination to secrete IgA antibodies [77-79]. Elevated secreted IgA production is required for the protection against food allergic responses [80-86]. Additionally, the synthetic TLR-2 ligands used as treatments can significantly suppress inflammation in the mucosal tissues by up-regulating the tight-junction of the intestinal epithelium [87-88]. As a result, the activation of TLR-2 is important to promote IgA secretion, B cell maturation, intestinal barrier function, mucosal homing as well as Treg differentiation during oral tolerance.

Besides oral tolerance, TLR-2s are subject to gastrointestinal inflammation during pathogenic infection. For example, during experimental colitis in mice, TLR-2 expression and activation are sufficient to facilitate eosinophil recruitment [90]. Similarly, during parasitic *Schistosoma mansoni* infection in mice, eosinophil recruitment to the large intestine and the subsequent chronic inflammatory responses require TLR-2 activation [89]. Sometimes, commensal bacteria protect the host from autoimmune disease by exerting their effects on the TLR-2 function to colonize the host tissues. For example, both symbionts *Bacillus fragilis* and *Bacillus breve* promote anti-inflammatory response through TLR-2 signaling within the host [91-93]. The commensal bacteria produce a polysaccharide on their surfaces called polysaccharide A (PSA) to interact with TLR-2. Such interaction with TLR-2s allows for commensal bacteria to resist in host tissues and inhibit the host inflammatory immune responses [91-93]. The suppression of host

inflammatory responses is due to the presence of Tregs since the administration of purified PSA can directly induce the T regulatory cell recruitment (Tregs) within the intestine [94]. Furthermore, the production of PSA secreted from *B. fragilis* can initiate the proliferation of FoxP3+Treg cells to produce IL-10 cytokines [91] in which these cytokines have a function to suppress pro-inflammatory host responses. On the other hand, probiotic strain *B. breve* promotes the induction of IL-10 via TLR-2 signaling on CD103+ DCs to promote the development of Tr1 cells, another subset of regulatory T cells [93]. Overall, these studies indicate that TLR-2 is a major factor that directs the immunological balance between tolerance and active immune responses during oral tolerance to food antigens as well as gastrointestinal infection from pathogens.

TLR-4s play a role in recognition of LPS or endotoxins on Gram (-) bacteria in the intestinal mucosa [55]. Upon activation by lipopolysaccharide (LPS) or endotoxin from Gram (-) bacteria, TLR-4 dimerizes with CD14 and MD-2 and subsequently induces the signaling cascade that ultimately leads to the activation of a pro-inflammatory response during host innate immunity [95-98]. During homeostatic condition in the gut, TLR-4s are normally anergic to stimuli, and they are expressed at low levels on the surface of gut epithelial cells [59-61]. This anergy of TLR-4s is due to the presence of ST2, a transmembrane protein that can sequester MyD88 and TIRAP, adaptor proteins associated with TLRs [99] and antagonize TLR-4 functions, allowing TLR-4 to become hyporesponsiveness to the commensal microbiota in the GI tract. Activation of TLR-4s on Tregs is critical for the proliferation and survival of Tregs [100]. Together, TLR-4s are important to maintain host tolerance toward commensals as to they are important for

active immune responses against pathogenic infection in the host gastrointestinal system.

B. Nod-like receptors. Nod-like receptors (NLR) are multiple sub-intracellular receptors that are expressed by many immune and non-immune cells. These intracellular receptors can recognize diverse microbial ligands or host cellular components released during cell damage. NLRs work together with TLRs to regulate the innate immune responses [101-102]. NLRs play key roles in the development of mucosal-associated lymphoid tissues, regulation of intestinal homeostasis, and maintenance of the gastrointestinal microbiota [101-102]. For example, Nod1 can be found in most immune cell types. These receptors can recognize peptidoglycan from Gram(-) bacteria, stimulate the pathogen-killing ability of bone marrow-derived neutrophils residing at a distance from the gut, and enhance lymphoid follicle development in a CCL20-CCR6 dependent manner, which is in turn necessary to maintain a stable state of microbial communities in the intestine [102]. Like Nod1, Nod2 has important roles in innate immunity during bacterial infection. Unlike Nod1, Nod2 is only expressed in some immune cells such as Paneth cell, dendritic eclls, macrophages, and IECs [103-107]. Nod2 promotes antibacterial activity of Paneth cells by secreting antibacterial molecules [108] such as α-defensins that are important molecules that shape the composition of the commensal communities [112]. However, such antimicrobial activity is altered due to the loss of function mutation on Nod2 resulting in altered interaction between host and commensal species [108]. Deficiency of Nod2 in mice leads to the altered microbiota composition [109] and appears to reduce expression of antimicrobial α-defensins produced by Paneth cells as well as antimicrobial peptide-secreting cells found in small intestinal crypts [110-

111].

Other NLR proteins (NLRPs) can form a cytoplasmic complex called the inflammasome [113] that upon activation, caspase-1 is catalytically auto-cleaved into IL-1β and IL-18 cytokines, these cytokines play major roles in pro-inflammatory responses of host immunity [113]. NLRP6 deficiency in mice results in reduced IL-18 levels in colonic epithelial cells and altered microbiota characterized by the outgrowth of commensal species such as *Bacteroidetes prevotellaceae* [114]. The altered microbial communities lead to dysbiosis that may predispose the host to inflammatory bowel disease or even colitis-associated colorectal cancer [114-115].

Cooperative PRR signaling at mucosal surfaces is important to maintain the mutualism between host and microbiota. For example, NLRP3 and NLRC4 both activate caspase-1 [116]. Also, the NLRC4 inflammasome mediates the production of mature IL-1β in intestinal phagocytes after infection with pathogens, but not with commensal bacteria [117]. Altogether, the functions of NLR and other NLR subfamilies represent an innate immune response that allows the host to respond to complex microbial challenges and to discriminate pathogenic from commensal bacteria.

C. C-type lectins. In the host, C-type lectin receptors function to help the host recognize numerous fungal species. For example, Dectin-1 is the β-glucan receptor that can recognize fungal wall components. Apparently, deficiency of Dectin-1 in mice causes such an increased susceptibility to colitis that the host fails to cope with fungal colonization [118]. Still, it is unknown whether altered fungal populations can lead to changes in commensal bacteria that subsequently affect the host and result in intestinal

inflammation.

1.2 Commensal bacteria with mucosal barrier structure.

A. The structure of mucosal barrier and its defense mechanisms against microbial approachment. The gastrointestinal mucosal structure is a complex structure that consists of both a biochemical barrier as well as a physical barrier. The physical barrier is shaped by a single layer of the intestinal epithelial cells (IECs) that separates the inside lamina propria from the intestinal lumen. Additionally, epithelial cells are closely bound by the tight junctions that allow for paracellular transport between the epithelium. These tight junctions also help to keep the intestinal epithelium intact. Mainly, the IECs consist of many non-immune cells such as absorptive enterocytes, goblet cells, and Paneth cells [154]. All of these IECs have a digestive function. However, some of these IECs have other specialized functions in addition to their digestive functions. Specifically, absorptive enterocytes play a role in metabolic function [155] while other secretory IECs, including Paneth cells, and goblet cells, provide the biochemical barrier of the mucosal structure [155-157]. This biochemical barrier protects the host from the pathogenic approachment and maintains the homeostatic balance of the gut microbiota. For example, both Paneth cells and Goblet cells secrete mucins to enforce the physical barrier, and they secrete antimicrobial peptides (AMPs) to maintain the biochemical barrier of the mucosal structure and protect the host from the pathogenic approachment [156-157].

Among mucins secreted from the Goblet cells, a mass production of mucin 2 (Muc2) is necessary to protect the host colon by the establishment of a physiological

barrier of colonic epithelium against pathogenic invasion [158]. In mucin2-deficient mice, these mice are predisposed to colitis and subsequently inflammation-induced colorectal cancers [159-160].

As mentioned before, besides mucins, Goblet cells secrete many AMPs, such as resistin-like molecule-β (RELMβ) and trefoil factor3 (TFF3). These AMPs protect the host from the pathogenic infection and maintain a physical barrier of the intestinal epithelium [161-164]. The function of TFF3s is to provide the cross-linked structure of mucin that enforces the mucus integrity and to stimulate epithelial reconstruction as well as the recruitment of IECs along with the counteraction to apoptosis [161-162,165]. On the other hand, RELMβs induce the secretion of Muc2, mediate the immune responses from macrophage as well as adaptive T cell responses during inflammation, and directly inhibit parasite chemotaxis [164-164].

In the small intestine and colon, other AMPs are produced by other IECs besides Goblet cells. For instance, regenerating -islet-derived protein IIIγ (REGIIIγ) secreted by enterocytes and Paneth cells have a function to shape the intestinal barrier function throughout the small intestine and colon so that the host intestinal epithelial layer are physically separated from the commensal microbiota [156-157,166-168]. Small AMPs secreted by Paneth cells from the crypts of the small intestinal such as defensins, cathelicidins, and lysozymes [156-157,166-168] aim for the bacterial cell wall. These AMPs are essential to shaping the composition of gut microbiota, and promoting mucosal innate immunity [156-157,166-168]. With the aid of AMPs secreted by host IECs, a complex antimicrobial network is formed on the surface of the gastrointestinal epithelium

to protect the host from pathogenic invasion and to prevent an inflammatory response activated by commensal species within the GI tract.

Another function of IECs is the transport of secretory IgAs from the epithelium to the host lumen [169]. In the lamina propria, plasma cells secrete dimeric IgAs, which subsequently bind to the polymeric-immunoglobulin-receptor (pIgR) at the basolateral membranes of the intestinal epithelium [169]. In here, the transcytosis of the whole complex of IgA with pIgR across the epithelium happens [169]. The secretion of IgAs across the epithelial cell barrier is necessary to protect the host from pathogenic infection by binding to the bacteria surface and to prevent bacteria from having a direct interaction with the host epithelium [173]. Regulation of IgAs is dependent on the programmed-cell-death-protein 1 (PD-1); these PD-1s have a role in the selection of IgA-specific plasma cells. In mice that have a PD-1 deficiency, these mice appear to have an aberrant overproduction of follicular T-helper cells (TFH) with an altered phenotype [174]. This overproduction of altered TFH affects the IgA selection process [174]. As a result, impaired PD-1 leads to dysbiosis in the GI tract due to the altered microbial compositions [174]. Together, the secretion of PD-1 regulated IgAs in the GI tract is important because it maintains the intact mucosal intestinal barrier, keeps track of the commensal population, and lastly regulates intestinal homeostasis.

Finally, microfold cells (M cells), a type of IECs, are necessary for immune-surveillance posts in the intestinal epithelium. These IECs are mostly found in Peyer's Patches, and their function is to facilitate the access of antigens or live bacteria in the lumen, and subsequently transport them to macrophages and other lymphocytes to initiate

the immune responses [175].

Overall, the role of IECs is important in the host GI tract because they provide a gastrointestinal barrier to separate the host lamina propria from the intestinal lumen, maintain the microbial populations within the gut via the secretion of AMPs and IgA, and lastly regulate the host immune responses.

B. Commensal bacteria shape mucosal barrier structure. Besides host immunity, commensal bacteria shape mucosal barrier structure. For example, commensal *Bifidobacteria* spp. secrete short-chain fatty acids (SCFAs) to rescue host mucosal epithelium from apoptosis caused by pathogenic infection or idiopathic inflammation. These *Bifidobacteria* spp. also suppress the activation of the NF-κB pathway along with the secretion of pro-inflammatory cytokines by IECs [176,177] (Fig. 1A). For further protection from pathogenic infection, microbiotas also induce the production of AMPs secreted by Paneth cells that require the action of TLR signaling to target on the pathogens [178-180] (Fig. 1E). Additionally, commensal bacteria are involved in the process of fucosylation of IECs in which the surface of epithelial cells is modified by the addition of fucose sugar group. This modification helps the host to prevent the pathogenic infection, such as *Salmonella typhimurium* [181-182] (Fig. 1C).

As mentioned before, the secretory IgA dimers (SIgA) produced by mucosal plasma cells are released into the lumen [169]. Here, SIgAs subsequently bind to the luminal microbes, neutralize specific mucosal pathogens, and limit the direct contacts of microbes on the epithelial surface. SIgAs also regulate intestinal homeostasis by extenuating the overall inflammatory responses in the mucosa and maintaining the

commensal population within particular intestinal regions [183-185]. In return, commensal bacteria take part in the process of IgA selection as well as IgA expression [186-188]. For instance, live commensal bacteria can be found in the intestinal dendritic cells (DCs) for a couple days, and these bacteria are involved in IgA class switching and expression [188]. As a result, the secretory IgAs protect the intestinal epithelium from the microbial invasion [188] (Fig. 1B).

Additionally, commensal bacteria have been shown to be involved in the generation of new IECs. For example, germ-free mice (GF) exhibit a slower turnover rate of IECs compared to the conventional mice [190-194]. Pathogenic infection or uncontrolled inflammatory response of the host causes intestinal injury in the host. In this case, the commensal bacteria exert their effect on TLR signaling for the proliferation of new IECs, and meanwhile, restore gastrointestinal homeostasis [189].

As mentioned before, each epithelial cell of the intestinal epithelium is bound by the tight junctions. These TJs require the action of the commensal bacteria to maintain their overall integrity. For instance, commensal bacteria induce the up-regulation of the tight junction proteins ZO-1 in a TLR-2-dependent manner [88,189]. Furthermore, when GF mice are colonized with commensal *B. thetaiotaomicron*, the intestinal tight junctions become up-regulated. This up-regulation of TJs is due to the effect of commensals on many intestinal mucosal genes including TJ proteins to promote the expression of tight junctions [88,189, 195] (Fig. 1D).

Lastly, commensal bacteria also contribute to mucus production. For example, secreted products of commensal bacteria such as lipopolysaccharide (LPSs) and

peptidoglycans (PGNs) can promote mucus production in GF mice upon oral administration [196] (Fig. 1F). Interestingly, commensal bacteria can further reduce IBD susceptibility. For example, bacterial products such as LPS (TLR-4 ligands) and CpGs (TLR-9 ligands) can stimulate the intestinal epithelial layer to mass producte mucus to rescue mice from the ulcerative colitis [189,197-198].

Overall, microbiota contribute many aspects of the intestinal structure and function. Specifically, commensal bacteria take part in the process of maintenance and shaping of the host mucosal barrier structure as well as host intestinal homeostasis through the up-regulation to the tight junctions, the induction of mucus production and the secretion of IgAs. Lastly, commensal bacteria play a role in the regeneration and proliferation of IECs and rescue IECs from the apoptosis caused by pathogenic infection.

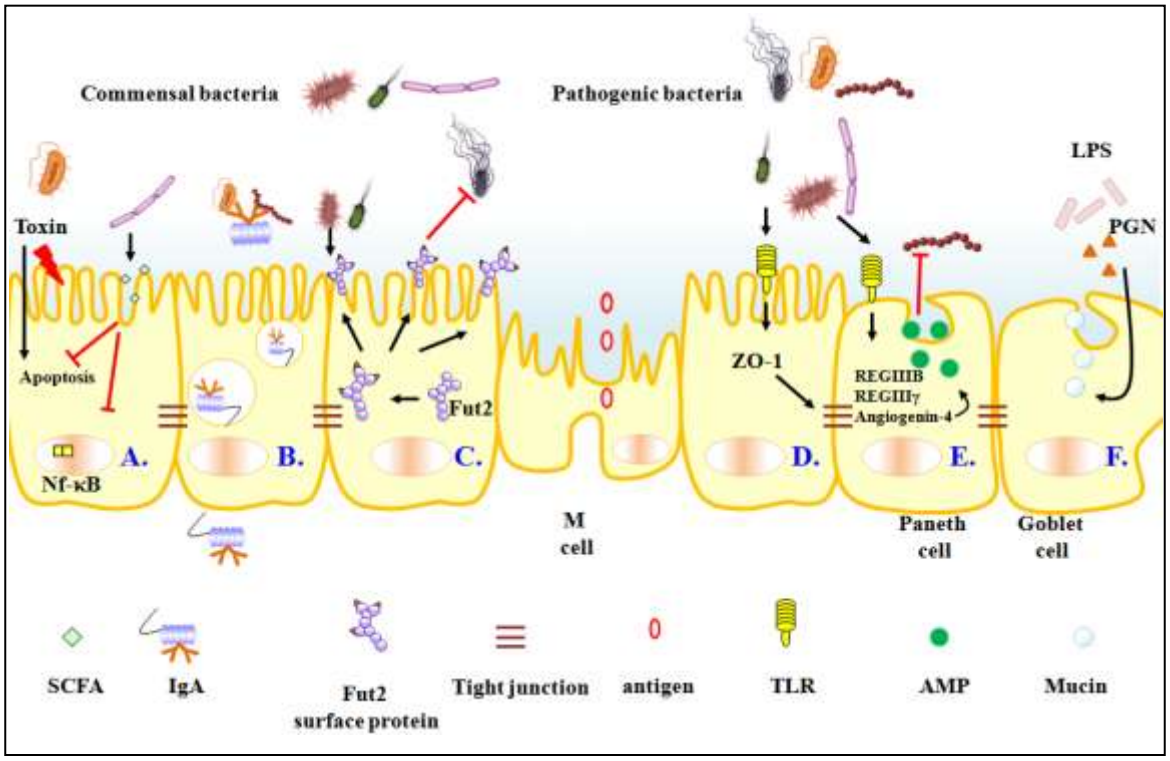

Figure 1: The role of microbiota on Mucosal Barrier Structure. Microbiota induced epithelial barrier mechanisms: (A) Microbiota secrete SCFAs and suppress the activated NF-κB signaling to rescue host mucosal epithelium from apoptosis caused by pathogenic infection of *E. coli* (B) The secretions of microbiota- induced dimeric IgAs released from the basolateral membranes of the intestinal epithelium and transcytosed into host lumen. In host lumen, IgAs subsequently bind to the luminal microbes, neutralize specific mucosal pathogens, and limit the direct contacts of microbes on the epithelial surface. (C) Microbiota induces the fucosylation on the surface of epithelial cells by the addition of fucose sugar group Fut2 to prevent the pathogenic infection such as *Salmonella typhimurium*. (D) Microbiota induces the up-regulation of the tight junction proteins ZO-1 in a TLR-restricted manner to shape the epithelium. (E) Microbiota induces the production of AMPs secreted by Paneth cells in a TLR-dependent manner to target the harmful pathogens. (F) Lipopolysaccharide (LPS) and peptidoglycan (PGN) secreted by microbiota promote mucus production from Goblet cells. Credit: An Nguyen

1.3 Commensal bacteria and food allergy.

Food allergies are a major problem in humans. This happens when the host immunity becomes over-reactive toward oral food allergens and considers food allergens as a threat. For example, consuming peanut, wheat, soy or egg may elicit such a severe, sometimes fatal allergic reaction. The overreaction of host immunity to food allergens is

due to the sensitization of the immunity to a particular food allergen. In this case, the host T cells recognize the food allergen and become activated. Upon activation, these T cells activate allergen-specific B cells that subsequently differentiate into allergen-specific plasma cells and secrete IgEs, an antibody that majorly participates in allergic reactions [119-121]. These allergen-specific IgEs later attach themselves to the surface of mast cells or basophils and prime the individual host immune system to react to the offending allergen. Upon subsequent exposure to certain allergenic food, the allergens cross-link with IgEs on the surface of the mast cells or basophils [128-130] and cause these cells to release various mediators such as histamines that mediate the allergic reaction in the bloodstream and tissues [122-127]. It is possible that gastric acid or enzymatic digestion fails to break down the potential of food allergy to bind IgEs [131]. Another possibility is that the food allergy may bypass the gastric acid or digestive enzymes by entering through intra-ileal route [131]. For example, experiments on mice have shown that mice fed with encapsulated food allergens can have the gastric acid or digestive enzymes bypassed [132]. This bypass causes the disturbance of oral tolerance and provokes an inflammatory IgE reaction [132]. Certain foods like eggs or peanut can resist degradation by gastric acid or digestive enzymes [133]. Lastly, an increased risk of allergic sensitization is associated with defects in gut barrier function [134].

Since there is an abundance of bacteria colonies in the GI tract, it is possible that microbiota may regulate the food allergen sensitization. Previous studies have suggested that certain diet or hygienic practices at an early age may reduce the exposure to infectious microbes that possibly disrupt the normal commensal microbiota [135].

Altered microbiota compositions are associated with the overuse of certain antibiotics, a change in formula-feeding, overconsumption of high-fat diet, and lastly a shift from vaginal birth toward to Caesarean births [136-143,275]. Antibiotic use during infancy is primarily concerned with the correlation of immune-allergies and the disruption of the homeostatic balance of bacteria [137]. Experiments on neonatal mice demonstrate that antibiotic exposure can alter the commensal microbiota and enhance food allergen sensitization [144]. Apparently, antibiotic-treated mice or even germ-free mice display a much higher elevated food allergen-specific IgE compared to the conventional mice [144] due to a disturbance of commensal bacteria [144]. However, the resolution of IgE-mediated- food allergy was significantly associated with the reestablishment of gastrointestinal commensal species by transferring feces from conventional mice into antibiotic-treated mice. For instance, when symbiont *Clostridia* bacteria were re-introduced to antibiotic-treated mice or germ-free mice, these mice were protected from food allergic reaction [144]. The underlying mechanisms that directly result in the suppression of allergic response are due to the induction of FoxP3+Tregs and IgA in the colonic lamina propria that have suppressive functions and promote oral tolerance in the host [144]. The presence of FoxP3+Tregs induced by *Clostridia* can efficiently suppress the allergen-specific Th2 responses in the small intestines [145]. Meanwhile, these Tregs induced by *Clostridia* also enrich the gut environment within the colon with their cytokine production of TGF- β, IL-10, and IL-35 to exert their anti-inflammatory properties in the host gastrointestinal tract [146]. Besides Tregs and IgA production, commensal bacteria also induce the production of IL-22 cytokines from hematopoietic

cells such as CD4+ T cells and RORγt+ innate lymphoid cells (ILCs) to enhance the host intestinal barrier and decrease the intestinal epithelial permeability of food allergen [144]. Specifically, the production of IL-22 promotes intestinal epithelial proliferation [151-152] and induces mucus secretion from Goblet cells [153]. Furthermore, these cytokines mediate the innate immune response through their interactions with IL-22 receptors on the intestinal epithelium [147-148]. As a result of the interaction between IL-22s and their receptors on intestinal epithelial cells (IECs), the secretion of antimicrobial molecules such as RegIIIb, RegIIIc, S100A8s, and S100A9s from IECs are essential to protect the host from pathogenic infection and keep microbiota in check [149-150]. After all, commensal bacteria and host immunity toward food allergen sensitization have a tight connection to each other. While commensal bacteria promote oral tolerance in the host by the induction of FoxP3+Tregs and IgA and keep the intestinal barrier intact as well as decrease the intestinal epithelial cell permeability of food allergen by the induction of IL-22 cytokines, the host immunity relies on IL-22 to induce antimicrobial molecules to prevent pathogenic infection and shape the microbiota composition.

Overall, the presence of food allergy is due to defects in the intestinal barrier or food allergens that can bypass the gastric acid or digestive enzyme, resulting in the allergen-specific IgE immune responses to certain foods. Another factor that may participate in food allergy is disturbance of the normal microbiota. The role of commensal microbiota to protect the host from food allergy is imperative because they can promote oral tolerance in the host by the induction of FoxP3+Tregs and IgA and induce the production of IL-22 cytokines to maintain the gastrointestinal barrier intact

and inhibit the permeability to the food allergen.

1.4 Commensal bacteria together and their metabolites on host immune system.

During normal homeostasis, the commensal bacteria are present in the intestinal lumen, and they do not directly interact with the intestinal epithelium due to the thick layer of mucus secreted from IECs, meaning that the commensals do not directly interact with resident intestinal macrophages. The absence of direct interaction of commensals on immune cells poses a question: how can commensal bacteria be able to regulate the host immune response without inducing an inflammatory response from macrophages? Previous studies have focused only on the metabolites secreted by commensal bacteria.

A. Overview of the roles of commensal bacteria with immune cells. In the GI tract, it is unknown how commensal microbiota can directly exert their effects on immune cells. However, many immune cells such as neutrophils, macrophages, innate lymphoid cells (ILCs), B cells as well as T cells require the presence of commensal bacteria for their activation. Specifically, the commensal bacteria can stimulate PRRs on the IECs to induce thymic stromal lymphopoietins (TSLPs) secretion along with retinoic acids from dendritic cells (DCs) [199]. TSLPs, in return, stimulate the production of B-cell-activating factors (BAFFs) along with proliferation-inducing ligands (APRILs) [200-201]. Besides TSLPs, retinoic acids secreted by DCs upon the stimulation of commensal bacteria are capable of inducing IgA selection during class-switch recombination of the plasma cells [202]. Additionally, these retinoic acids which are induced by commensal bacteria act as an important factor for Treg differentiation in which these Tregs can subsequently suppress theTh17cellular activation [203].

Commensal bacteria participate in the differentiation process of Tregs and maintain the immune-tolerance in the gastrointestinal mucosa. Notably, commensal bacteria promote transforming growth factor-beta (TGF-β) secreted by the intestinal epithelium. Interestingly, TGF- βs are capable of driving the successful differentiation of Tregs from naïve CD4+T cells [204-205]. Additionally, commensal bacteria induce IL-10 secretion from DCs and macrophages after receiving the intestinal antigens from the Goblet cells [206-208]. These IL-10s can aid further the process of Tregs differentiation [209]. For instance, commensal *B. fragilis* can prevent such an uncontrolled intestinal inflammation by directing the development of Tregs and suppressing the activation of pro-inflammatory Th17 cells which are induced by pathogens [210-211, 213]. In a similar manner, oral administration of symbiotic bacteria such as symbiotic *Clostridia* can induce the development of Tregs, and rescue mice from the ulcerative colitis and other allergic inflammatory diseases [145]. These reflect the ability of commensals to promote immuno-tolerance in the GI tract and suppress the host inflammatory responses.

Finally, the commensal bacteria can maintain intestinal homeostasis. For instance, they can induce IL-25secretions from endothelial cells. These IL-25 cytokines exert their effects on a subset of DCs to suppress IL-22 production by RORγt+ innate lymphoid cell group3 (ILC3) [214-215]. This suppression of IL-22 can inhibit the secretion of AMPs that target the microbial communities, thereby, maintaining the intestinal homeostasis as well as the commensal population.

B. Gut metabolites shape the intestinal immune system.

a. SCFAs. Recent studies have discovered that commensal bacteria can communicate

with the host via the secretion of the microbial products to regulate the host immune response. Previous studies have shown that microbiota metabolites contribute a significant role in host physiological homeostasis development, metabolism, and immunity. The microbial metabolites exert their effect on the host signaling pathways to regulate host immunity. For instance, commensal bacteria secrete many different bile acids and short -chain fatty acids (SCFAs) [8-9].

SCFAs are known to have many beneficial effects on many host metabolic pathways as well as abilities to suppress the pro-inflammatory responses from various immune cells [10-16]. Acetate, butyrate, and propionate are the major SCFAs that are present in the GI tract [216]. During many host metabolic pathways, these SCFAs are used as a source of energy, and they are taken up by organs and used as substrates or signal molecules to regulate lipid, glucose, and cholesterol metabolism in various tissues after they are delivered into the bloodstream [217-221]. As mentioned before, SCFAs can suppress many host pro-inflammatory responses to prevent an uncontrolled inflammation. For instance, oral administration of acetate via drinking can rescue mice from the development of the chemically DSS -induced inflammatory colitis [16]. The resolution of a chemically-induced-inflammatory colitis is due to the interaction of SCFAs with G-protein coupled receptors 43 (GPR43s) that profoundly suppress such an immune inflammatory response [16]. In the same colitis experiment, mice that lack GPR43 appear to have an exacerbated inflammation [16]. Additionally, SCFAs can induce the differentiation of FoxP3+Tregs from naïve CD4+T cells upon the administration of SCFAs into either GF mice or specific-pathogen mice (SPF) [9,222-223]. Here, the

presence of Treg cells protects the host from such an inflammatory response [9,222-223].

Among SCFAs, butyrates secreted by probiotics can efficiently convert naïve CD4+T cells into Tregs cells *in vitro* [9,223]. In the presence of butyrates, these SCFAs exert their effects on gene expressions of CD4+T cells and facilitate the FoxP3+ expression [224]. Additionally, butyrates can directly induce the production of IL-10 from Tregs cells [225] in which IL-10 cytokines have an anti-inflammatory effect. The hallmark of the inhibitory effect of butyrates is through the induction of TGF-β secreted by epithelial cells that can suppress the generation of the cancerous epithelium [212]. Altered microbial composition affects the level of butyrates in the GI tract and exposes the host to many inflammatory diseases. For example, IBD patients appear to have a lesser number of butyrate-producing bacteria comparing to healthy individuals [226].

Unlike butyrates, other SCFAs, such as acetate and propionate, participate in the process of Treg recruitment rather than Treg differentiation. Mice that are fed with propionate and acetate appear to have such an induction of gut-homing molecules on Tregs and subsequently the accumulation of Tregs in the GI tract system [223]. Additionally, acetate can enhance the gastrointestinal epithelial barrier and suppress the host inflammation upon pathogenic infection [176].

In conclusion, the role of SCFAs to protect the host from such an inflammatory response is due to their abilities to promote and induce FoxP3+Treg differentiation and recruitment. Furthermore, they are responsible for the production of IL-10 cytokines to inhibit inflammation, the secretion of TGF-β to suppress such an uncontrolled proliferation of cancerous epithelium, and lastly, the reinforcement of the gastrointestinal

barrier during pathogenic infection.

b. ESPs. Besides SCFAs, exopolysaccharide (EPS) secreted from commensal bacteria have been brought to attention due to their abilities to suppress the inflammatory response. The structure of EPS consists of carbohydrate polymers that form a layer on the extracellular surfaces of many different microorganisms. EPS have been known for their anti-inflammatory function. Specifically, EPS secreted by the human microbiota *B. fragilis* (also called polysaccharide A (PSA)) can suppress the pro-inflammatory responses of IL-17 A by directly inducing IL-10 producing-Tregs and limit the resistance of the pathogenic infection via TLR-2 signaling pathway [91-94,227-232]. Additionally, in GF mice, administration of EPS results in the expansion of the T-cell population and resolution of the aberrant TH1/TH2 imbalances [230].

EPS secreted by *B. fragilis* also can induce a unique subset of M2 macrophages that can promote the secretion of inhibitory cytokine IL-10 that in turn suppresses the inflammatory responses via the TLR-2 signaling pathway and protects the host from the chemically-induced colitis [233].

Sphingolipids, a particular type of EPS secreted by *B. fragilis*, can rescue mice from the chemically-induced colitis. The development of colitis is attributed to the accumulation of the invariant natural killer T cells (iNKTs) [237]. In the presence of sphingolipids, the number of iNKT cells is reduced. As a result, the host is protected from such an inflammatory-mediated colitis.

Interestingly, despite the fact that EPS secreted by commensal bacteria can elicit such an anti-inflammatory response to against the action of host immunity, EPS secreted

by non-commensal species may result in the activation of host immunity. For example, EPS secreted by *B. breve* can inhibit the antibody production [234]. In contrast, EPS secreted by *Lactobacillus kefiranofacien* can promote the proliferation of plasma blasts and induce the activation of the pro-inflammatory subset of macrophages [235-236]. It is believed that the different effects of EPS secreted by various microbial species are due to the nature of that microbial species. For a species that participate in the host gastrointestinal microbiota, it is possible that *B. brevis* has developed several microbial products to restrict host immune response such as antibody secretion so that these commensal bacteria can be able to resist for their colonization in the host gastrointestinal lumen. On the contrary, *L. kefiranofaciens* are just transient microbial species since they only briefly pass the GI tract without continual colonization. As a result, the host immunity mistakenly considers *L. kefiranofaciens* as a threat and provokes such an inflammatory response to target on these microbes.

Collectively, EPS secreted by commensal bacteria are capable of suppressing the immune responses of the host and maintaining the homeostasis of the host GI tract system.

c. Vitamins and amino acids. Microbiota are also responsible for the production of vitamins as well as amino acids. The presence of vitamins together with amino acids is essential for host lipid metabolic pathways as well as host immune systems. For instance, Vitamin B-based metabolites (such as riboflavin) secreted by *Bifidobacterium* spp. can activate the MHC-related protein 1 (MR1)-dependent mucosal associated T cells (MAITs) [238,239]. Upon activation, MAIT cells then elicit pro-

inflammatory cytokines such as TNF- α and IFN-γ [239]. It is believed that such an interaction between vitamin-B metabolites secreted by commensals and MAIT cells helps to reinforce the host immune surveillance across the epithelium and modulate the host immune response within the GI mucosal region.

d. Indoles. The secretion of microbial-indoles during quorum sensing process of the commensal bacteria contributes to the reinforcement of the gastrointestinal barrier. For instance, indole-production induces the up-regulation of the epithelial tight junctions [240,241]. Thereby, the secretion of indoles by commensal species helps to shape the intestinal mucosal barrier.

C. Altered level of microbial metabolic profile leads to host inflammation and other inflammatory diseases. The cross-talk between host immunity and commensal bacteria is attributed to the action of microbial metabolites/products that control and regulate the host immune system and homeostasis. Disturbances of the microbial metabolites that are associated with the overuse of certain antibiotic drugs, change in diet, altered microbiota composition, or pathogenic infection predisposes the host to many inflammatory immune diseases and metabolic disorders, such as IBDs, diabetes, obesity, ulcerative colitis and behavioral disorders. For example, mice fed with high fat diets appear to have altered microbiota communities such as an outgrowth of deoxycholic acid–producing bacteria. Subsequently, deoxychloic acid causes altered phenotypes of hepatic stellate cells (HSC), which then triggers the production of pro-inflammatory cytokines and ultimately leads to cancerous liver tumors [243]. Other studies involve the injection of polyinosinic-polycytidylic acid in mice that leads to the production of 4-ethylphenylsulfate (4EPS) and

indole-pyruvate [244]. These molecules are associated with autism spectrum disorder (ASD)-like behavior in the mouse model [244].

Overall, it is important to understanding how microbial metabolites impact the host immune system. Especially, it may provide useful insights for the improvement of potential therapeutics against individual host immune diseases as well as other metabolic disorders.

II. INTESTINAL MACROPHAGES

The GI tract encounters more foreign substances than any other part of the body, and, therefore, it harbors the largest compartment of the immune system. Intestinal immune cells include a major reservoir of tissue macrophages that participate in the first line defense of the GI immune system. Particularly, intestinal macrophages can be found in the lamina propria region, just beneath the intestinal epithelium. Here, the intestinal macrophages play a central role in initiating and sustaining protective immune responses mounted against a vast number of potentially harmful bacteria and antigenic stimuli present in the intestinal lumen. Meanwhile, the intestinal macrophages must ensure the continuation of immune tolerance toward innocuous antigens such as dietary proteins or commensal bacteria. Thereby, it is important for intestinal macrophages to be able to distinguish non-harmful antigens from others that are considered as a threat to the host. In particular cases where the host is disturbed by pathogenic infection or altered microbiota composition, such tolerance of intestinal macrophages may breakdown, and it may result in the development of immune allergies and other host inflammatory diseases. Consequently, the intestinal macrophages must maintain the harmonious balance between

their abilities to elicit such an immune response toward antigenic stimulation and their tolerance toward the non-harmful species or antigens. The function of intestinal macrophages depends on the conditional state of the GI tract: steady state versus intestinal inflammation. Upon the conditional states of the GI tract, the characteristics of intestinal macrophages display differently and will be further discussed here below:

A. The features of intestinal macrophages during the steady state of GI tract.

During steady state, intestinal macrophages play a central role in performing housekeeping functions. Favorably located beneath the intestinal epithelium, the intestinal macrophages function to capture and phagocytose any foreign substances that breach the epithelial barrier [250]. This bactericidal activity allows intestinal macrophages to protect the host from pathogenic infection or antigenic stimulation that may be considered as a threat to host. Furthermore, intestinal macrophages are responsible for the production of pro-IL-1β in the presence of commensal bacteria [117]. The production of pro-IL-1β is beneficial to the host for clearance of enteric pathogens in the NLRC4 inflammasome –restricted manner. On the other hand, intestinal macrophages are also responsible for the clearance of apoptotic or senescent cells and promote tissue remodeling [245-248]. Additionally, the intestinal macrophages secrete various cytokines and several soluble factors that are necessary for tissue-remodelling. For instance, the production of PGE2s secreted by intestinal macrophages can induce generation of the epithelial precursor cells to maintain the gastrointestinal epithelial barrier [249]. Thus, it is believed that intestinal macrophages play a key in host immune recognition to distinguish the commensal bacteria or dietary antigens from the pathogenic bacteria or

harmful substances.

Interestingly, unlike other tissue macrophages, the intestinal macrophages are inflammation-anergic. In this case, the intestinal macrophages must maintain tolerance toward to non-harmful dietary antigens or commensal bacteria. Immune tolerance of the intestinal macrophages is attributed to the low expression of toll-like receptors (TLRs) in macrophages as well as the failure of the TLR downstream signaling to elicit such an immune response [37,250,268]. As a result, intestinal macrophages fail to trigger pro-inflammatory cytokines to elicit host inflammation during the steady state [250-252]. Furthermore, intestinal macrophages also do not release any reactive oxygen species nor generate nitric oxide upon ingestion of live bacteria [253]. Like other tissue macrophages, intestinal macrophages possess a high expression of MHC class II [246,256-258]; however, they do not migrate to meet naïve CD4+ T cells for CD4+T activation in the resting condition [259-260]. Interestingly, these intestinal macrophages are responsible for the differentiation of FoxP3+Treg cells and the maintenance for the survival of Treg cells by constitutively secreting anti-inflammatory IL-10s [254]. The production of the intestinal macrophages-derived IL-10s is necessary to promote the secondary-expansion and the survival of Tregs [73]. As mentioned before, the Treg population has an essential function to suppress inflammatory immune responses and promote oral tolerance in the host.

Intestinal macrophages also contribute to the maintenance of host immune homeostasis and intestinal epithelial barrier. Intestinal macrophages secrete a small number of pro-inflammatory TNF- α cytokines that have a wider effect on the host

immunity [35, 36, 254]. During the steady state, TNF- α can modulate the proliferation of enterocytes and maintain the intestinal epithelial permeability by stimulating the activity of the matrix metalloproteinases and several tissue-remodeling enzymes [254,255].

Intestinal macrophages also take part in the regulation of other immune cells in the gastrointestinal immune system. Specifically, the secretion of microbiota-induced IL-1β by intestinal macrophages is responsible for the proliferation of Th17 cells [264]. Additionally, there is an intrinsic cross-talk between macrophages and innate lymphoid cell group 3 (ILC3). In this case, microbiota-regulated macrophages stimulate the secretion of granulocyte-macrophage-colony-stimulating factors (GM-CSFs) secreted by ILC3. GM-CSFs subsequently provide feedback to dendritic cells (DCs) and macrophages to produce other inhibitory factors such as IL-10 and retinoic acid [265] which is necessary for Treg differentiation and expansion.

Intestinal macrophages also participate in the sampling process of the luminal contents by extending trans-epithelial dendrites (TED) toward the lumen to capture any antigenic stimuli or live bacteria [266]. Subsequently, these samples are then transported to the neighboring DCs for the T-cell priming process [266-267], suggesting a mechanism where by the host can induce oral tolerance and suppress allergic/ inflammatory reactions by exerting the sampling function of TED on macrophages.

B. Immuno-anergy of intestinal macrophages during the steady state.

The intestinal macrophages display an immune-anergy toward the commensal bacteria or dietary antigens. This hyporesponsiveness is a distinctive feature of intestinal macrophages and is attributed to the down-regulation of toll-like receptors (TLRs) on

macrophages together with an unsuccessful TLR downstream signaling pathway to trigger host immune responses [37,250,268]. Consequently, during the steady state of the intestinal macrophages, the host fails to elicit pro-inflammatory cytokines to provoke such an inflammatory reaction in the GI tract system [250-252].

Additionally, the continuous secretion of IL-10 cytokines is also responsible for the immune-anergy of the intestinal macrophages [254]. These IL-10 cytokines, by their action, induce the naïve CD4+T cells for FoxP3+Treg differentiation and support the survival of Tregs [73] in which the presence of Tregs are necessary to suppress the pro-inflammatory responses in the host and induce oral tolerance. Together with Tregs, IL-10 cytokines exert their effects on other effector cells to suppress the host immune responses. Interestingly, the secretion of IL-10 is dependent on the modulation of the commensal bacteria. For instance, in the absence of microbiota in GF mice, macrophages fail to secrete IL-10s [261]. Interestingly, IL-10R deficient mice are predisposed to the chronic GI inflammatory diseases [269-270]. Lastly, the secretion of TGF-β by Treg cells also involves the hyporesponsiveness of the intestinal macrophages to a variety TLR ligands [37].

In conclusion, several factors are accountable for the anergic responses of the intestinal macrophages. Notably, these factors involve the small presence of TLRs on macrophages as well as the unsuccessful TLR downstream signaling, and the mass production of IL-10 from macrophages that leads to the differentiation of Tregs to secrete other inhibitory factors such as TGF-β to suppress the host immune responses. These findings suggest a possible link between commensal bacteria and immuno-anergy of host

intestinal macrophages. However, the impact of microbiota on intestinal macrophages remains unclear, and the mechanisms of how microbiota exert on intestinal macrophages needs to be further studied (Fig. 2).

C. Functions of macrophages in the intestinal inflammation.

In the presence of external factors that may disturb the host intestinal homeostasis, such as the overuse of the antibiotic drugs, change in diet, or pathogenic invasion, the intestinal macrophages elicit a series of inflammatory responses that cause inflammation in the host GI tract system. For instance, intestinal macrophages up-regulate co-stimulatory molecules and secrete a mass number of pro-inflammatory cytokines which involve the participation of IL-1β, TNF- α, IL-6 cytokines, reactive oxygen intermediaries, and nitric oxide to promote a severe inflammation in the gut [271-272, 268]. These pro-inflammatory cytokines induce the secretion of chemokine attractants to facilitate the recruitment of other effector innate immune cells including Th1 cells, Th17 cells, and eosinophil cells at the site of inflammation. Notably, the inflamed intestinal macrophages still maintain the secretion of anti-inflammatory IL-10 cytokines to promote tissue repair in the host GI tract [251, 268,272-273]. Perhaps, it is an important feature of intestinal macrophages to restore homeostasis after the clearance of infection or inflammatory agents to prevent chronic inflammation. Failure to restore homeostasis leads to chronic inflammatory disease in the GI system (Fig.2).

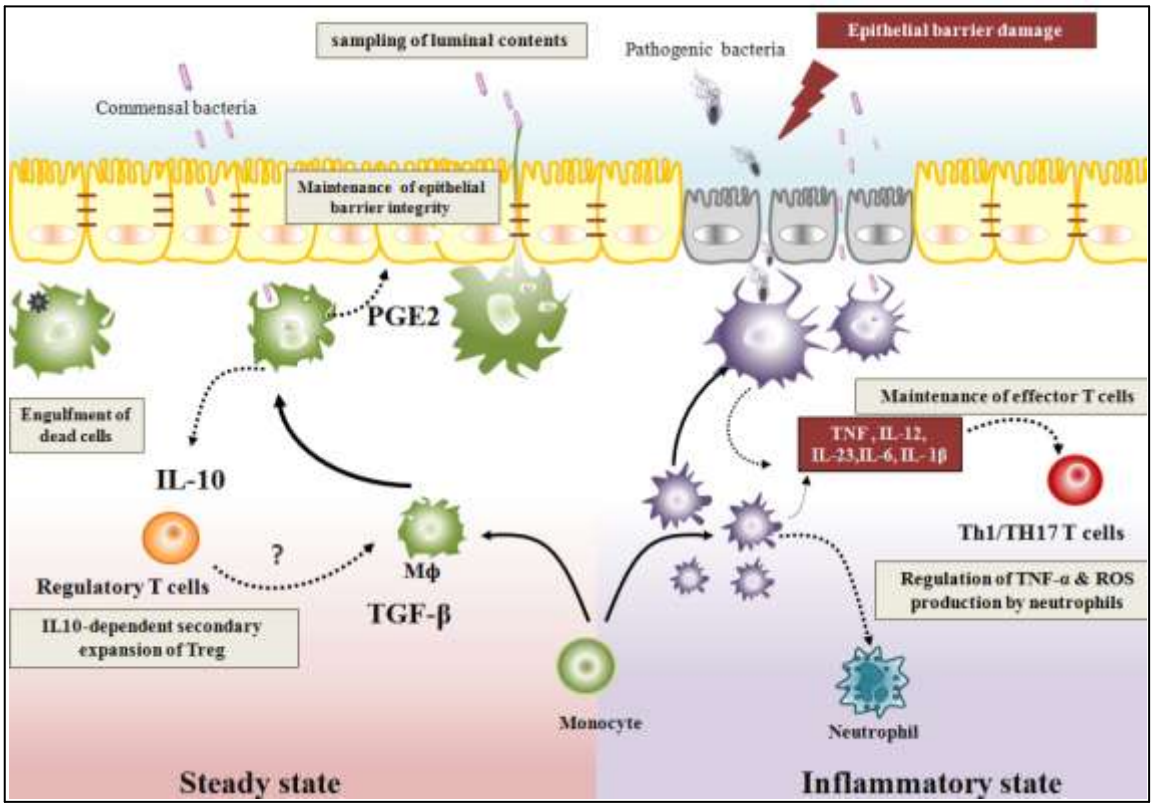

Figure 2: The functions of macrophages during steady state and inflammatory state. During steady state, intestinal macrophages play a role in a) engulfment of apoptotic or senescent cells, b) elimination of invading bacteria, c) maintenance of host immune homeostasis and intestinal epithelial barrier by the secretion of PGE2 to promote tissue-remodeling, d) sampling of luminal contents by extending TEDs toward the lumen to capture any antigenic stimuli or live bacteria, e) secretion of IL-10 constitutively to facilitate the secondary-expansion and the survival of FoxP3+ Tregs. In a presence of such external factors that may disturb the host intestinal homeostasis, the intestinal macrophages elicit a series of inflammatory responses such as the production of pro-inflammatory cytokines and other chemokine attractants to facilitate the recruitment of effector innate immune cells including Th1 cells, Th17 cells, and eosinophil cells at the site of inflammation. Intestinal macrophages also recruit neutrophils to regulate TNF-α and ROS species such as nitric oxide. Credit: An Nguyen

III. THE STATEMENT OF PROBLEM AND HYPOTHESIS

In humans the major populations of commensal bacteria reside in the gastrointestinal system where they exert their influences on host immunity and maintain gastrointestinal homeostasis. Such influences from commensal bacteria contribute in protecting the host from pathogenic invasion and developing food allergies, as well as maintaining the host mucosal structure together with host intestinal homeostasis. Recent studies have discovered that the secreted products/ bacterial metabolites from commensal bacteria provide a crosstalk between host and microbe interactions [8-9, 10-16, 91-94,176,216-241]. This complex crosstalk plays an important role in regulating host immunity, host physiological homeostasis development, and host metabolism. So far, little is known about the mechanisms of how secreted bacterial products/ metabolites take part in regulating host immune systems.

In the gastrointestinal tract, the intestinal macrophages, unlike other regional macrophages, appear to have tolerance towards the commensal bacteria despite their phagocytic and bactericidal activities that allow them to actively participate in host first-line defense against pathogenic invasion and/or antigenic stimuli. Since the intestinal macrophages are located in close proximity to the commensal bacteria, just beneath the epithelial layers, it is possible the presence of commensal bacteria in gut lumen may somehow alter the functions of intestinal macrophages and contribute to the anergic characteristics of the intestinal macrophages. Given commensal bacteria crosstalk with host immunity through their secreted microbial products/ metabolites, even though it is unknown whether these secreted microbial products/ metabolites directly exert their

effects on immune cells, we believe that commensal bacteria communicate with intestinal macrophages through their secreted microbial products/ metabolites. In this study, we sought to understand the involvement of the secreted commensal bacterial factors in response to the anergic function of intestinal macrophages. We hypothesized that the secreted bacteria factors/metabolites present in the bacterial supernatant can suppress macrophage activation upon the induction of TLR or NLRP3 agonists on macrophages.

CHAPTER TWO

EXPERIMENTAL DESIGN AND METHOD

Commensal bacterial culture and supernatant collection.

Four different commensal strains found in human GI tract were gifted from Dr. Schreckenberger at Loyola Medical Center. These four strains including *Lactobacillus delbrueckii (LD), Lactobacillus rhamnosus (LR), Lactobacillus fermentum* (LF) and *Streptococcus oralis (SO)* were cultured separately in tryptic soy (TS) broth, pH: 6.0 – 6.4 at 37˚ C, 5% CO_2 for three weeks until they reached an OD_{600} of 1.0 to have approximately 10^9 cells per mL. The bacteria cultures were centrifuged at 10,000 rpm for 15 minutes, collected and then filtered through a 0.22 µ m filter (Millex) to remove any whole bacterial cells. The bacteria supernatants were stored at -20˚ C for future use.

Mice.

C57BL/6 mice were purchased from Jackson Laboratories and maintained by Dr. Qiao at Loyola University Chicago. $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were a generous gift from Dr. Knight. All mice were bred and cared for according to protocols approved by the Institutional Animal Care and Usage Committee at Loyola University Medical Center, Maywood, IL. C57BL/6, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice (6 weeks old) were utilized for the experiments and sacrificed by CO_2 inhalation.

Bone Marrow Cell isolation and Characterization of Murine bone-marrow derived macrophages with conjugated antibodies.

Bone marrow cells were isolated from mice and grown in culture cell flask in the presence of macrophage colony stimulating-factor (M-CSF), which was secreted by L929 cells and was used in the form of L929-conditioned medium [311]. Phenotypic characterization of BMDMs with conjugated antibodies was acquired by flow cytometry. The 0.25 x 10^6 cell suspensions were stained with Pacific blue Annexin V (Biolegend), PE-conjugated anti-mouse F4/80 Ab (BM8, Biolegend), APC-conjugated anti-mouse CD11b Ab (M1/70,Biolegend), FitC-conjugated anti-mouse CD11c Ab (N418, Biolegend), isotype control anti-mouse CD16/32 Ab (93, Biolegend). Data were acquired by LSRFortessa flow cytometer (BD Biosciences) and analyzed with Flowjo software (Treestar).

Cell Culture.

Different macrophage cell lines were used in this experiment, including murine B6 macrophage cell lineand THP-1 human cell line. Both B6 mouse cell line and THP-1 human cell line were maintained by Dr. Qiao's lab. Primary cells were also used in this study. Murine bone-marrow derived macrophages (BMDM) were first isolated from bone marrows and then treated with L929 conditioned medium to differentiate into macrophages as mentioned before.

For B6 and BMDM, they were grown and cultured in a complete DMEM cell culture media (DMEM+ 10% fetal bovine serum (FBS) + 1% Penicillin-Streptomycin). Both B6 and BMDM cells were placed into a 24 well plate (BD Biosciences) with a concentration of 0.5 x 10^6 and 0.25 x 10^6 cells, respectively.

As for human THP-1 cells, they were maintained in complete RPMI medium 1640 (RPMI + 10% FBS + 1% Penicillin-Streptomycin). For THP-1 cells to differentiate into macrophages, THP-1 cells were stimulated with PMA (100 nM) for three hours at 37˚C, 5% CO_2 until cells adhered to the plates. After that, the adherent cells were collected and plated at 0.25 x 10^6 cells per well in 24-well plates in the absence of FBS, and incubated overnight at 37˚C, 5% CO_2.

Enzyme-treated bacteria supernatant.

The bacterial supernatant (either LD, SO, LR or LF) was treated either with RNase A (5U/mL, Thermo Scientific) or DNase I (5U/mL, Thermo Scientific) or proteinase K (10 ug/mL, Thermo Scientific) for at least 30 minutes at 37˚C followed by heat inactivation at 95˚C for 5 minutes. TS, bacteria culture media exposed either with DNase I, RNAse or proteinase K alone was used as controls. Both enzyme-treated bacterial supernatants (either LD, SO, LR or LF) and TS bacterial culture media were stored at -20˚ C until use.

Heat-treated bacteria supernatant.

The bacterial supernatants (either LD, SO, LR or LF) were incubated at 95^{o}C for 5 minutes. TS heat treated was used as a control. After that, both heat deactivated bacterial supernatants (either LD, SO, LR or LF) and TS were cooled down and stored at at -20˚ C for future use.

Fractionate of bacteria supernatant by ultracentrifugation.

The bacteria supernatants (either LD, SO, LR or LF) were fractionated into above 10KDa (>10KDa) and less than 10KDa (<10KDa) fractionates by Amicon Ultra

centrifugal filters (Millipore) at 5000 rpm for 30 minutes. For the control, TS bacterial culture media was also fractionated into <10KDa and >10KDa fractionates. Both differential fractionates of bacterial supernatants (either LD, SO, LR or LF) and TS bacterial culture media were stored at -20^{o}C until use.

Sugar isolation from bacteria supernatants by Chloroform/Phenol extraction.

The bacteria supernatants (either LD, SO, LR or LF) or TS were first treated with DNase I, RNase A and proteinase K for 30 minutes at 37˚C, followed by heat inactivation at 95˚C for 5 minutes. After that, an equal volume of phenol: chloroform (1:1) was added into the treated-bacterial supernatants (either LD, SO, LR or LF) or TS control. The mixtures were centrifuged at 2,000 rpm for 5 minutes until the organic and aqueous phases were well-separated. The aqueous phases were pipetted to fresh tubes and stored at -20^{o}C until use.

Macrophages treated with bacterial supernatants.

The optimal condition for macrophage survival in the presence of the bacterial supernatants was when the bacterial supernatants (either LD, SO, LR or LF) were incubated with macrophages overnight in a ratio of 1:2 (v/v) at 37˚C, 5% CO_2. The ratio 1:2 (v/v) was used for all *in vitro* experiments except those are involved in the transwell system experiments. Cells exposed to media DMEM, TS bacteria culture media or LPS (50ng/mL) alone were used as controls in the same volume ratio as mentioned above. After overnight incubation, cell supernatants were collected and the pro-inflammatory TNF-α, IL-1β, and IL-6 cytokines were measured by ELISA assay. Cellular surfaces TLR-2 and TLR-4 were characterized by LSRFortessa flow cytometer (BD Biosciences)

using the conjugated antibodies FitC-conjugated anti-TLR-2 Abs and PE-conjugated -anti TLR-4 Abs (eBioscience). The data were analyzed by Flowjo software (Treestar).

Suppression of bacteria supernatants on stimulated macrophages.

A. LPS stimulation. <10KDa fractionates or TS culture media were first incubated overnight with macrophages (either mouse BMDMs or human THP-1 cells) in a ratio of 1:2 (v/v) at 37˚C, 5% CO_2. After overnight incubation, cells were treated with LPS (50ng/mL) at 37˚C, 5% CO_2 for 6 hr. Finally, cell supernatants were collected, and the pro-inflammatory TNF-α and IL-6 cytokines were measured by ELISA (Fig. 3).

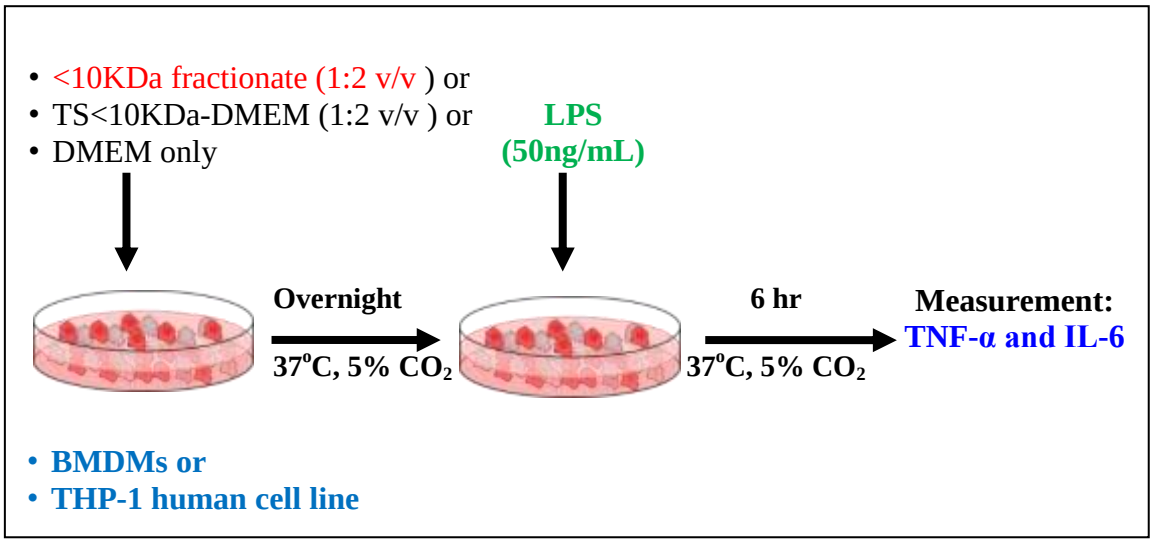

Figure 3: Outline of LPS stimulation on macrophages *in vitro*. The <10KDa fractionates or TS culture media were incubated overnight with macrophages (either mouse BMDM or human THP-1 macrophages) in a ratio of 1:2 (v/v) at 37˚C, 5% CO_2. After overnight incubation, cells were treated with LPS (50ng/mL) at 37˚C, 5% CO_2 for 6 hr. Finally, cell supernatants were collected, and the pro-inflammatory TNF-α and IL-6 cytokines were measured by ELISA. Credit: An Nguyen

B. Mono sodium urate (MSU) crystal stimulation. Macrophages (either mouse BMDMs or human THP-1 cells) were first primed with LPS (50 ng/mL) overnight at 37˚C, 5% CO_2. After that, <10KDa fractionates were incubated with LPS-primed cells in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2 for 6 hr. Cells exposed to TS<10KDa culture

media were used as control in the same volume ratio. After 6 hour incubation, cells treated with MSU crystals (250 µg/well) were again incubated overnight at 37˚C, 5% CO_2. Finally, cell supernatants were collected and the pro-inflammatory IL-1β cytokine was measured by ELISA (Fig. 4).

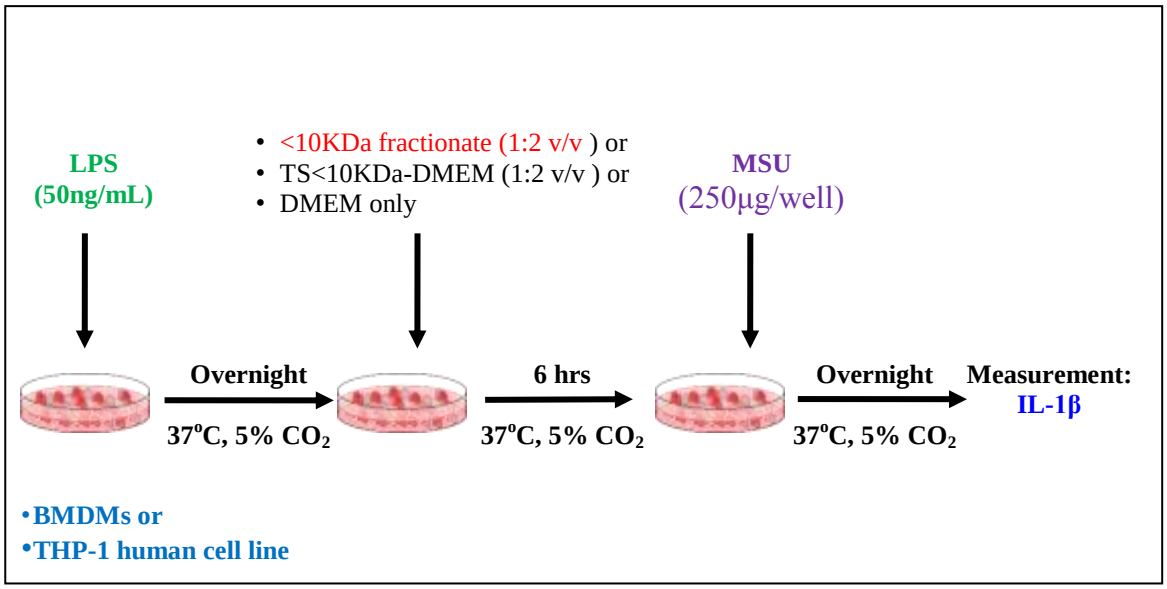

Figure 4: Outline of MSU crystal stimulation on macrophages *in vitro*. Macrophages (either mouse BMDMs or human THP-1 cells) were first primed with LPS (50 ng/mL) overnight at 37˚C, 5% CO_2. After that, <10KDa fractionates were incubated with LPS-primed cells in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2 for 6 hr. Cells exposed to TS<10KDa culture media were used as control in the same volume ratio. After 6 hour incubation, cells treated with MSU crystals (250µg/well) were again incubated overnight at 37˚C, 5% CO_2. Finally, cell supernatants were collected and the pro-inflammatory IL-1β cytokine was measured by ELISA. Credit: An Nguyen

In vivo mouse peritonitis model.

Peritonitis induced by the intraperitoneal injection of LPS stimuli has been described previously [303]. Briefly, 6-week old mice were intraperitoneally injected with <10KDa fractionate (500 µL/mouse) per day for two consecutive days. Mice intraperitoneally injected with TS culture media or PBS vehicle were used as controls. Two days later, all mice were intra-peritoneally injected with LPS (10 µg/mouse) or PBS

vehicle as control. Four hours later after LPS injection, mice were then sacrificed by exposure to CO_2 and the peritoneal cavities were washed with 5 ml cold PBS. The recruited polymorphonuclear neutrophils present in the peritoneal lavage fluid were quantified by flow cytometry using the neutrophil marker Ly6G (BD Bioscience). The samples were acquired on a FACSCantoflow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Treestar) (Fig.5).

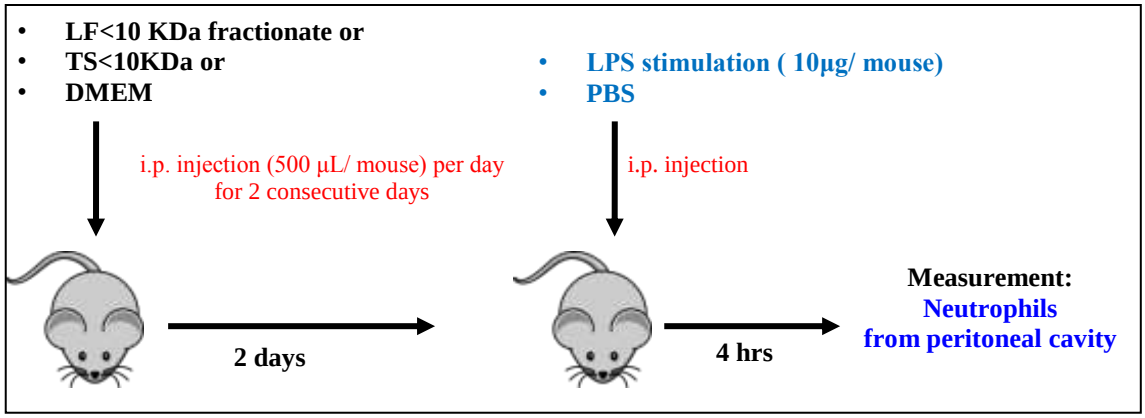

Figure 5: Outline of the i.p injection of mouse peritonitis model *in vivo*. 6-week old mice were intraperitoneally injected with <10KDa fractionate (500 µL/mouse) per day for two consecutive days. Mice intraperitoneally injected with TS culture media or PBS vehicle were used as controls. Two days later, all mice were intra-peritoneally injected with LPS (10 µg/mouse) or PBS vehicle as control. Four hours later after LPS injection, mice were then sacrificed by exposure to CO_2 and the peritoneal cavities were washed with 5 ml cold PBS. The recruited polymorphonuclear neutrophils present in the peritoneal lavage fluid were quantified by flow cytometry using the neutrophil marker Ly6G (BD Bioscience). The samples were acquired on a FACSCantoflow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Treestar). Credit: An Nguyens

ELISA.

Paired (capture and detection) antibodies and standard recombinant proteins for mice and humans of IL-1β, TNF-α and IL-6 (from Biolegend) were used to quantify the cytokine levels in cell culture supernatants according to the manufacturer's instructions.

Trans-well experiments.

Twenty four-transwell system plates with collagen-coated insert membranes

(Corning) were used for this assay. The culture plate inserts consisted of an upper chamber and bottom chamber. The chambers are separated by a microporous transparent Biopore membrane (0.4 μm). In this experiment, human differentiated epithelial colorectal adenocarcinoma CACO-2 cells were required to form an artificial epithelial layer that mimics the gastrointestinal systems.

Briefly, CACO-2 cells were used to differentiate into epithelial cells for the trans-well experiment on the upper chamber while the THP-1 macrophage cells were placed on the bottom chamber (Fig. 6).

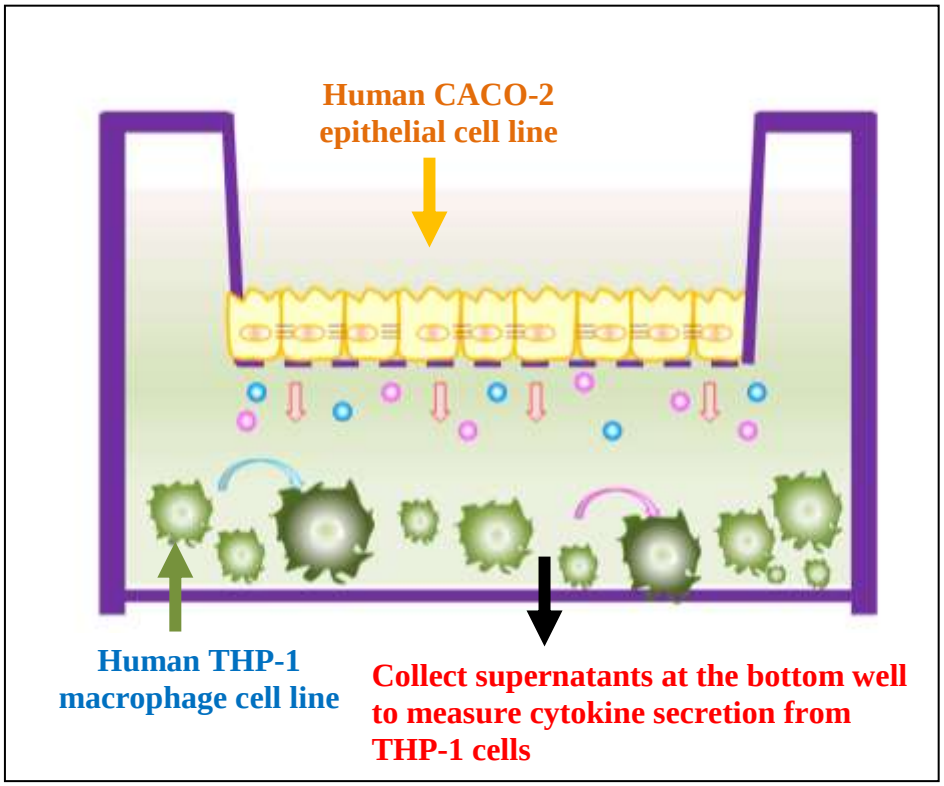

Figure 6: Outline of a transwell system. Twenty four-transwell system plates with collagen-coated insert membranes (Corning) were used for this assay. The culture plate inserts consisted of an upper chamber and bottom chamber. The chambers are separated by a microporous transparent Biopore membrane (0.4 μm). In this experiment, human differentiated epithelial CACO-2 cells were required to form an artificial epithelial layer that mimics the gastrointestinal systems. Briefly, 0.5 x 10^6 human CACO-2 cells were used to differentiate into epithelial cells for the trans-well experiment on the upper chamber while 0.25 x 10^6 human THP-1 macrophage cells were placed on the bottom chamber. Credit: An Nguyen

To form the artificial epithelial layer, 0.5 x 10^6 CACO-2 cells were seeded in the upper chambers in the presence of complete DMEM media (DMEM+ 10% fetal bovine serum(FBS) + 1% Penicillin-Streptomycin). CACO-2 cells were grown at least for 2 weeks until they become confluent. Once the CACO-2 became confluent, THP-1 cells (cultured and maintained as described above) were then placed into the bottom chamber of the transwell system at a density at 0.25 x 10^6 cells per well.

A. LPS stimulation. Because the upper chamber of the transwell system could not hold a large enough volume, in this experiment, the sample test was added into CACO-2 cells on the upper chamber with a volume ratio of 1:1 (v/v), instead of 1:2 (v/v) as we previously used. Specifically, the <10KDa bacterial LF was added into the upper chamber of the transwell system with a volume ratio of 1:1 (v/v). In different wells, LF whole bacterial supernatant (without fractionate) or >LF 10KDa fractionates were also added in to the CACO-2 cells on the upper chamber of trans-well in the same volume ratio of 1:1 (v/v). CACO-2 cells exposed with TS media or DMEM cell culture media were used as controls. Both samples and controls were incubated overnight at 37˚C, 5% CO_2. After overnight incubation, LPS (50ng/mL) was added to the bottom chambers which contained THP-1 cells. For control, LPS was also added into the upper chamber. Finally, the whole trans-well systems were incubated for six hours at 37˚C, 5% CO_2. Cell supernatants at the bottom chamber were collected and the level of TNF-α was measured by ELISA (Fig. 7).

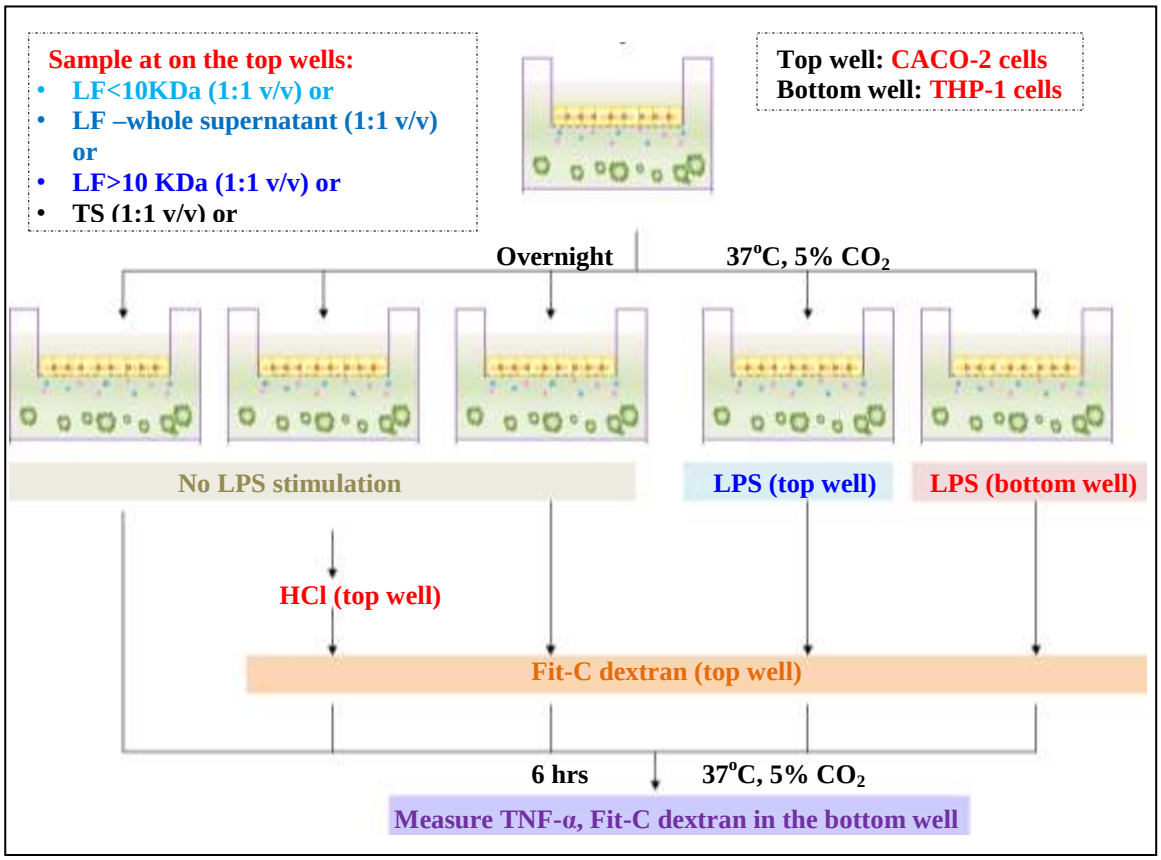

Figure 7: Outline of LPS stimulation on macrophages in transwell system. 0.5 x 10^6 human CACO-2 cells were used to differentiate into epithelial cells for the trans-well experiment on the upper chamber while 0.25 x 10^6 human THP-1 macrophage cells were placed on the bottom chamber. In different wells, <10KDa fractionates of LF or the whole, unfractionated bacterial supernatant of LF (without fractionate) or >LF 10KDa fractionates were also added in to the CACO-2 cells on the upper chamber of trans-well in the same volume ratio of 1:1 (v/v). CACO-2 cells exposed with TS media or DMEM cell culture media were used as controls. Both samples and controls were incubated overnight at 37˚C, 5% CO_2. After overnight incubation, LPS (50ng/mL) was added to the bottom chambers which contained THP-1 cells. For control, LPS was also added into the upper chamber. Finally, the whole trans-well systems were incubated for six hours at 37˚C, 5% CO_2. Cell supernatants at the bottom chamber were collected and the level of TNF-α was measured by ELISA. To examine the paracellular permeability of CACO-2 epithelial cells, internal control FitC- dextran (4KDa) was added into the upper chamber simultaneously with the sample tests were added. A high concentration of hydrochloric acid (HCl) 6.63 mM was added to the upper chamber to disrupt the tight junctions as a control of the internal control. FitC-dextran, collected from the upper chamber and bottom chamber, was measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). The concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100. Credit: An Nguyen

B. MSU crystal stimulation. LPS (50ng/mL) was first added to the bottom chamber for THP-1 cell priming for at least an overnight incubation. The next day, <10KDa bacterial supernatant LF, or the whole bacteria supernatant LF (without fractionate), or >10KDa fractionate of bacteria supernatant LF were then added to the upper chamber which contained CACO-2 cells in the volume ratio of 1:1 for six hours at 37˚C, 5% CO_2. (As mentioned before, because the upper chamber of the transwell system could not hold a large enough volume, in this experiment, the sample test was added into CACO-2 cells on the upper chamber with a volume ratio of 1:1 (v/v)). In different wells, CACO-2 cells on the upper chamber exposed with TS bacteria culture media or cell culture media DMEM were used as controls. After 6 hour incubation, MSU crystals (250μg/well) were added directly to CACO-2 cells at the bottom chamber and the whole trans-well systems were then incubated overnight at 37˚C, 5% CO_2. After overnight incubation, cell supernatants at the bottom chamber were collected, and the levels of IL-1β were measured by ELISA (Fig. 8).

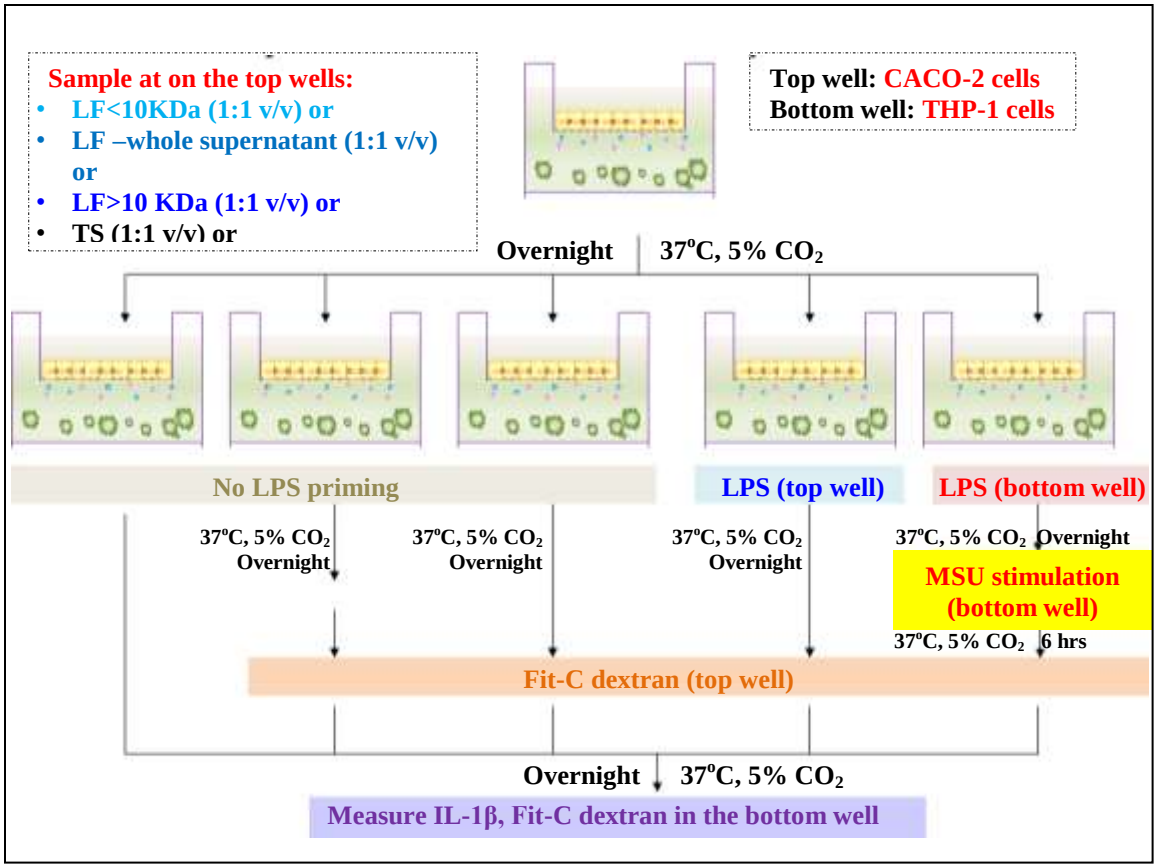

Figure 8: Outline of LPS stimulation on macrophages in transwell system. 0.5 x 10^6 human CACO-2 cells were used to differentiate into epithelial cells for the trans-well experiment on the upper chamber while 0.25 x 10^6 human THP-1 macrophage cells were placed on the bottom chamber. LPS (50ng/mL) was first added to the bottom chamber for THP-1 cell priming for at least an overnight incubation. The next day, <10KDa bacterial supernatant LF, or the whole bacteria supernatant LF (without fractionate), or >10KDa fractionate of bacteria supernatant LF were then added to the upper chamber which contained CACO-2 cells in the volume ratio of 1:1 for six hours at 37˚C, 5% CO_2. In different wells, CACO-2 cells on the upper chamber exposed with TS bacteria culture media or cell culture media DMEM were used as controls. After 6 hour incubation, MSU crystals (250 µg /well) were added directly to CACO-2 cells at the bottom chamber and the whole trans-well systems were then incubated overnight at 37˚C, 5% CO_2. After overnight incubation, cell supernatants at the bottom chamber were collected, and the levels of IL-1β were measured by ELISA. To examine the paracellular permeability of CACO-2 epithelial cells, internal control FitC-dextran (4KDa) was added into the upper chamber simultaneously with the sample tests were added. A high concentration of hydrochloric acid (HCl) 6.63 mM was added to the upper chamber to disrupt the tight junctions as a control of the internal control. FitC-dextran, collected from the upper chamber and bottom chamber, was measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). The concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100. Credit: An Nguyen

C. Assessment of epithelial permeability. An internal control was required to ensure that the bacteria supernatant does not alternate tight junctions between epithelial cells or promote inter-cellular transport. For internal control, 0.5 mg/mL FitC- dextran (4KDa) was used to examine the paracellular permeability of CACO-2 epithelial cells because FitC- dextran is hydrophilic and can diffuse between narrow intercellular spaces. FitC- dextran (4KDa) was added into the upper chamber simultaneously with the sample tests were added (Fig. 7, and 8). For control, 6.63 mM HCl was added to the upper chamber to disrupt the tight junctions (Fig. 7, and 8). FitC-dextran, collected from the bottom chamber, was measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). The concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100.

Lactate dehydrogenase (LDH) cytotoxicity measurement.

The LDH citotoxicity kits were purchased from Roche. The cell supernatants after overnight incubation with the <10KDa fractionates of bacterial supernatants were collected and the cytotoxicity assay was measured according to the Roche's instruction.

Statistical analyses.

All data are shown as mean ± s.d. Statistical analysis was performed using a two-tailed Student's t-test for all studies. For all tests, p-values less than 0.05 were considered statistically significant.

CHAPTER THREE

RESULTS

Preliminary studies of bacterial supernatants on macrophage surface markers and pro-inflammatory cytokine secretion from macrophages.

It was unknown whether microbial products secreted from the commensal bacteria had any effects on the characteristics and functions of macrophages. We hypothesized that the secreted microbial products/ metabolites of the commensal bacteria had a suppressive function on macrophage activation. The question on whether secreted products of commensal bacteria present in the bacterial culture supernatants might suppress the level of TLR-2/TLR-4 markers on macrophages as well as their abilities to inhibit TNF-α and IL-6 secretions had not yet been addressed.

In our study, we used four different commensal bacteria that were isolated from the human GI tract. Those were *Lactobacillus delbrueckii (LD), Lactobacillus fermentum (LF), Lactobacillus rhamnosus (LR),* and *Streptococcus oralis (SO).* The genus *Lactobacillus* is represented by a large number of commensal bacteria species that inhabit the mammalian GI tract [312,313]. They can be found in the intestines of various mammals, including mice, horses, non-human primates, and humans and have been used as probiotic agents for the treatment of IBD and colitis [312-316]. In both mice and humans, *Lactobacillus* species form stable microbiota populations in the intestinal tract, especially in the intestines where they adhere to host epithelial cells [317-318].

We first treated macrophages with bacterial supernatants (LD/SO/LR/LF) or with bacterial culture media TS or with macrophage cell culture media DMEM overnight. After overnight treatment, we determined the levels of TLR-2/TLR-4 present on macrophage surfaces as well as the level of TNF-α and IL-6 present in the collected cell supernatant. Our preliminary data found that after macrophages had been directly treated with each bacterial supernatant (LD/SO/LR/LF), macrophages appeared to express a significant level of TLR-2 surface markers as well as TNF-α and IL-6 cytokine secretions compared to those that were treated with bacterial culture medium TS alone or with cell culture DMEM alone (data not shown). However, we did not detect any TLR-4 surface markers (data not shown). Since there were possibly many components present in the bacterial supernatants (including DNA, RNA, protein, carabonhydrate, lipid/fatty acids) that could be responsible for this induction of inflammatory cytokines (TNF-α, IL-6) as well as TLR-2 upregulation on macrophages, we next determined which components were responsible for the cytokine secretions of macrophages. We first determined which components (either DNA/RNA/protein) present in the bacterial supernatants may contribute to the high secretion of TNF- α and IL-6 from macrophages. We treated our bacterial supernatnats (either LD/SO/LR/LF) with enzymes (either with Dnase, RNase or proteinase) for 30 minutes followed by 5 minutes- heat deactivation. Enzyme –treated bacterial supernatants (LD/SO/LR/LF) or enzyme treated-TS were then cultured together with macrophages overnight and cell supernatants were collected to measure TNF-α cytokines. Our data indicated that there was no significant difference in the level of TNF-α secretion from macrophages after exposure to either enzyme-treated

(Dnase/RNase/proteinase) bacterial supernatants or untreated-bacterial supernatants. Similarly, enzyme-treated TS did not alter the level of TNF-α secretion from macrophages compared to those cells that were treated with untreated TS (Fig. 9). This indicated that the secreted products present in the supernatants of commensal bacteria that were responsible for the secretion of TNF-α were not DNA, RNA or protein.

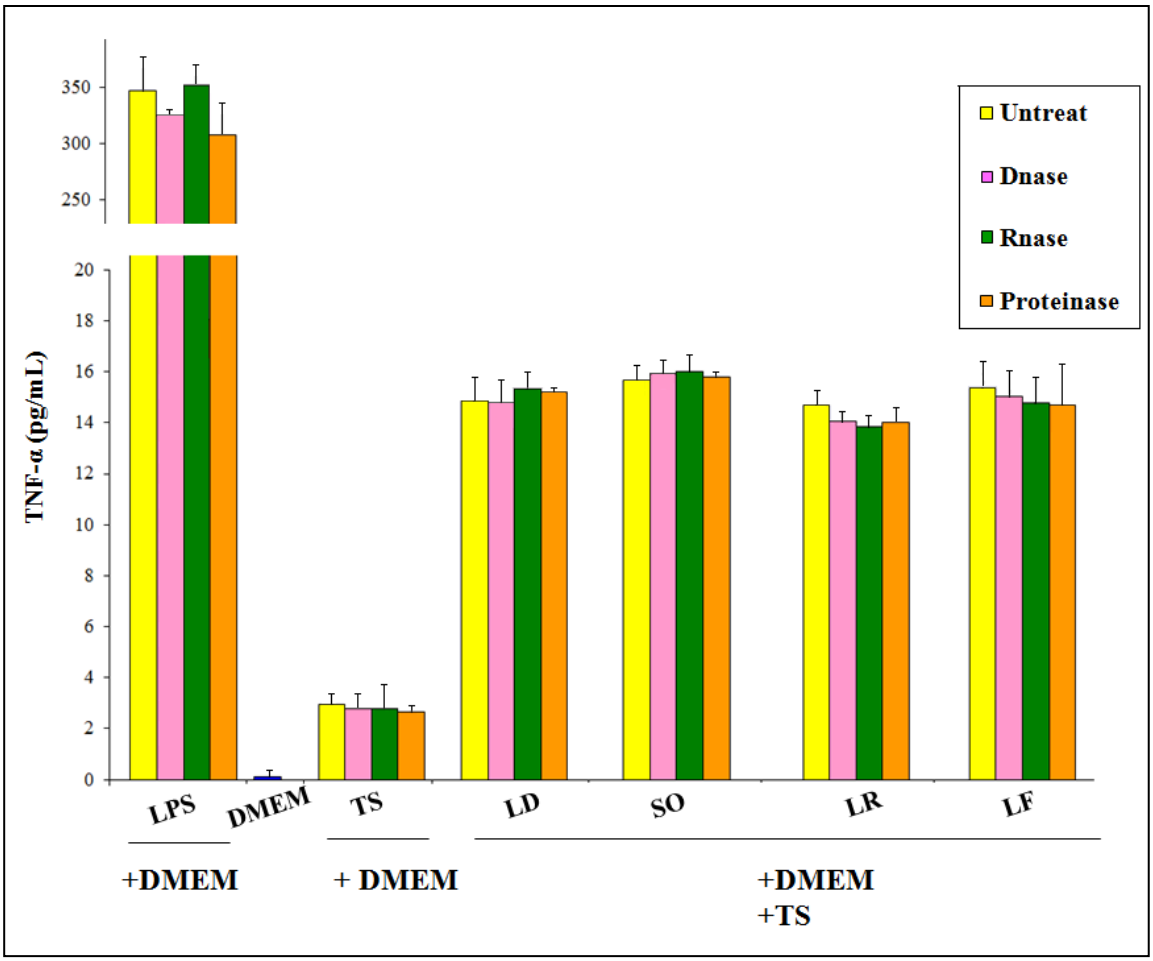

Figure 9: TNF-α secretion from BMDM macrophages after cells are incubated with enzyme-treated bacteria supernatants. The bacterial supernatant (either LD, SO, LR or LF) was treated either with RNase A (5U/mL, Thermo Scientific) or DNase I (5U/mL, Thermo Scientific) or proteinase K (10 ug/mL, Thermo Scientific) for at least 30 minutes at 37˚C followed by heat inactivation at 95˚C for 5 minutes. TS, bacteria culture media exposed either with DNase I, RNAse or proteinase K alone was used as controls. The enzyme treated- bacterial supernatants were incubated overnight with 0.25 x 10^6 BMDMs in the volume ratio of 1:2 (v/v) for overnight. Cells exposed with enzyme-treated TS bacteria culture media, or DMEM or LPS (50ng/mL) were used as controls in the same volume ratio. Finally, cell supernatants were collected, and the TNF-α secretion was measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d.

To confirm that the unchanged level of TNF-α secreted from macrophages after an overnight incubation either with enzyme-treated bacterial supernatants or untreated bacterial supernatant was not due to the heat deactivation after enzyme treatment, we heat-treated the bacterial supernatant (either with LD/SO/LR/LF) or with bacterial culture media,TS. Macrophages were then either treated with heat-treated bacterial supernatant (LD/SO/LR/LF) or heat-treated bacterial culture media, TS, or untreated bacterial supernatant (LD/SO/LR/LF) or untreated-bacterial culture media TS for overnight incubation. As a result, there was no significant difference of TNF-α secreted from macrophages after treatment with either heat-treated or untreated-bacterial supernatants (LD/ SO/LR or LF). Since DNA, RNA or proteins were not responsible for the induction of TNF-α from macrophages, we next determined if the sugar groups/carbonhydrates present in bacterial supernatants were responsible for the secretion of TNF-α from macrophages. Normally, sugar groups/carbonhydrates have a polar characteristic and they can dissolve in aqueous phase. We employed phenol/chloroform treatment to collect the sugar groups/ carbonhydrates from aqueous phase after we completely removed DNA, RNA, proteins in our bacterial supernatants. We first treated bacteria supernatants (either LD, SO, LR or LF) or bacterial culture mediaTS with DNase I, RNase A and proteinase K for 30 minutes at 37˚C, followed by heat inactivation at 95˚C for 5 minutes. After that, an equal volume of phenol: chloroform (1:1) was added into the treated-bacterial supernatants (either LD, SO, LR or LF) or TS control. The mixtures were centrifuged at 2,000 rpm for 5 minutes until the organic and aqueous phases were well-separated. The aqueous phases were pipetted to fresh tubes and treated

with our macrophages. As a result, phenol-chloroform treatment did not alter the level of TNF-α secretion from macrophages (data not shown). We then realized that the structures of the microbial metabolites are far more complex since not all polysaccharides are polar and possibly they may conjugate with the fatty acid groups to form a complex structure. This led us to believe that it is possible that different molecular weights of the microbial products may contribute to the different level of TNF-α secretion. We next determined if different molecular weights of bacterial products may be responsible for the different level of TNF-α secreted from macrophages. We first separated our bacterial supernatants (LD/SO/LR/LF) or bacterial culture media TS into different fractionates: less than 10KDa fractionates (<10KDa fractionates) and greater than 10KDa fractionates (>10KDa fractionates). We then treated <10KDa fractionates, or the whole, un-fractionated or >10KDa fractionates of bacterial supernatants (either LD/SO/LR/LF) or different fractionates of bacterial culture media TS (either <10KDa fractionates, or the whole unfraction, >10KDa fractionates) with macrophages for an overnight incubation. The overnight cell culture supernantants were then collected to measure TNF-α by ELISA. Interestingly, we found that there were significantly different levels of TNF-α secretion from macrophages (Fig. 10). Specifically, when we treated macrophages with>10KDa fractionates of the bacterial supernatants (either LD, SO, LR, or LF), the level of TNF-α secretions were above 50 (pg/mL) (yellow columns). However, the levels of TNF-α were significantly reduced by more than half (~ 20 pg/mL) when cells were treated with the whole, un-fractionated bacterial supernatants that contained both <10KDa fractionates and >10KDa fractionates (green columns) of the bacterial supernatants (either LD, SO,

LR or LF) (Fig. 10). Cells that were treated with <10KDa bacterial fractionates (LD/SO/LR/LF) had significantly lower profiles of TNF-α compared to those cells that were treated with the whole, untreated bacterial supernatants (LD/SO/LR/LF). Furthermore, cells that were exposed to different fractionates of TS bacterial culture media or DMEM as controls had no change on the levels of the TNF-α secretion with a relatively low TNF-α level (<10 pg/mL).

We observed that macrophages treated with >10KDa fractionates secreted a higher level of TNF-α. The high profile of TNF-α was significantly diminished when macrophages were treated with the whole, un-fractionated bacterial supernatants which contained both >10KDa fractionates and <10KDa fractionates. This indicated that the additional presence of <10KDa fractionates in the whole, un-fractionated bacterial supernatants (either LD/SO/LR/or LF) caused a reduction of TNF-α secreted from macrophages. We hypothesized that <10KDa fractionates had a suppressive effect on the macrophage response while the >10KDa fractionates had an inductive role in promoting macrophage activation. We then focused on the role of <10KDa fractionates on macrophage function. We hypothesized that the <10KDa could suppress macrophage activation upon the stimulation of TLR or NLRP3 agonists on macrophages.

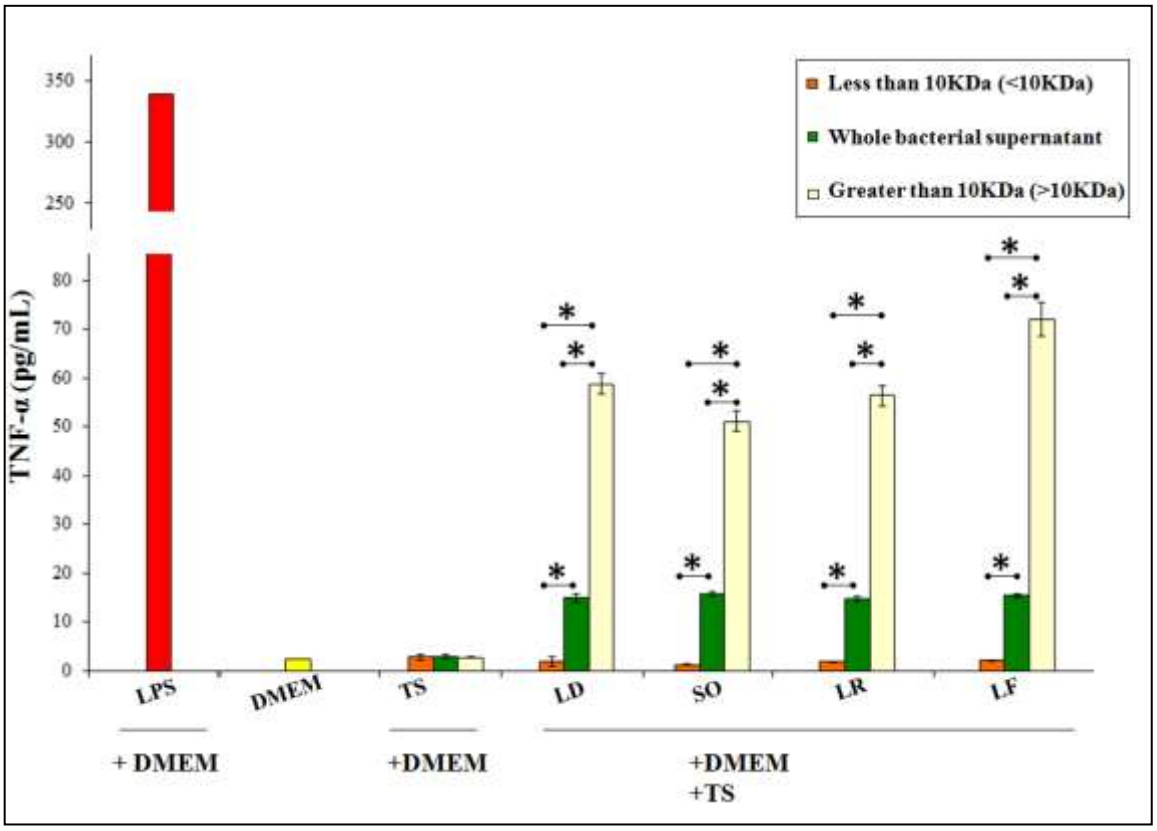

Figure 10: TNF-α secretion from BMDM macrophages after cells are treated with different bacterial fractionates: >10KDa, <10KDa and whole. The bacteria supernatants (either LD, SO, LR or LF) were fractionated into above 10KDa (>10KDa) and less than 10KDa (<10KDa) fractionates by Amicon Ultra centrifugal filters (Millipore) at 5000 rpm for 30 minutes. For the control, TS bacterial culture media was also fractionated into <10KDa and >10KDa fractionates. Different fractionates of the bacterial supernatants (either LD, SO, LR or LF) were incubated overnight with 0.25 x 10^6 BMDMs in the volume ratio of 1:2 (v/v). Cells exposed to different fractionates (>10KDa, <10KDa, or whole) of TS bacterial cell culture media, DMEM cell culture media, or LPS (50ng/mL) were used as controls in the same volume ratio. After overnight incubation, cell supernatants were collected, and the TNF-α secretion was measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

The effects of <10KDa fractionates on macrophage activation *in vitro*.

Our preliminary data showed that different molecular weights of microbial products may contribute to the secretion of TNF-α level (Fig. 10). Particularly, macrophages treated with >10KDa fractionates of bacterial supernatants (LD/SO/LR/LF) had significantly higher levels of TNF-α compared to those that were treated with the

whole, unfractionated bacterial supernatants (LD/SO/LR/LF). This suggested that the presence of <10KDa fractionates in the whole, unfractionated bacterial supernatants potentially reduced the level TNF-α induced by >10KDa fractionates and that the <10KDa fractionates of bacterial supernatants may have a suppressive function on macrophage activation. To determine if <10KDa fractionates of bacterial supernatants can actually have a suppressive function, we first wanted to determine if <10KDa fractionates could suppress macrophage activation *in vitro* upon the presence of stimulation.Two different agonists for macrophage activation were used: LPS and MSU crystals.

Macrophages can become activated upon one of these two scenerios: 1) TLR pathway via LPS stimulation, or 2) inflammasome NLPR3 pathway via monosodium urate (MSU) crystal stimulation. Upon activation, depending on the pathways, macrophages then secrete inflammatory cytokines that elicit host inflammatory response. Here, we tested both scenarios of macrophage activation upon treated with <10KDa fractionates.

A. LPS stimulation. We first studied the effects of <10KDa fractionates on macrophage activation by LPS. Previous studies had shown that macrophages treated with LPS stimuli secrete TNF-α and IL-6 pro-inflammatory cytokines [304]. To determine whether <10KDa fractionates of bacterial supernatants could suppress macrophage activation upon LPS stimulation, we first pre-treated macrophages (250,000 cells per well) *in vitro* with <10 KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) or with bacterial culture media (TS<10KDa) or cell culture media

(DMEM) overnight. (Note: we used both murine macrophages, BMDMs and human macrophages, THP-1 for this experiment). The reason that we pre-treated the <10KDa bacterial supernatants instead of a combination of <10KDa bacterial supernatants and LPS stimuli at the same time is due to the presence of the intestinal epithelial layer in the GI tract. The layer of epithelium prevents direct interaction of immune cells in the lamina propria from live bacteria or antigenic stimuli present in the gut lumen [154-175]. Only small molecules such as food antigens, carbohydrates e.g. glucose, ions or vitamins are small enough to pass between intestinal epithelial cells into the blood vessels [154]. We assumed that only small secreted products of commensal bacteria could be able to enter into the lamina propria in the same manner. Another possible route for the transport of molecules or live bacteria into the lamina propria is through the extending of the trans-epithelial dendrites (TED) of the intestinal macrophages. However, ir is unlikely to happen because only small groups of intestinal macrophages which express CX_3CR1 can promote transepithelial uptake from the lumen [318], and these cells may mainly function as a transporter of antigenic stimuli or live bacteria from gut lumen to the neighboring DCs for the T-cell priming process [266-267]. Yet, it is unknown whether TEDs are involved in the process to uptake molecules or antigenic stimuli from gut lumen into lamina propria.

Here we pre-treated macrophages with the <10KDa fractionates of the bacterial supernatants. After overnight incubation of macrophages either with <10 KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) or TS<10KDa or DMEM overnight, macrophages are stimulated with LPS for 6 hours. After 6 hours,

TNF-α and IL-6 secretion were measured by ELISA and compared to one another (Fig. 3). If <10KDa fractionates of bacterial supernatants were capable of suppressing macrophage activation upon LPS stimulation, then we expected macrophages after treatment with <10KDa fractionates (either LD/SO/LR/LF) to have a significantly reduced level of TNF-α as well as IL-6 cytokines.

As predicted, we found that macrophages pre-treated with <10KDa of bacterial supernatants (either LD, SO, LR or LF) resulted in a significant decrease in the secretion of TNF-α as well as IL-6 from macrophages. Specifically, upon LPS stimulation, bone marrow-derived macrophages (BMDMs) treated with < 10KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) exhibited, on average, a 4-fold decrease in TNF-α and 3-fold decrease IL-6 secretion, respectively, relative to the cytokine secretion of BMDM cells pre-treated with TS alone or DMEM alone (Fig. 11a, and 11b). A similar result was also observed with the human macrophage line, THP-1. Normally, upon LPS stimulation, THP-1 cells typically produce TNF- α, yet they do not produce IL-6 cytokine [305]. In a similar experiment, we also treated THP-1 cells with the <10KDa fractionates before LPS stimulation and then measured TNF-α secretion. Similar to BMDM cells, we found that upon LPS stimulation, THP-1 cells that were pre-treated with <10KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) exhibited, on average, a 3-fold decreasein TNF- α compared to the cytokine secretion of THP-1cells pre-treated with TS or DMEM alone (Fig. 12)

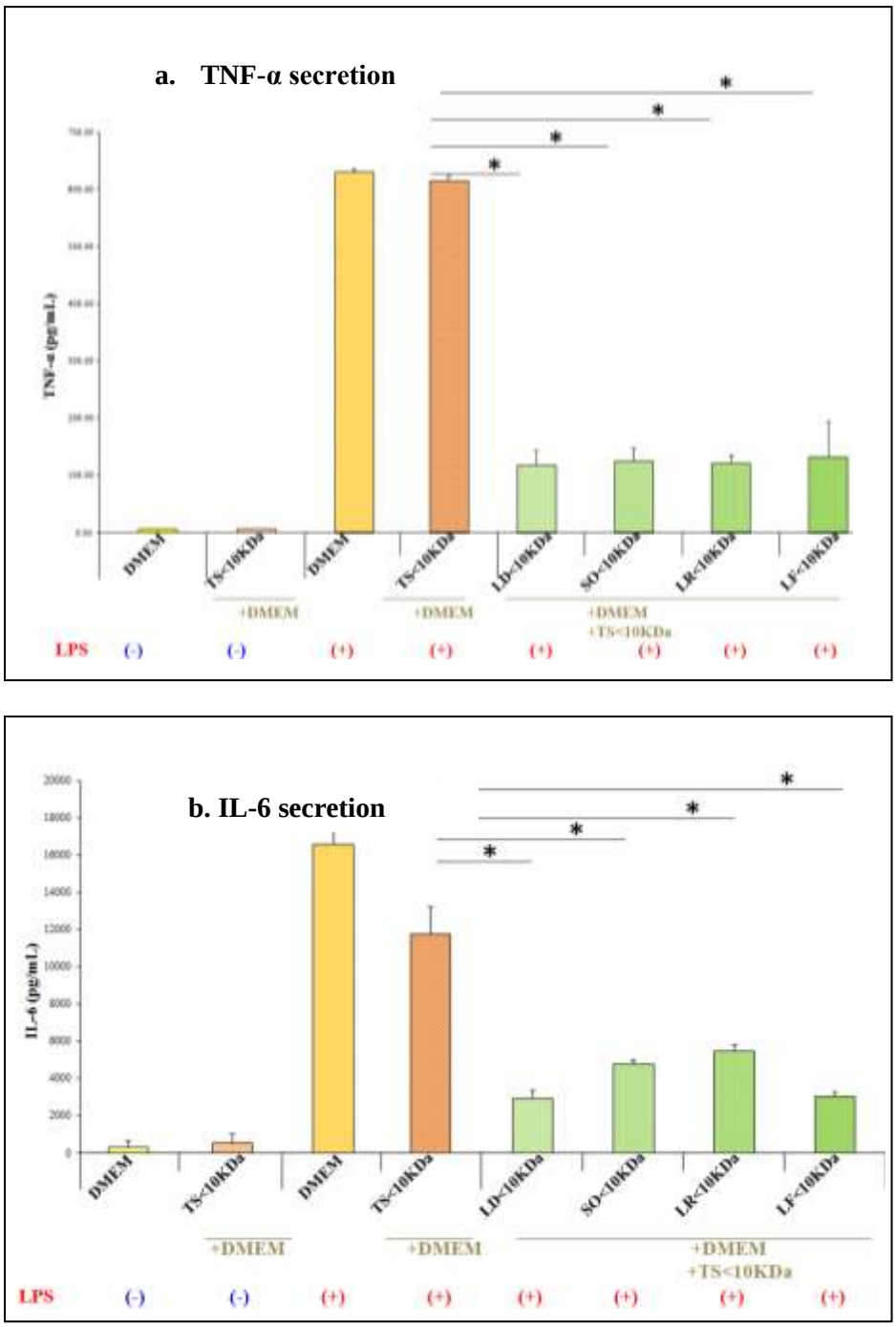

Figure 11: The effects of <10KDa fractionates on murine BMDM upon LPS stimulation *in vitro*. The <10KDa fractionates were incubated overnight with mouse BMDMs in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2.BMDMs exposed to TS<10KDa or DMEM were used as controls in the same volume ratio. After overnight incubation, cells were treated with LPS (50ng/mL) at 37˚C, 5% CO_2 for 6 hr. Finally, cell supernatants were collected, and the pro-inflammatory TNF-α and IL-6 cytokines were measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

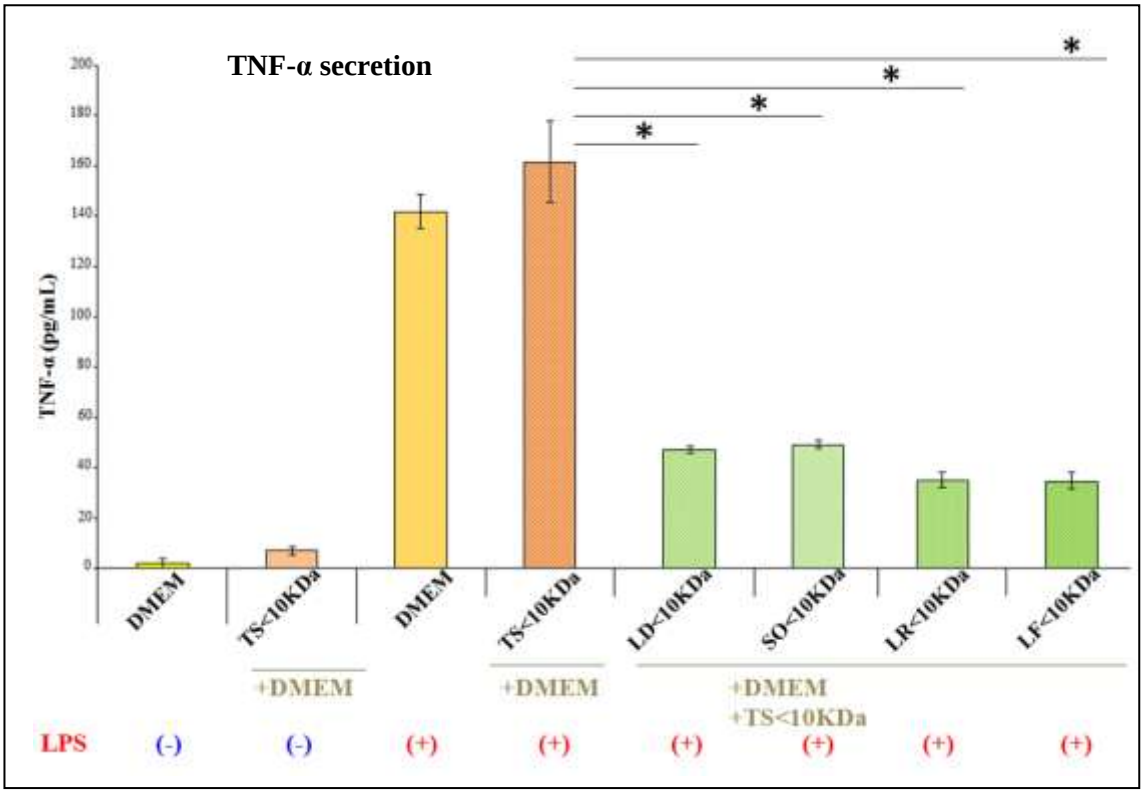

Figure 12: The effects of <10KDa fractionates on TNF-α secreted byTHP-1 macrophages upon LPS stimulation *in vitro*. The <10KDa fractionates were incubated overnight with human THP-1 cells in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2. THP-1 cells exposed to TS<10KDa or DMEM were used as controls in the same volume ratio. After overnight incubation, cells were stimulated with LPS (50ng/mL) at 37˚C, 5% CO_2 for 6 hr. Finally, cell supernatants were collected, TNF-α and IL-6 cytokines were measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

B. **MSU crystal stimulation.** As mentioned before, there are two distinct pathways for macrophage activation. The first one is through the TLR signaling upon LPS stimulation, and the second one is throught the inflammasome NLPR3 pathway in the presence of monosodium urate (MSU) crystal stimulation. Since our previous experiences showed that our <10KDa fractionates can suppress both mouse and human macrophages upon LPS stimuli, we next determined if our <10KDa fractionates of bacterial supernatants (either LD/SO/LR/LF) can suppress macrophage activation upon MSU crystal stimuli.

When macrophages become activated via inflammasome NLRP3 pathway upon MSU ctystal stimulation, the cells then secrete pro-inflammatory IL-1β cytokines which act as an important mediator for the host inflammatory responses. There is a "two-step" process that is necessary for NLRP3 inflammasome-mediated IL-1β release from macrophages. The first step requires NF-κB activation to induce synthesis of pro-IL-1β. Pro-IL-1 β then requires the mature form of caspase-1 to cleave into IL-1β. For caspase-1 activation, it requires a second signaling that involves assembly of a large cytosolic protein complex, termed inflammasomes [306-307]. Previous studies have shown that mono sodiumurate (MSU) crystal is one of the stimuli that can provide the second signaling to induce the mature form of caspase-1 while LPS-primed macrophages promote the induction of pro-IL-1β.

To determine whether <10KDa fractionates can suppress macrophage activation upon MSU crystal stimulation, we first primed our macrophages either murine BMDMs macrophages or human THP-1 macrophages (250,000 cells per well) with LPS (50ng/mL) overnight. After that, cells then treated with <10KDa fractionates of bacteria supernatant (either LD, SO, LR or LF) or with TS<10KDa or with DMEM for at least 6 hours. After 6 hours, MSU crystals were added directly to the cells and stimulated overnight. The next day, cell culture supernatants were collected, and IL-1βs from macrophages were measured by ELISA and compared to one another (Fig. 4). If <10KDa fractionates of bacterial supernatants were capable of suppressing LPS-primed macrophage activation upon MSU crystal stimulation, then we expected LPS-primed macrophages to have a significantly reduced level of IL-1β.

The data show that without MSU crystal stimuli, the levels of IL-1β were very low (< 50 pg/mL) when macrophages are primed with or without LPS (Fig.13). However, in the presence of MSU crystal stimulation, the level of IL-1β secretion from LPS-primed macrophages was significantly increased, especially when cells were pre-treated either with DMEM or TS<20KDa. Interestingly, when LPS-primed macrophages were pre-treated with <10KDa fractionates of bacterial supernatants (either LD, SO, LR or LF) prior to MSU crystal stimulation, the levels of IL-1βs from LPS-primed BMDMs were significantly reduced compared to those LPS-primed BMDM cells that were pre-treated either with TS<10KDa fractionates or DMEM alone (Fig. 13). Consistently, a similar result was also observed from THP-1 human macrophages (Fig. 14). Upon MSU crystal stimulation, LPS-primed THP-1 cells that had been pre-treated with <10KDa fractionates of bacterial supernatnats (either LD/SO/LR/or LF) appeared to have a significantly lowered level of IL-1β compared to those that were pre-treated either with TS <10KDa or cell culture media alone, DMEM. (Fig. 14)

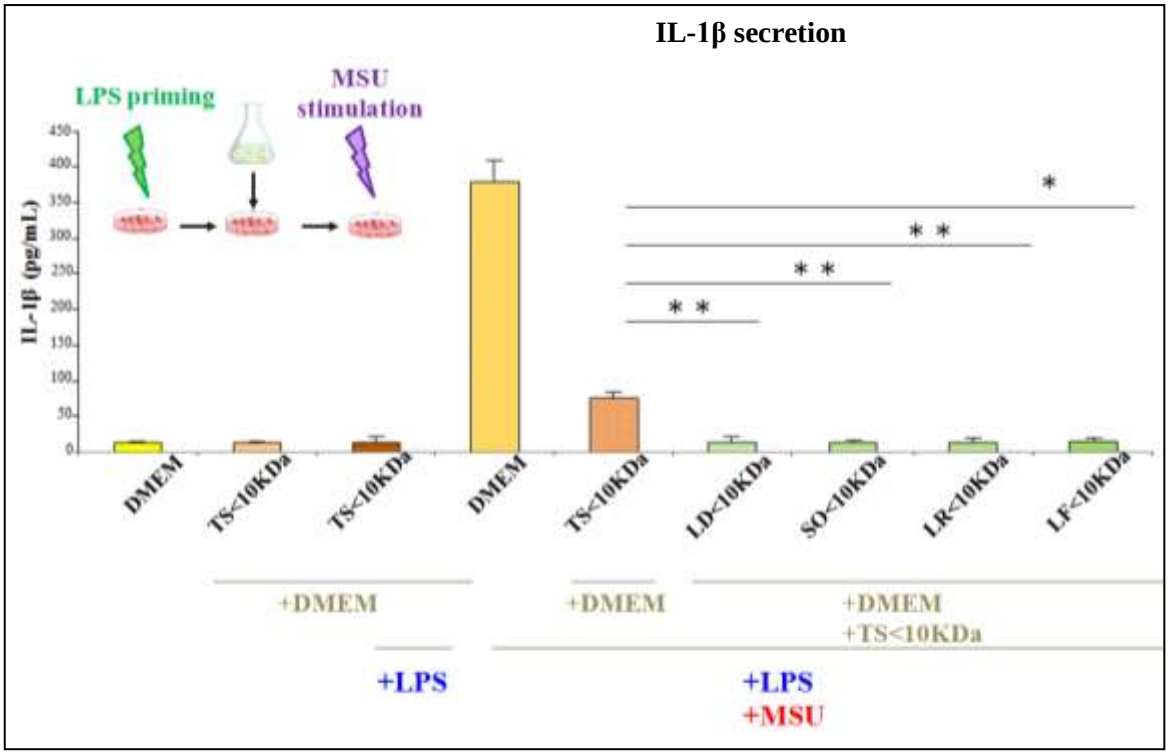

Figure 13: The effects of <10KDa fractionates on IL-1β secreted by murine BMDM upon MSU crystal stimulation *in vitro*. 0.25 x 10^6 murine BMDMs were first primed with LPS (50 ng/mL) overnight at 37˚C, 5% CO_2. After overnight incubation, the <10KDa fractionates were incubated with LPS-primed BMDMs in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2 for 6 hrs. LPS-primed BMDMs exposed to TS<10KDa or DMEM were used as controls in the same volume ratio. After 6-hour incubation, LPS-primed BMDMs were stimulated with MSU crystals (250 μg /well) overnight. Finally, cell supernatants were collected and the pro-inflammatory IL-1β cytokine was measured by ELISA Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$, ** $p<0.01$.

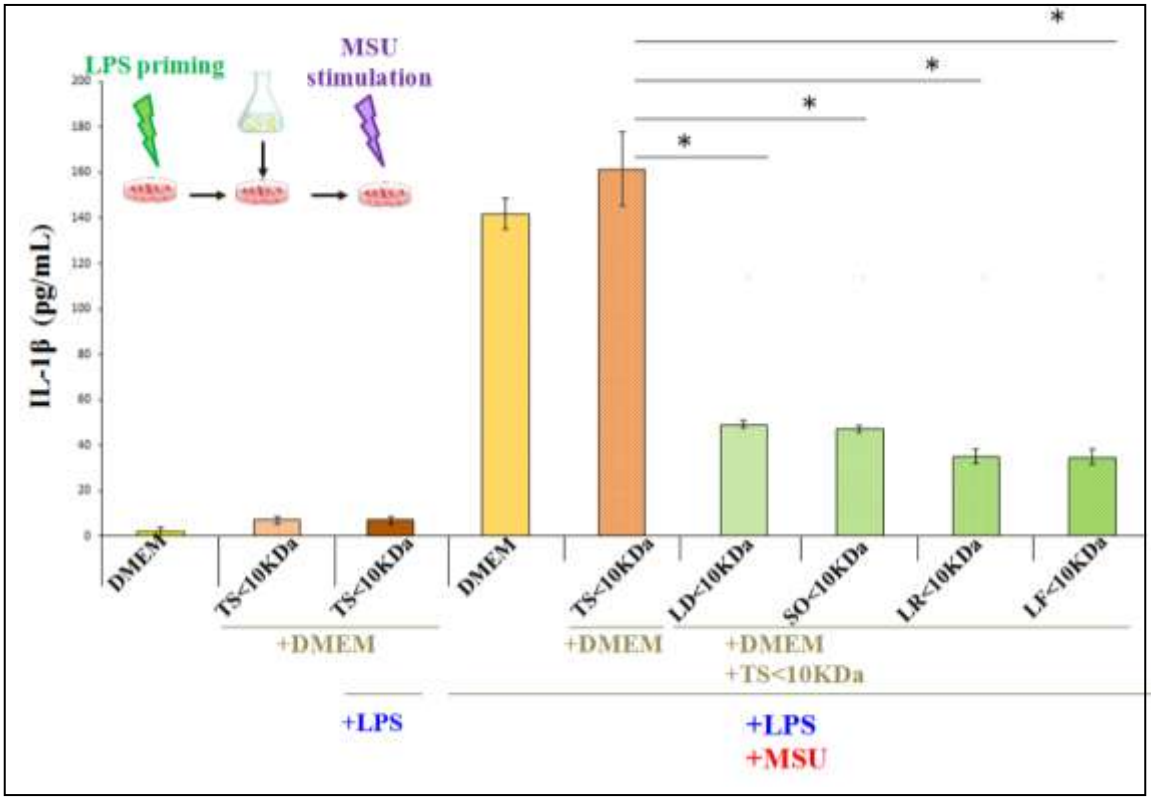

Figure 14: The effects of <10KDa Fractionates on IL-1β secreted by human THP-1 macrophages upon MSU crystal stimulation *in vitro.* 0.25 x 10^6 human THP-1 cells were first primed with LPS (50 ng/mL) overnight at 37˚C, 5% CO_2. After overnight incubation, the <10KDa fractionates were incubated with LPS-primed THP-1s in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2 for 6 hrs. LPS-primed THP-1 cells exposed to TS<10KDa or DMEM were used as controls in the same volume ratio. After 6-hour incubation, LPS-primed THP-1s were stimulated with MSU crystals (250 µg /well) overnight. Finally, cell supernatants were collected and the pro-inflammatory IL-1β cytokine was measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

The effects of <10KDa fractionates on mouse peritonitis model *in vivo*.

Previous experiments showed that <10KDa fractionates of bacterial supernatants (either LD/SO/LR/ or LF) can suppress TNF-α, IL-6 secretion (Fig. 11&12) or IL-1β (Fig.13 & 14) from macrophages upon LPS stimulation or IL-1β MSU crystal stimulation, respectively, compared to those that were-pretreated with TS<10KDa fractionates or with DMEM. To ensure the suppression of <10KDa fractionates of bacterial supernatants (either LD/SO/LR/LF) did not promote cell death, we next

determined the lactate dehydrogenase (LDH) levels present in the cell supernatants after cells were treated with <10KDa fractionates of bacterial supernatant (either with LD/SO/LR/LF). We employed the LDH assay to measure the cellular cytotoxiciy after our macrophages were treated with <10KDa fractionates of bacterial supernanats (either LD/SO/LR/LF). We treated macrophages with <10KDa fractionates of bacterial supernatants (either LD/SO/LR/or LF) or with bacterial culture media, TS<10KDa fractionates, or with cell culture media, DMEM overnight. After that, the supernatants of cell culture were collected and the percentage of cytotoxicity was measured (according to Roche's manual). Macrophages treated with the <10KDa bacterial fractionates or TS<10KDa fractionates did not significantly promote cellular cytotoxicity (Fig. 15). This indicated that the suppression of <10KDa fractionates of bacterial supernatnats (either LD/SO/LR or LF) on macrophages was not due to cell death.

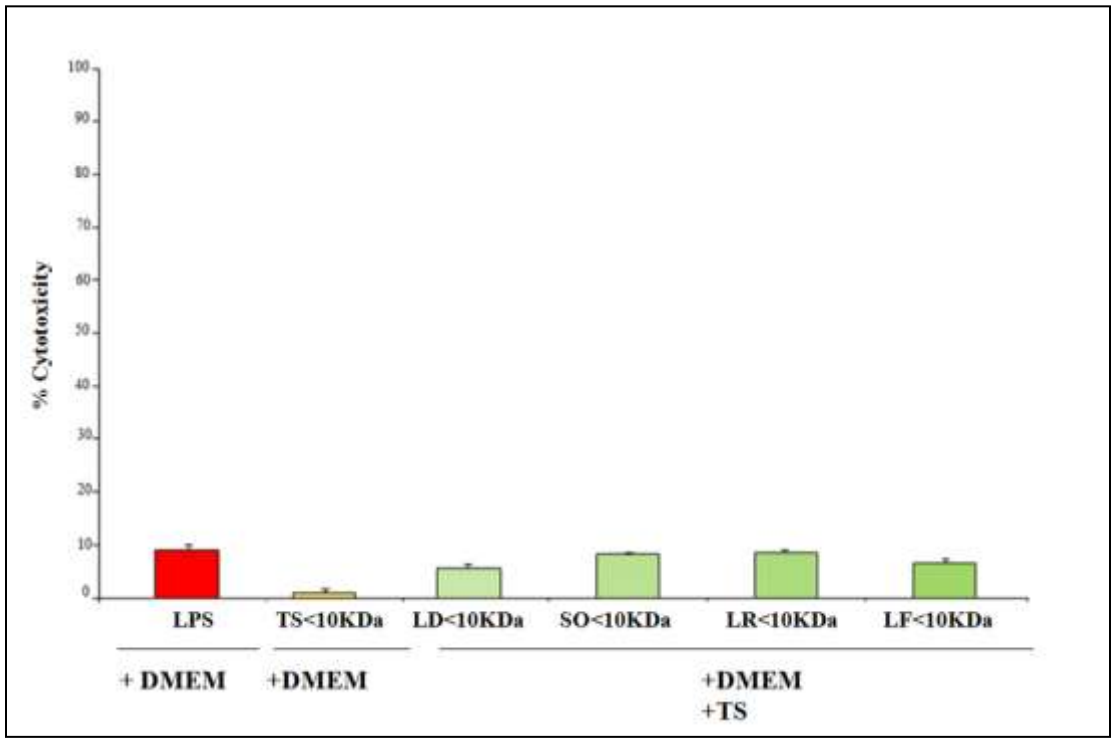

Figure 15: Macrophage viabilities after treatment with the <10KDa fractionates. The <10KDa fractionates were incubated overnight with 0.25 x 10^6 mouse BMDMs in a volume ratio of 1:2 (v/v) at 37˚C, 5% CO_2. BMDMs exposed to TS<10KDa or DMEM or LPS (50ng/mL) were used as controls in the same volume ratio. After overnight incubation, cell supernatants were collected and the percentage (%) of cytotoxicity was measured according to the Roche's manual. Data are representative of at least three independent experiments and are shown as mean ± s.d.

Since <10Da fractionates were capable to suppress macrophage activation upon LPS stimulation or MSU crystal stimulation *in vitro* (Fig. 11-12, Fig,13-14, respectively) and this suppression did not promote cellular cytotoxicity (Fig.15), we further validated the suppressive role of <10KDa fractionates *in vivo* upon LPS stimulation. Specifically, we wanted to determine whether <10KDa fractionates of bacterial supernatant can suppress neutrophil recruitment to the peritoneal cavity after intraperitoneal injections of LPS stimuli in the mouse peritonitis model.

In humans and other mammals, the peritoneal cavity is a large, hollow space within the body. The lining of the peritoneal cavity is known as peritoneum which is formed by a single layer of mesothelial cells. The structure of the peritoneum is supported by a very thin layer of connective tissues as well as lymphatic vessels [319]. The function of the peritoneum is to give support to the abdominal organs, absorp the peritoneal fluid, and prevent significant cell leakage if the GI tract is damaged [320-321]. Furthermore, the peritoneum plays as a key role in a local defence against pathogenic invasion. The defense mechanisms of the peritoneum rely on the activation of the local immune cell population as well the recruitment of circulating immune cells [322-323]. Specifically, resident macrophages control the early stage of neutrophil recruitment during tissue inflammation or pathogenic invasion. Following LPS stimulation, peritoneal macrophages become activated and secrete neutrohpil chemoattractants CXCL1/CXCL2 to recruit neutrophils at the site of inflammation [324-326]. Depletion of macrophages in mice impairs LPS-induced neutrophil extravasation. Here we determined whether the <10KDa fractionates of bacterial supernatant LF can suppress macrophage activation *in vivo* upon LPS stimulation. If this were true, we would expect mice that were pre-treated with LF<10KDa fractionates to have a significantly reduced number of neutrophils collected from the mouse peritoneal cavity upon LPS stimulation.

We first intra-peritoneally injected <10KDa fractionates of bacterial supernatant (in this case, LF) or with bacterial culture media TS or PBS vehicle into mice at a volume of 500 μL in two consecutive days (500 uL per day). Two days later, we intra-peritoneally injected LPS stimuli or PBS into mice for 4 hours. After 4 hours, mice were

sacrificed by exposure to CO_2 and the peritoneal cavities were washed with 5ml cold PBS. The recruited neutrophils present in the peritoneal lavage fluid were quantified by flow cytometry using neutrophil marker Ly6G (Fig. 5). The average numbers of peritoneal cells and the numbers of neutrophils collected are shown in Table 1. If the LF<10KDa fractionates were able to suppress macrophages *in vivo* upon LPS stimulation, then we expected that mice that were pre-treated with LF<10KDa fractionates to have a significantly lower number of collected neutrophils compared to those that were pre-treated with TS or PBS vehicle after intra-peritoneal LPS treatment.

As predicted, we found that in the presence of LPS stimuli, both PBS- and TS<10KDa-pre-treated mice exhibited a high numbers of neutrophils present in the peritoneal lavage fluid. However, in mice that were pre-treated LF <10KDa fractionates, the number of neutrophils was significantly reduced compared to mice that were pre-treated either with TS bacterial culture media or with PBS vehicle upon LPS stimuli (Fig. 16 & Fig.17)

Table 1: The number of peritoneal cells and neutrophils collected from 5mL PBS cold wash collected from LPS-induced peritonitis mouse model. Data are shown as mean ± s.d. and the data are representative of at least two independent experiments with 3 mice per group.

Pre-treatment	Stimulation	# peritoneal cells	# neutrophils
PBS	PBS	2.78×10^6	$8.96 \times 10^4 \pm 1.24 \times 10^5$
	LPS	3.89×10^6	$1.91 \times 10^6 \pm 2.46 \times 10^5$
TS<10 KDa	PBS	1.33×10^6	$8.96 \times 10^3 \pm 1.36 \times 10^3$
	LPS	6.94×10^6	$2.15 \times 10^6 \pm 2.43 \times 10^5$
LF<10 KDa	PBS	3.41×10^6	$6.07 \times 10^4 \pm 6.10 \times 10^4$
	LPS	2.11×10^6	$2.63 \times 10^5 \pm 1.28 \times 10^5$

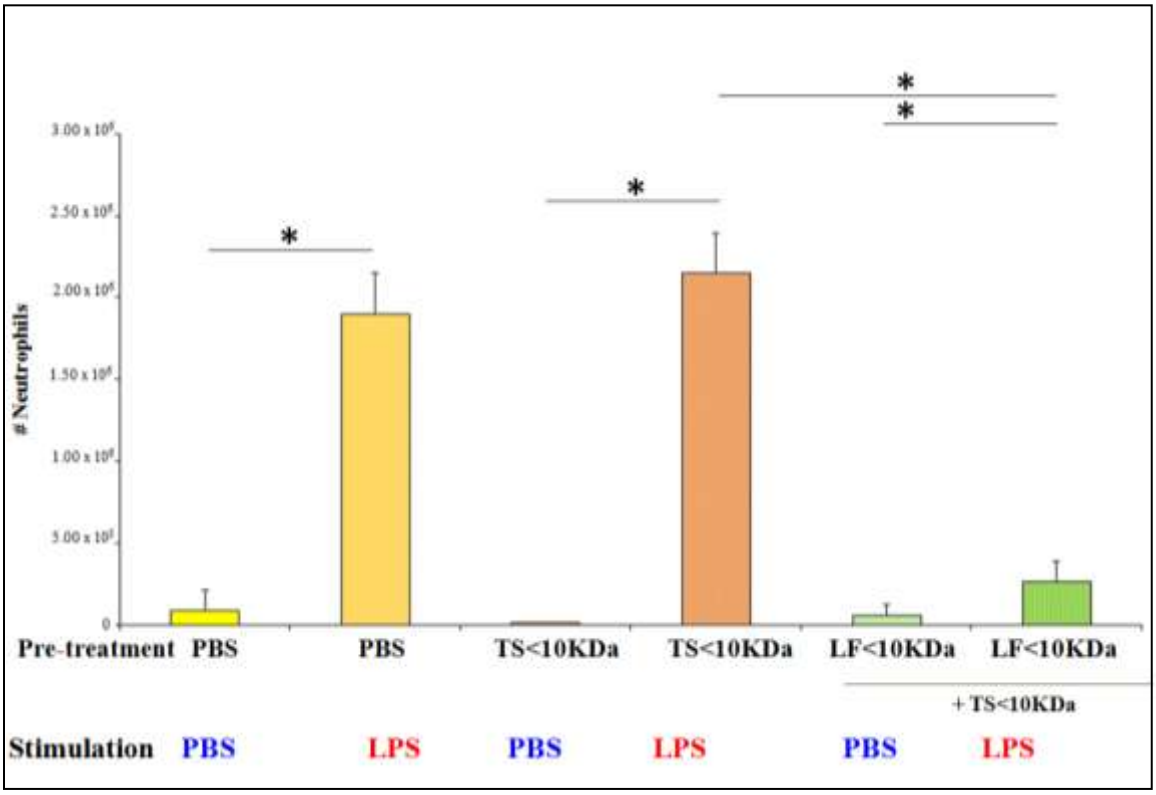

Figure 16: The effects of <10KDa fractionates on mouse peritonitis model upon LPS stimulation *in vivo.* 6-week old mice were intraperitoneally injected either with <10KDa fractionate (500 µL/mouse) for two consecutive days. Mice intraperitoneally injected with TS culture media or PBS vehicle were used as controls. Two days later, all mice were intra-peritoneally injected with LPS (10 µg/mouse) or PBS vehicle as control. Four hours later after LPS injection, mice were then sacrificed by exposure to CO_2 and the peritoneal cavities were washed with 5 ml cold PBS. The recruited polymorphonuclear neutrophils present in the peritoneal lavage fluid from 5 mL cold PBS wash were quantified by flow cytometry using the neutrophil markers Ly6G (BD Bioscience). The samples were acquired on a FACSCantoflow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (Treestar). Data are representative of at least two independent experiments with 3 mice per group and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

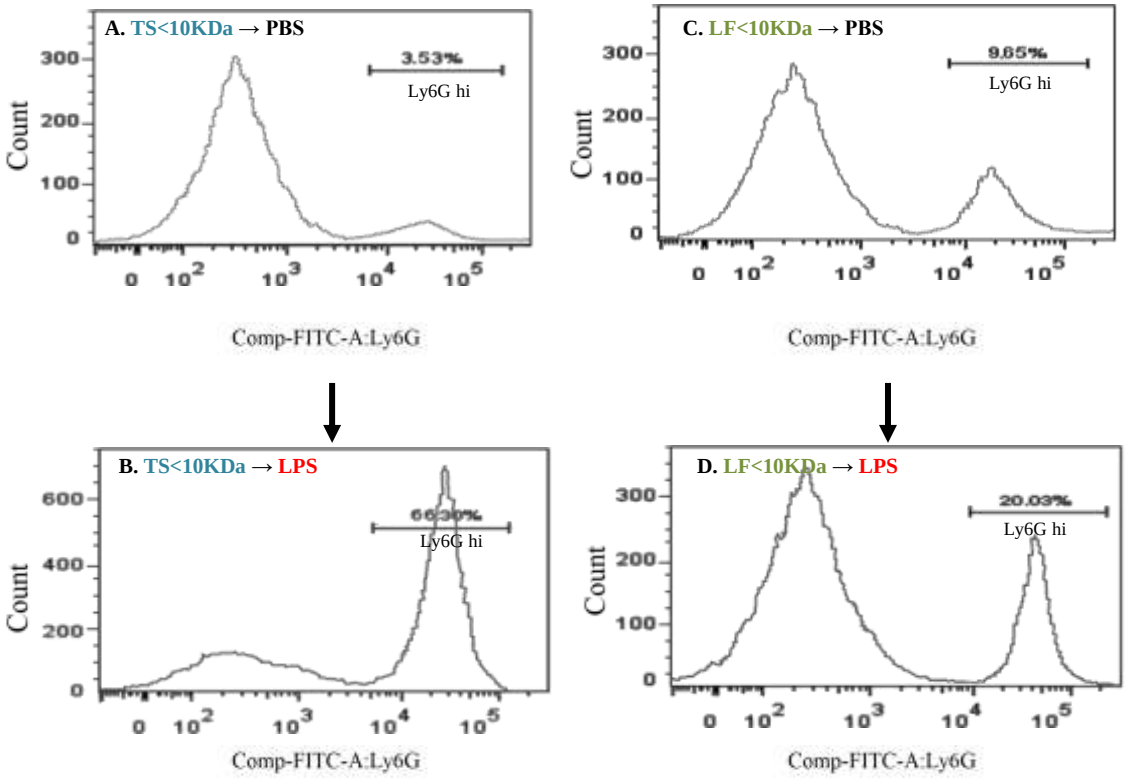

Figure 17: FACS representation of peritoneal cells in mouse peritonis model. These are representations of FACS gating of cells collected from peritoneal cavity. Panels (A) and (B) are mice that were first pre-treated with TS<10KDa while panels (C) and (D) are mice that were pre-treated with LF<10KDa fractionates for two consecutive days. Two days later, mice were stimulated either with LPS (10µg per mouse) or PBS vehicle for 4 hours. After that, peritoneal cells were harvested. The collected peritoneal cells wer blocked with Fc bloking antibody and then stained with neutrophil marker FitC-anti mouse Ly6G. Histograms of FACS staining for Ly6G are shown in panel A-D. Mouse neutrophils were quantified by FACS based on high expression of Ly6G neutrophil marker which was indicated in each panel.

The effects of <10KDa fractionates on macrophage activation in transwell system.

From previous experiments, although <10KDa fractionates of commensal bacteria supernatants (LD/SO/LR/LF) were able to suppress TNF-α, IL-6 secretion or IL-1β from macrophages upon LPS or MSU crystal stimulation respectively *in vitro* and inhibited neutrophil recruitment into peritoneal cavity upon LPS stimulation *in vivo*, it was impossible to conclude that <10KDa fractionates could actually suppress macrophage activation in the gastrointestinal system due to the complex structure of GI tract. In the gut, commensal bacteria are present in the gut lumen, which is separated from intestinal

macrophages by the complex layer of intestinal epithelium. The presence of intestinal epithelial layer prevents the direct interaction of commensal bacteria with intestinal macrophages. We did not know whether <10KDa fractionates were small enough to directly pass between intestinal epithelial cells to enter into the lamina propria where they directly interact with macrophages. Another possible scenario might be that <10KDa fractionates could directly intereact with the intestinal epithelium and promote the intestinal epithelium to secrete other cytokines that may provide signaling to regulate macrophage activation. Lastly, the trans-epithelial dendrites (TED) of macrophages accross the intestinal epithelial cells may gain direct access for both antigenic stimuli and the <10KDa secreted molecules from commensal bacteria into the lamina propria. However, not all intestinal macrophages possess the TED function. Only small groups of intestinal macrophages that express CX_3CR1 [318] can promote transepithelial uptake from the lumen, and these cells may mainly function as a transporter of antigenic stimuli or live bacteria from gut lumen to the neighboring DCs for the T-cell priming process [266-267]. The extending of TED has been shown to be involved in both TLR-dependence and indepdence on intestinal epithelial cells [267]. However, it remains unclear what functional significance TED plays in the GI physiology, and how different molecular weights of commensal products have an impact on TED's function. Overall, it was unknown whether <10KDa fractionates can actually suppress macrophage activation in the presence of epithelial cells. Here, we wanted to determine if the <10KDa fractionates can suppress macrophages in the presence of epithelial layer.

In this experiment, we employed the transwell system which mimics the

gastrointestinal system [328-329]. The transwell system consists of two chambers: upper chamber and bottom chamber. We then used differented CACO-2 human cells to generate an artificial epithelial layer that were present in the upper chamber of the transwell system. The bottom chamber of the transwell system represented the lamina propria where we used differented THP-1 cells to generate macrophage populations. Specifically, in transwell system, the upper chambers contained differentiated CACO-2 cells (250, 000 cells per well), and the bottom chambers contained THP-1 cells (250,000 cells per well) (Fig.6). We then placed bacterial supernatants on the upper chamber that were directly incubated with differentiated CACO-2 epithelial cells and determined whether the treatment of different bacterial supernatnats (either <10KDa fractionates, unfractionates, or >10KDa fractionates) could suppress macrophages activation upon stimulation. We hypothesized that both <10KDa fractionates or the whole, unfractionated bacterial supernatants (in this case, LF) on the upper chamber were able to suppress macrophage activation upon the induction of TLR or NLRP3 agonists on macrophages while >10KDa fractionates of bacterial supernantants would fail to suppress macrophage activation. As mentioned before, there are two scenarios for macrophage activation to promote pro-inflammatory responses: 1/ TLR pathway via LPS stimulation, or 2/ inflammasome NLPR3 pathway via MSU crystal stimulation. Here, we tested the roles of <10KDa fractionates to suppress macrophage activation upon LPS stimulation or MSU crystal stimulation in the transwell system. We used FitC-dextran as an internal control to ensure that our <10KDa supernatants did not promote paracellular transport by altering the tight junctions between epithelial cells. For negative control (as a part of internal control), in a

separate well, we added HCl acid (6.63 mM) in the upper chamber together with the FitC-dextran. The optical density (OD) of FitC-dextran was measured by fluorescence spectrometer. The actual concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100.

A. LPS stimulation. In this experiment, we had three different samples we wanted to test: <10KDa fractionates of LF, or the whole, unfractionated bacterial supernatant LF, or >10KDa fractionates of LF. Here, we wanted to determine whether <10KDa fractionates could suppress THP-1 macrophage activation upon LPS stimulation. Also, we would expect that the presence of <10KDa fractionates in the whole, unfractionated bacterial supernatant of LF could also suppress LPS-stimulated THP-1 activation in the transwell system. Before LPS stimulation, we first pre-treated CACO-2 cells with <10KDa fractionate of bacterial supernatant LF or the whole, un-fractionated bacteria supernatant LF (without fractionate) or >10KDa fractionate of LF supernatant in the volume ratio of 1:1. For control, in a different well, we added either TS bacteria culture media or DMEM cell culture media in the same volume ratio of 1:1. Both samples and controls were incubated overnight. The next day, we stimulated cells with LPS stimulation (50ng/mL) by adding LPS either in the top chamber or bottom chamber of the transwell system. At the same time, we also added FitC-dextran as an internal control. The transwell system was again incubated for 6 hours. After 6 hours we collected

cell supernatants at the bottom chamber and measured the level of TNF-α from the collected supernatants by ELISA (Fig. 7).

If <10KDa fractionates could suppress THP-1 macrophage activation upon LPS stimulation, we expected that upon LPSstimulation, CACO-2 cells that had been pre-treated either with LF<10KDa fractionates or the whole LF at the upper chamber to have a significant decrease of TNF-α secretion from THP-1 cells at the bottom chamber compared to the trans-well that had CACO-2 cells exposed with TS control or DMEM control.

Our data found that in the absence of LPS stimulation, when the trans-well system was pre-treated with TS bacteria culture media or DMEM cell culture media in the upper chamber, they appeared to have a low level of TNF-α secretion. Also, when LPS was added into the upper chamber for stimulation, the level of TNF-α secretion was also low. This level of TNF-α was soon significantly increased when LPS was directly treated with THP-1 cells at the bottom chamber (Fig. 18)

As expected, in the transwell system that had <10KDa fractionates or the whole, un-fractionated bacterial supernatant LF-pre-treated CACO-2 cells present on the upper chamber, the levels of TNF-α secretion were significantly reduced compared to the transwell that had CACO-2 cells that were first exposed with either bacterial culture media, TS<10KDa or cell culture media, DMEM upon LPS stimulation at the bottom chamber. More importantly, we did not observe such a reduction of TNF-α secretion from the transwell system that had >10KDa fractionates of LF pre-treated-CACO-2 cells upon LPS stimuli at the bottom chamber (Fig. 18)

As for internal control, when the transwell was pre-treated with either sample (>10KDa fractionate of LF, or the whole LF supernatant or <10KDa fractionate of LF) or controls (DMEM or TS). We did not detect any FitC- dextrans present in the sample test supernatants collected from the bottom chamber. However, FitC- dextrans were detected in the bottom chamber when the transwell system was treated with 6.63 mM HCl (Fig. 19)

Collectively, our data demonstrated the presence of <10KDa fractionates of bacterial supernantants (either <10KDa fractionates of LF alone or their presence in our whole, un-fractionated supernatants of LF) can suppress macrophage activation upon LPS stimulation (Fig. 20), and the presence of bacterial supernatants do not promote cellular permeability.

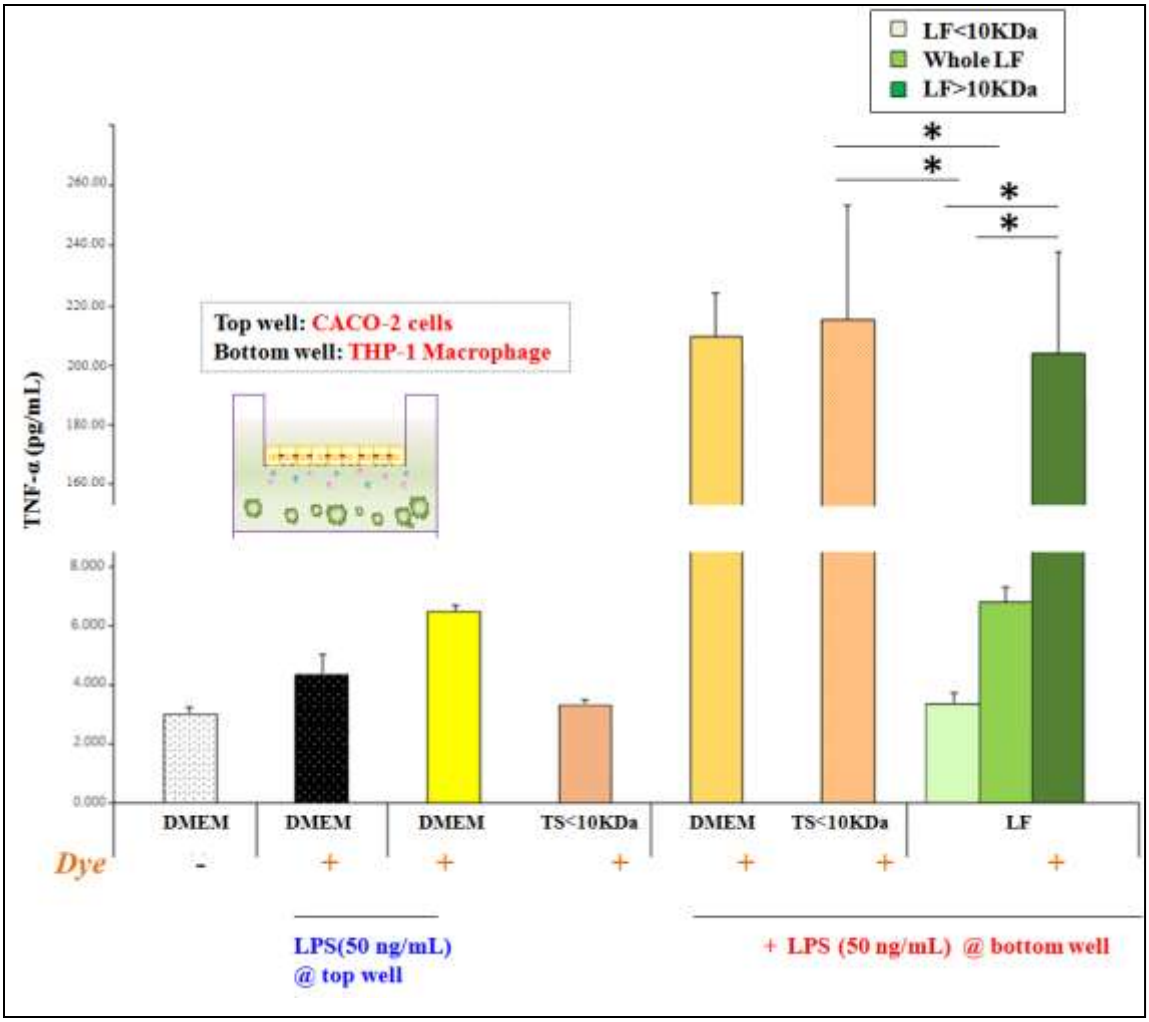

Figure 18: The effects of different fractionate LF (>10KDa, <10KKDa, or whole) on human THP-1 macrophage activation in transwell system upon LPS stimulation. 0.5 x 10^6 CACO-2 cells were seeded in the upper and grown at least for two weeks until they become confluent. Once the CACO-2 became confluent, THP-1 cells (cultured and maintained as described above) were then placed into the lower chamber of the transwell system at a density at 0.25 x 106 cells per well. The <10KDa bacterial supernatant LF was added to CACO-2 cells in the upper chamber with a volume ratio of 1:1. Furthermore, in different wells, LF whole bacterial supernatant (without fractionate) or >LF 10KDa fractionates were also added to the CACO-2 cells in the upper chamber of transwell in the same volume ratio of 1:1 (v/v). CACO-2 cells exposed to TS media or DMEM cell culture media were used as controls. Both samples and controls were incubated overnight at 37˚C, 5% CO2. After overnight incubation, LPS (50ng/mL) was added to the lower chambers that contained THP-1 cells. For control, LPS was also added into the upper chamber. Finally, the whole trans-well systems were incubated for six hours at 37˚C, 5% CO2. Cell supernatants at the bottom chamber were collected, and the level of TNF-α was measured by ELISA. For internal controls, FitC-dextrans (4KDa) were added into the upper chamber simultaneously as the sample tests were added. A high concentration of hydrochloric acid (HCl) 6.63 mM as a control was added to the upper chamber to disrupt the tight junctions. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

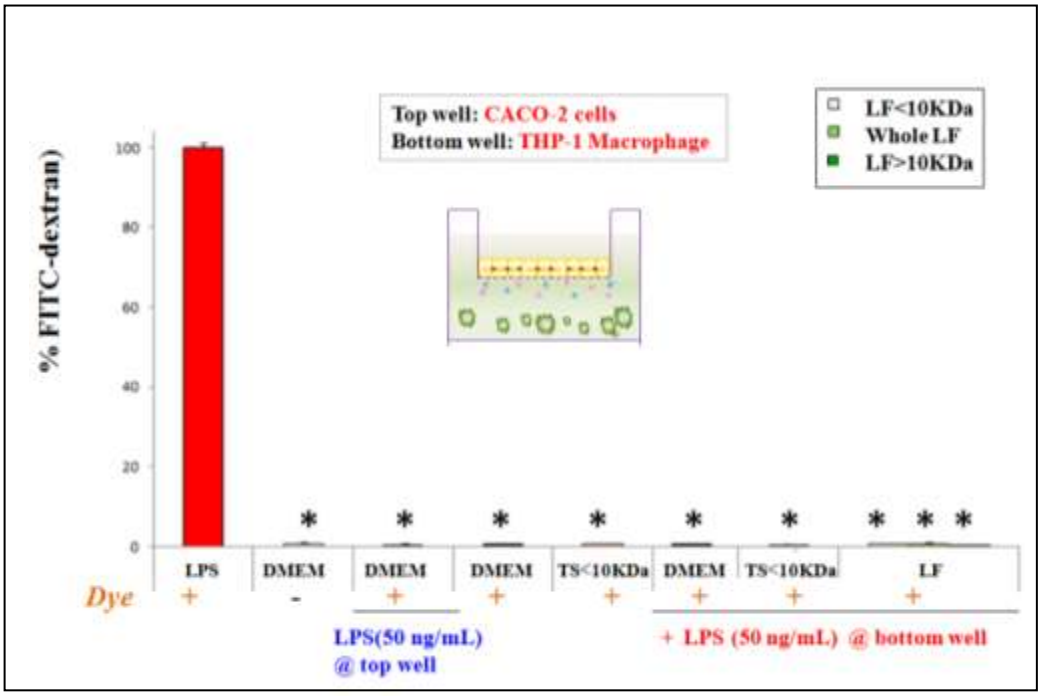

Figure 19: The assessment of the permeability of the differentiated CACO-2 epithelium cells after the treatment of different LF fractionates (>10KDa, <10KDKa, or whole) in transwell system upon LPS stimulation of THP-1 macrophages by measuring the percentage (%) concentration of FitC- dextran at the bottom chambers of a transwell system. 0.5 x 10^6 CACO-2 cells were seeded in the upper and grown at least for two weeks until they become confluent. Once the CACO-2 became confluent, THP-1 cells (cultured and maintained as described above) were then placed into the lower chamber of the transwell system at a density at 0.25 x 106 cells per well. The <10KDa bacterial supernatant LF was added to CACO-2 cells in the upper chamber with a volume ratio of 1:1. Furthermore, in different wells, LF whole bacterial supernatant (without fractionate) or >LF 10KDa fractionates were also added to the CACO-2 cells in the upper chamber of transwell in the same volume ratio of 1:1 (v/v). CACO-2 cells exposed to TS media or DMEM cell culture media were used as controls. Both samples and controls were incubated overnight at 37˚C, 5% CO2. After overnight incubation, LPS (50ng/mL) was added to the lower chambers that contained THP-1 cells. For control, LPS was also added into the upper chamber. Finally, the whole trans-well systems were incubated for six hours at 37˚C, 5% CO2. Cell supernatants at the bottom chamber were collected, and the level of TNF-α was measured by ELISA. For internal controls, FitC-dextrans (4KDa) were added into the upper chamber simultaneously as the sample tests were added. A high concentration of hydrochloric acid (HCl) 6.63 mM as a control was added to the upper chamber to disrupt the tight junctions. FitC-dextran collected from the bottom chamber were collected and measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). The concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

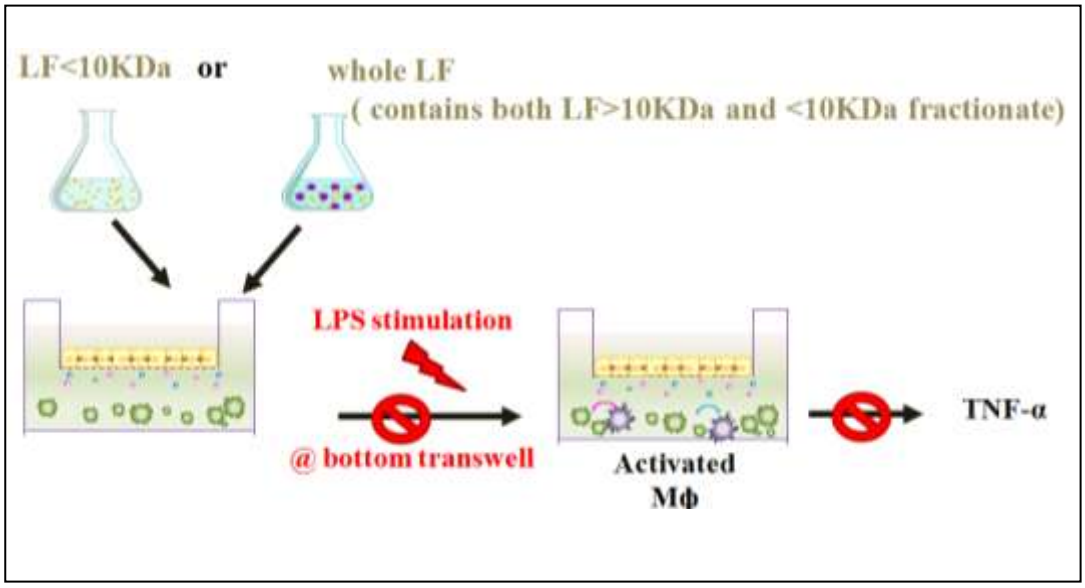

Figure 20: The effects of different LF fractionates (>10KDa, <10KKDa, or whole) on human THP-1 macrophage activation in transwell system upon LPS stimulation. Human CACO-2 cells at the top well pre-treated either with LF<10KDa fractionates or the whole LF bacterial supernatant can suppress the activation of LPS-stimulated THP-1 cells at the bottom well of the transwell system. As a result, the secretion of TNF-α was significantly inhibited.

B. **MSU crystal stimulation.** Since our <10KDa fractionates as well as the whole, un-fractionated bacterial supernatant of LF were able to suppress macrophage activation upon LPS stimulation in the transwell system that mimics the gastrointestinal system, we next determined whether <10KDa fractionates of bacteria supernatant could suppress macrophage activation in the transwell system upon MSU crystal stimulation. We had three different samples we wanted to test: <10KDa fractionate of LF, the whole bacterial supernatant LF (without fractionate), and >10KDa fractionate of LF. (Note: in a similar setup, we placed our differentiated CACO-2 human epithelial cells on the upper chamber while we placed our differentiated THP-1 human macrophages at the bottom chamber. The different bacterial fractionates of LF were separately placed on the upper chamber that contained CACO-2 cells).

If the <10KDa fractionate of LF could suppress macrophage activation upon MSU crystal stimuli, then we would expect that there would be a low level of IL-1β secretion collected from the bottom chamber. We would also expect that the whole bacteria supernatant of LF (which contains both <10KDa molecules and >10KDa molecules prior fractionate) also could suppress the macrophage activation by inhibiting IL-1β secretion from the THP-1 human macrophage collected from the bottom chamber.

As mentioned before, it requires "two step" process for the activation of inflammasome NLRP-3 in macrophages to secrete pro-inflammatory cytokine, IL-1βs. The first step requires LPS priming of macrophages that promotes the formation of pro-IL-1β via the activation of NF- κB. The second step requires the assembly of inflammsome that requires MSU crystal stimuli to promote a full form of caspase-1. This full-form of caspase-1 then cleaves pro-IL-1β into IL-1β cytokines that are necessary to elicit the inflamamtion [306-307].

We first added LPS at the bottom chamber for THP-1 priming overnight. After that, the sample test (either <10KDa fractionate of LF, or the whole LF supernatant, or >10KDa fractionate of LF) or the control (TS<10KDa bacteria culture media or DMEM cell culture media) was then added to the upper chamber of the transwell system in the volume ratio of 1:1 (v/v) and the CACO-2 cells were incubated together either with sample test or control for 6 hrs. After 6 hours, MSU crystals were added into the bottom chamber and at the same time, FitC-dextran dye as an internal control was added into the upper chamber. We incubated the whole transwell system for an overnight period. On the

next day, we collected the cell supernatant from the bottom chamber and measured the level of IL-1β secretion by ELISA (Fig. 8).

We found that in the absence of MSU crystal stimulation, when the CACO-2 cells of the upper chamber were pre-treated with TS<10KDa bacteria culture media or DMEM cell culture media, the level of IL-1β secretion from LPS-primed macrophages was low. This level of IL-1β secretion was also comparable to the THP-1 cells without LPS priming. However, upon the addition of MSU crystal stimuli at to the bottom chamber, the level of IL-1β from LPS-primed THP-1 cells was significantly enhanced in the transwell that had <10KDa or DMEM pre-treated CACO-2 cells at the upper chamber. Interestingly, when CACO-2 cells were pre-treated either with <10KDa fractionates of LF or the whole, unfractionated bacterial supernatant of LF, the level of IL-1β secretion from LPS-primed THP-1 macrophages was also significantly reduced upon MSU crystal stimulation. In contrast, when the CACO-2 cells of the upper chamber were pre-treated with >10KDa fractionate of LF, the level of IL-1β secreted from LPS-primed THP-1 macrophages was still high and comparable to those that had TS- or DMEM-pretreated CACO-2 cells of the upper chamber upon MSU crystal stimulation (Fig. 21).

Similar to LPS stimulation, as an internal control, when the transwell was pre-treated with either the sample (>10KDa fractionate of LF, or the whole, un-fractionated LF supernatant or <10KDa fractionate of LF) or the controls (DMEM or TS), we did not detect any FitC- dextran present in the sample test supernatants collected from the bottom chamber. However, FitC- dextran was detected in the bottom chamber when the transwell system was treated with 6.63mM HCl (Fig. 22).

Together, our data showed that in the presence of <10KDa fractionates of bacterial supernantants (either <10KDa fractionates of LF alone or their presence in our whole, un-fractionated supernatants of LF), this presence of <10KDa fractionates can suppress macrophage activation upon LPS stimulation (Fig. 23).

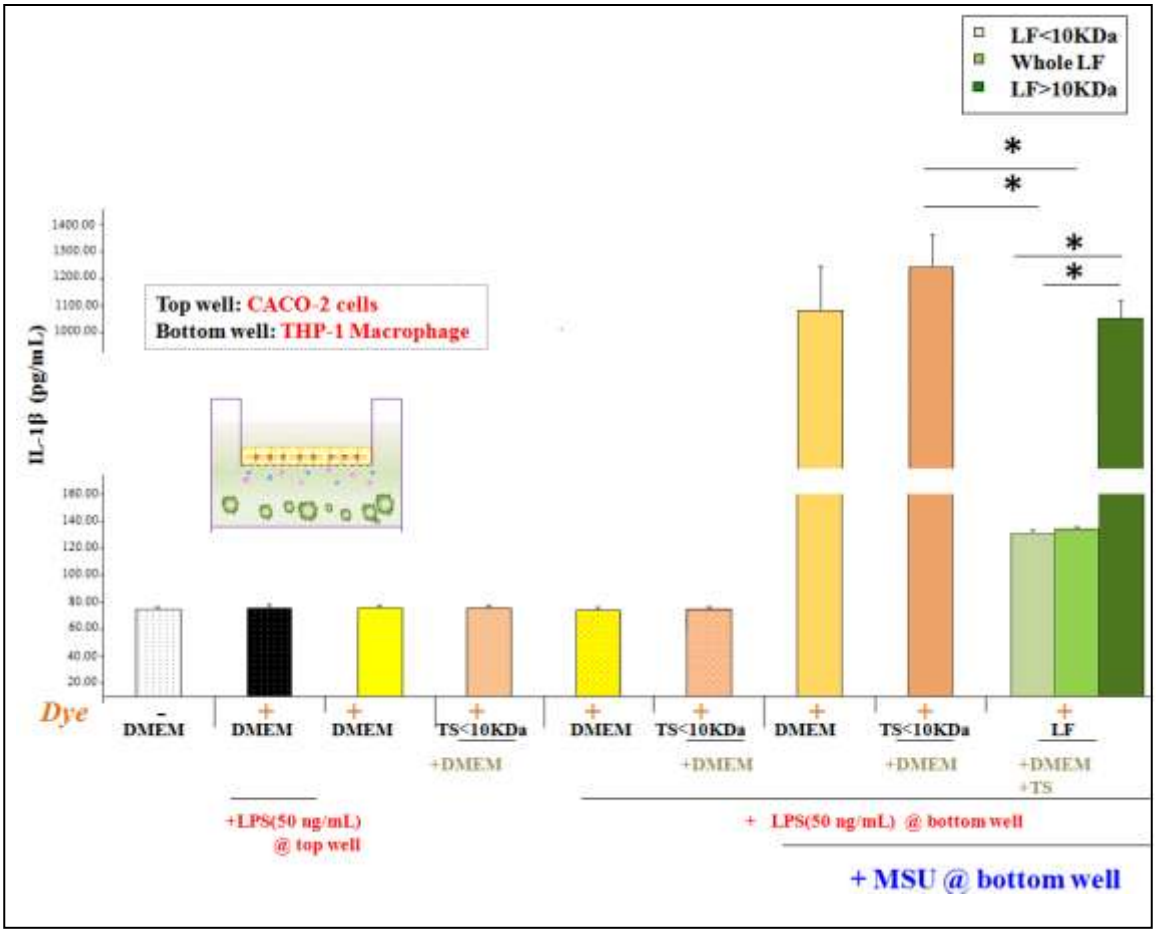

Figure 21: The effects of different fractionate LF (>10KDa, <10KKDa, or whole) on LPS-primed THP-1 macrophage in transwell system upon MSUcrystal stimulation. 0.5 x 10^6 CACO-2 cells were seeded in the upper and grown at least for 2 weeks until they become confluent. Once the CACO-2 became confluent, THP-1 cells (cultured and maintained as described above) were then placed into the lower chamber of the transwell system at a density at 0.25 x 106 cells per well. LPS (50ng/mL) was first added to the lower chamber for THP-1 cell priming for at least an overnight incubation. The next day, <10KDa bacterial supernatant LF, whole bacteria supernatant LF (without fractionate), or >10KDa fractionate of bacteria supernatant LF were then added to the upper chamber that contained CACO-2 cells in the volume ratio of 1:1 for six hours at 37˚C, 5% CO2. In different wells, CACO-2 cells on the upper chamber exposed to TS bacteria culture media or cell culture media DMEM were used as controls. After a 6 hour incubation, MSU crystals (250 µg/well) were added directly to CACO-2 cells in the bottom chamber, and the whole trans-well systems were then incubated overnight at 37˚C, 5% CO2. After overnight incubation, cell supernatants at the bottom chamber were collected, and the levels of IL-1β were measured by ELISA. For internal controls, FitC- dextrans (4KDa) were added into the upper chamber simultaneously as the sample tests were added. A high concentration of hydrochloric acid (HCl) 6.63 mM as a control was added to the upper chamber to disrupt the tight junctions. FitC-dextran collected from the bottom chamber were collected and measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

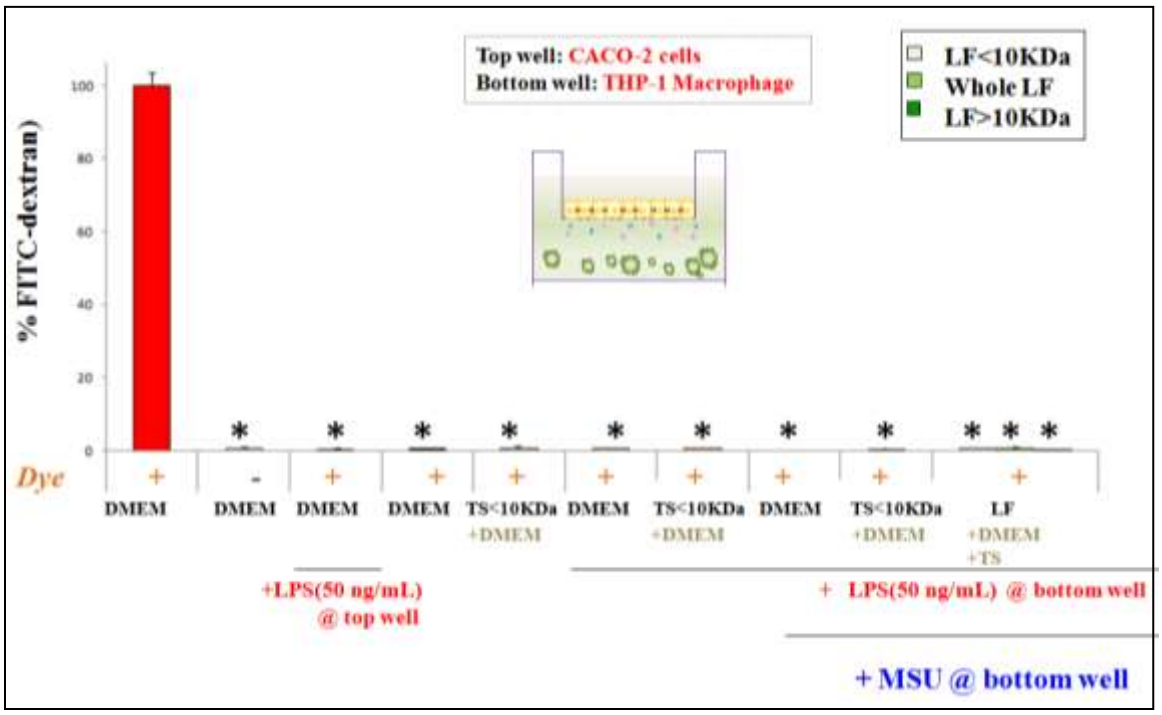

Figure 22: The assessment of the permeability of the differentiated CACO-2 epithelium cells after the treatment of different LF fractionates (>10KDa, <10KDKa, or whole) in transwell system upon MSU crystal stimulation of LPS-primed THP-1 cells by measuring the percentage (%) concentration of FitC- dextran at the bottom chambers of a transwell system. 0.5 x 10^6 CACO-2 cells were seeded in the upper and grown at least for 2 weeks until they become confluent. Once the CACO-2 became confluent, THP-1 cells (cultured and maintained as described above) were then placed into the lower chamber of the transwell system at a density at 0.25 x 106 cells per well. LPS (50ng/mL) was first added to the lower chamber for THP-1 cell priming for at least an overnight incubation. The next day, <10KDa bacterial supernatant LF, whole bacteria supernatant LF (without fractionate), or >10KDa fractionate of bacteria supernatant LF were then added to the upper chamber that contained CACO-2 cells in the volume ratio of 1:1 for six hours at 37˚C, 5% CO2. In different wells, CACO-2 cells on the upper chamber exposed to TS bacteria culture media or cell culture media DMEM were used as controls. After a 6 hour incubation, MSU crystals (250 µg/well) were added directly to CACO-2 cells in the bottom chamber and the whole trans-well systems were then incubated overnight at 37˚C, 5% CO2. After overnight incubation, cell supernatants at the bottom chamber were collected, and the levels of IL-1β were measured by ELISA. For internal controls, FitC- dextrans (4KDa) were added into the upper chamber simultaneously as the sample tests were added. A high concentration of 6.63 mM HCl as a control was added to the upper chamber to disrupt the tight junctions. FitC-dextran collected from the bottom chamber were collected and measured by a fluorescence spectrometer (excitation, 490 nm; emission, 520 nm). The concentrations of FitC–dextran at the bottom well were obtained from the standard curve of a known, purified FitC-dextran concentration. To calculate the percentage of FitC-dextran present at the bottom well, the amount of FitC-dextran at the bottom well was divided by the original amount of FitC-dextran present at the top well and then multiplied by 100. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

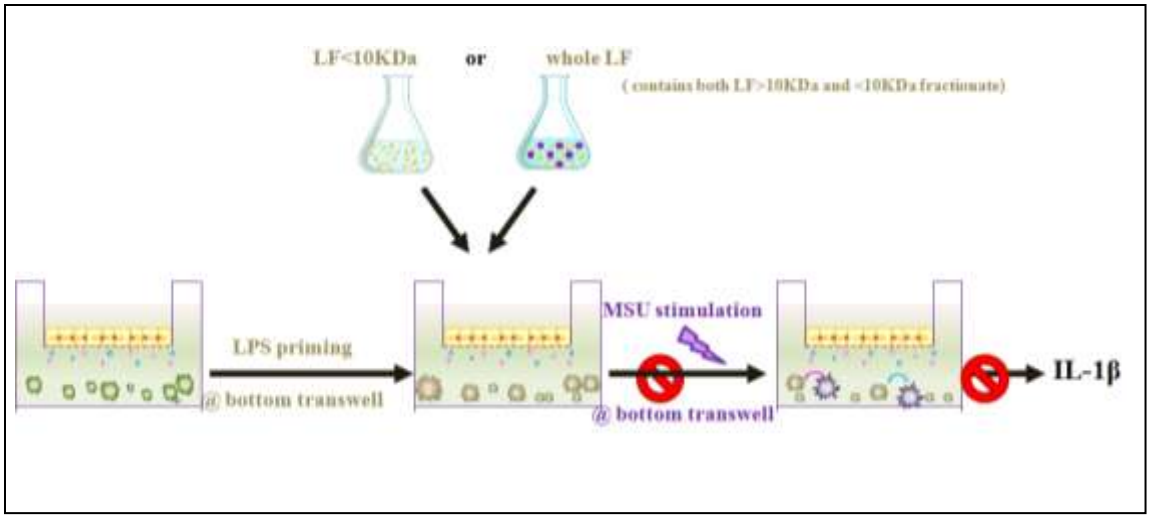

Figure 23: The effects of different LF fractionates (>10KDa, <10KKDa, or whole) on LPS-primed THP-1 macrophage activation in transwell system upon MSU stimulation. Human CACO-2 cells at the top well pre-treated either with LF<10KDa fractionates or the whole LF bacterial supernatant can suppress the activation of LPS-primed THP-1 cells upon MSU stimulation at the bottom well of the transwell system. As a result, the secretion of IL-1β was significantly inhibited.

Macrophage activation by >10KDa fractionates is TLR-2 dependent, not TLR-4 *in vitro*.

Our preliminary data showed that when macrophages were treated with the whole bacterial supernatants alone (either LD, SO, LR, or LF), they appeared to express a significantly higher level of TLR-2 surface markers compared to those that were treated with bacterial culture medium TS, or cell culture DMEM alone. However, we did not detect TLR-4 surface markers on macrophages after treatment with the whole bacterial supernatant (either LD, SO, LR, or LF). Also, our preliminary data showed that when macrophages were treated with different fractionates (either >10KDa, or <10KDa or the whole) of bacterial supernatants (either LD, SO, LR, or LF), there are different levels of TNF-α secretions from macrophages (Fig. 10). Specifically, the levels of TNF-α secreted by macrophages upon >10KDa fractionates of bacterial supernatants were significantly higher than those macrophages that were treated with the whole bacterial supernatants

(which contained both >10KDa fractionates and <10KDa fractionates). This suggests that that the presence of <10KDa fractionates in the entire supernatant suppressed the level of TNF-α of macrophages which were stimulated by >10KA fractionates of bacterial supernatants (Fig.10). Also, >10KDa fractionates of bacterial supernatants failed to suppress macrophage activation upon LPS or MSU crystal stimulation in the transwell-system (Fig. 18 & Fig. 21). This led us believe that >10KDa acted as stimulatory factors for macrophage activation. Furthermore, we believed that >10KDa fractionates were components from the bacteria cell walls since only bacteria cell walls can promote macrophage activation [308]. Additionally, macrophages can respond to bacterial cell walls through the recognition of TLR-2 and TLR-4. Thus, for further clarification, we next determined the mechanism that allows >10KDa fractionates to stimulate macrophage responses. Specifically, we tested whether >10KDa fractionates can activate macrophage *in vitro* in either TLR-2 or TLR-4 dependent manner.

For this experiment, we isolated BMDMs from $TLR2^{-/-}$ and $TLR4^{-/-}$ mice. After that, we treated the >10KDa fractionates of bacterial supernatants (either LD, SO, LR or LF) with macrophages overnight in a ratio of 1:2 (v/v) at 37˚C, 5% CO_2. BMDMs exposed either to LPS (50ng/mL), TS bacteria cell culture media or DMEM cell culture media were used as controls. After overnight incubation, we collected the cell supernatants, and we measured the TNF-α secretion by ELISA. If >10KDa fractionates acted as stimulatory factor for macrophage activation and this activation is dependent on TLR-2/TLR-4 manner, then we would expect that the level of TNF-α would be significantly reduced in macrophages that had $TLR2^{-/-}$ or $TLR4^{-/-}$ mice.

We found that the level of TNF-α secreted by macrophages was significantly reduced in TLR2$^{-/-}$ mice compared to the macrophages isolated from wild-type mice upon >10KDa fractionate treatment (Fig. 24). As for control cell culture media TS, we did not see such a change in the level of TNF-α in WT mice or TLR2$^{-/-}$ mice. However, we did not see such a reduction of the TNF-α level in TLR4$^{-/-}$ mice (Fig.25).

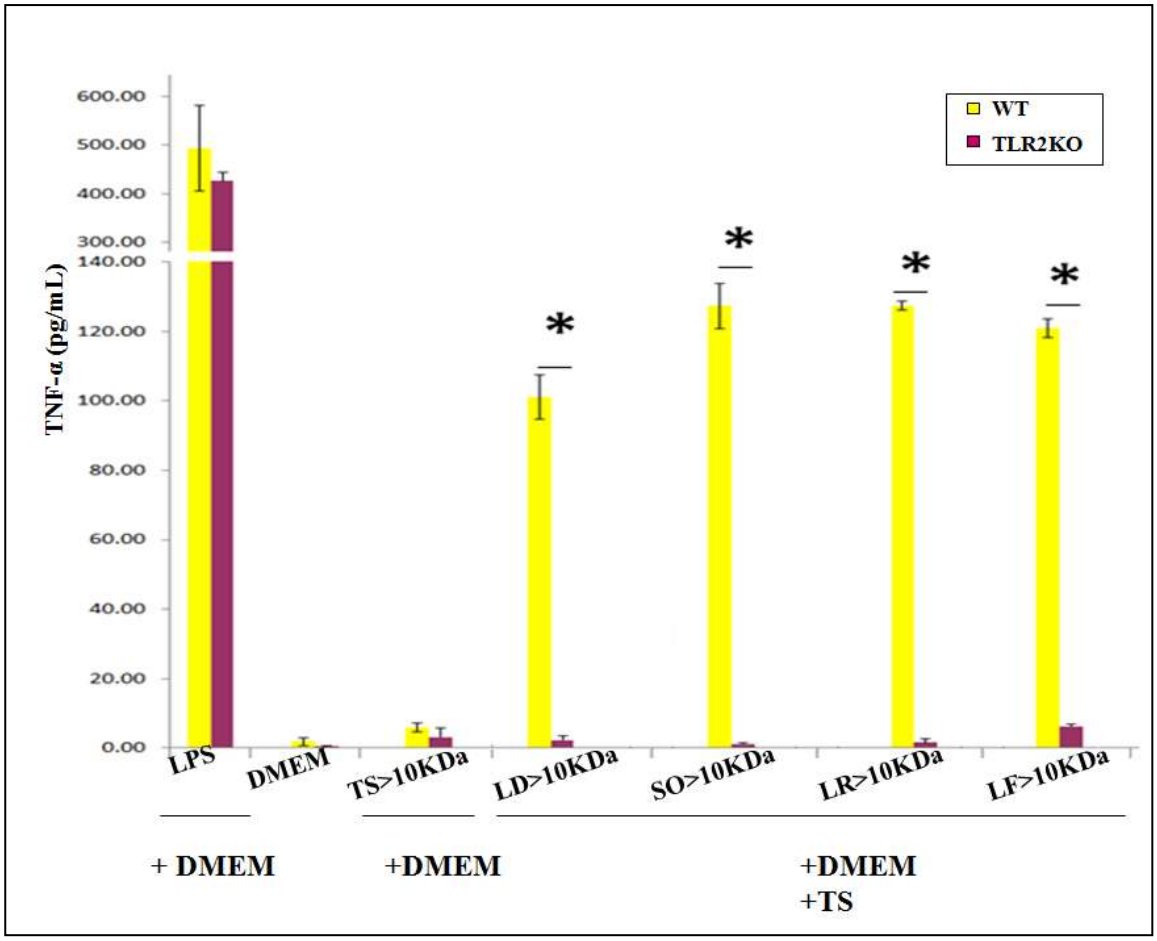

Figure 24: TNF- α secretion from WT vs TLR-2KO BMDM macrophages after murine BMDMs were treated with >10KDa fractionates of bacteria supernatant (either LD, SO, LR, or LF). 0.25 x 10^6 BMDMs isolated from TLR2$^{-/-}$ or wild type mice were then incubated with >10KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) in the volume ratior of 1:2 (v/v) for overnight. Cells exposed with TS bacterial cell culture media, DMEM cell culture media, or LPS (50ng/mL) were used as controls. Cell supernatants were then collected and the TNF-α secretion was measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$.

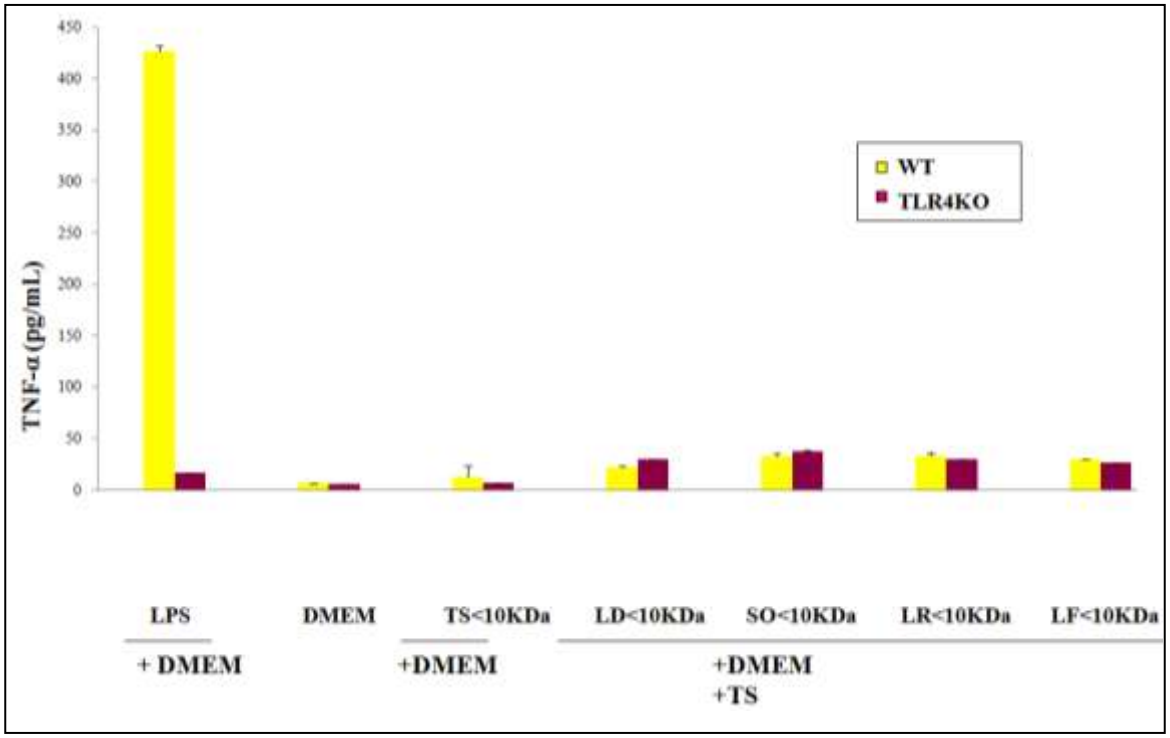

Figure 25: TNF- α secretion from WT vs TLR-4KO BMDM macrophages after murine BMDMs were treated with >10KDa fractionates of bacteria supernatant (either LD, SO, LR, or LF). 0.25 x 10^6 BMDMs isolated from $TLR4^{-/-}$ or wild type mice were then incubated with >10KDa fractionates of bacterial supernatants (either LD, SO, LR, or LF) in the volume ratior of 1:2 (v/v) for overnight. Cells exposed with TS bacterial cell culture media, DMEM cell culture media, or LPS (50ng/mL) were used as controls. Cell supernatants were collected and the TNF-α secretion was measured by ELISA. Data are representative of at least three independent experiments and are shown as mean ± s.d. Statistically significant differences were determined by the standard Student's t-test, * $p<0.05$

CHAPTER FOUR

DISCUSSION

The GI tract is home to a complex network of microbial communities [1,2,6]. The gastrointestinal mucosal structure is an intricate structure that consists of both a biochemical barrier as well as a physical barrier. The physical barrier consists of the intestinal epithelial cells (IECs) that separate the inside lamina propria from the intestinal lumen. The surface of the intestinal epithelium is coated with a thick layer of mucus which serves as a matrix to entrap microbes. Despite the abundant number of commensal bacteria present in the GI tract, the host is equipped with highly effective immune cells present in the lamina propria. These immune cells are ready to initiate the innate immune responses upon pathogenic infection. Among the immune cells found in the GI tract, a major reservoir of intestinal macrophages play a central role in initiating and sustaining protective immune responses mounted against a vast number of potentially harmful bacteria and antigenic stimuli present in the intestinal lumen. During steady state, intestinal macrophages play a central role in performing housekeeping functions. Favorably located beneath the intestinal epithelium, the intestinal macrophages function to capture and phagocytose any foreign substances that breach the epithelial barrier [250]. This bactericidal activity allows intestinal macrophages to protect the host from pathogenic infection or antigenic stimulation that may be considered as a threat to host. Despite having phagocytic activity and defensive functions, the intestinal macrophages

are inflammation-anergic toward non-harmful dietary antigens or commensal bacteria. Such an immune tolerance of the intestinal macrophages is attributed to the low expression of TLRs on macrophages as well as the unsuccessful TLR downstream signaling [37,250,268], the mass production of IL-10 [254] from macrophages that lead to the differentiation of Tregs [73] to secrete other inhibitory factors such as TGF-β to suppress the host immune responses [37]. However, when the intestinal homeostasis is disrupted, the intestinal macrophages switch to pro-inflammatory functions and elicit a series of inflammatory responses in the host GI tract system. Such an inflammatory response involves in the production of pro-inflammatory cytokines and other chemokine mediators to facilitate the recruitment of other innate immune cells at the site of inflammation [271-272, 268]. Commensal bacteria have been involved in many immune-anergic pathways of intestinal macropahges. However it is unknown how commensal bacteria can exert their effects on the intestinal macrophages. Since microbiota secrete many metabolites that play a central role in host immune system, it is possible that microbiota-derived metabolites have influence on the function of macrophages and the hypo-responsiveness of intestinal macrophages toward the commensal bacteria. Therefore, it is important to determine whether microbiota-derived metabolites can influence the immune response of macrophages. We hypothesized that those commensal bacteria were able to immuno-modulate macrophage function through their secreted metabolites. Specifically, we hypothesized that the secreted bacteria factors/metabolites present in the bacterial supernatant can suppress macrophage activation upon the induction of TLR or NLRP3 agonists on macrophages.

While evaluating the effects of different fractionates of our commensal bacteria on macrophage response, we unexpectedly discovered that different molecular weights of microbial products may contribute to the secretion of TNF-α level. Specifically, macrophages treated with >10KDa fractionates of bacterial supernatants (LD/SO/LR/LF) had significantly higher levels of TNF-α compared to those that were treated with the whole, unfractionated bacterial supernatants (LD/SO/LR/LF). This suggested that the presence of <10KDa fractionates in the whole, unfractionated bacterial supernatants potentially reduced the level TNF-α induced by >10KDa fractionates and that the <10KDa fractionates of bacterial supernatants may have a suppressive function on macrophage activation while the >10KDa fractionates had an inductive role in promoting macrophage activation. We hypothesized that the <10KDa could suppress macrophage activation upon the stimulation of TLR or NLRP3 agonists on macrophages.

We first focused on the role of <10KDa fractionates on macrophage function. Specifically, we wanted to determine whether <10KDa fractionates can suppress macrophage responses in the presence of stimuli. *In vitro* experiments, the <10KDa fractionates of bacterial supernatants can suppress macrophage responses in the presence of two different stimuli: LPS versus MSU crystals. LPS stimuli are well known to promote pro-inflammatory cytokines secreted from macrophages such as TNF-α, IL-6 through Toll-lie receptor pathway [309]. On the other hand, MSU crystals are well known to promote inflammasome cascade in macrophages to become activated and secrete pro-inflammatory cytokine IL-1β [310]. In both scenarios, <10KDa fractionates of bacterial supernatant (either LD, SO, LR, LF) can suppress macrophage responses to

LPS or MSU crystal stimuli to inhibit the secretion of pro-inflammatory TNF-α, IL-6 and IL-1β cytokines *in vitro*.

The suppression of <10KDa fractionates of bacterial supernatants was also successful in the mouse peritonitis model to determine the effects of <10KDa fractionates on neutrophil recruitment. Normally, the peritoneal cavity is sterile and small numbers of bacteria can be efficiently disposed of in the peritoneal cavity. However, peritonitis happens when the defense mechanisms are overwhelmed by massive or continued contamination by introduction of an infection into the sterile peritoneal environment through organ perforation. For example, peritoneal injection of LPS can promote peritonitis by inducing neutrophil recruitment to the peritoneal cavity [303]. The defense mechanisms rely on the activation of the resident macrophages in the peritoneal cavity. The peritoneal macrophages are responsible for recruiting neutrophils at the early stage of inflammation through the secretion of the neutrohpil chemoattractants CXCL1/CXCL2 [324-326]. We first treated mice with the <10KDa fractionate of bacteria supernatant prior to LPS stimulation to determine if the <10KDa fractionate of bacterial supernatant can suppress macrophage activation in the peritoneal cavity. Our findings reported herein suggest that the recruitment of neutrophils to the peritoneum after intraperitoneal administration of LPS was significantly attenuated in mice that previously received <10KDa fractionate of bacterial supernatant. However, the <10KDa fractionates of bacterial supernatant cannot completely abolish the neutrohpil recruitment upon the injection of LPS stimuli. This suggests that there is an alternative pathway for the migration of neutrophils which might not be influenced by the <10KDa fractionates of

bacterial supernatants. Besides macrophages, mast cells also participate in the rapid initial recruitment of neutrophil during tissue inflammation. Mast cells also secrete neutrophil chemokine-attractants CXCL1/CXCL2 [324,326]. Other studies have shown that microbiota strongly influence the neutrophil recruitment. Particuarly, in germ-free mice, the recruitment of neutrophils to the peritoneal cavity is markedly impaired upon injection of the chemical zymosan [327]. Future studies should focus on the effects of the <10KDa fractionates on other peritoneal cells and the role of microbiota in the process of the neutrophil recruitment.

Though our <10KDa fractionates of bacterial supernatants can suppress macrophage response *in vitro* as well as suppress the neutrophil recruitment *in vivo* experiments, we were uncertain whether <10KDa fractionates can indeed suppress macrophage responses in the gastrointestinal system since the gastrointestinal tract is a very complex system that consists of multi-layers that prevent the external factors from entering into the lamina propria. In the GI tract, during the steady state, both commensal and pathogenic bacteria are excluded from the lamina propria by the intestinal epithelium together with the thick layer of mucus. The presence of intestinal epithelium acts as an intestinal barrier and allows the host to absorb nutrients. We further tested the suppression of <10KDa fractionates on macrophage response to LPS or MSU crystal stimuli in a transwell system, a system that mimicked the GI tract in a very simplified way. The transwell system consists of upper chamber and bottom chamber. In this transwell system, we cultured differentiated CACO-2 cells at the upper chamber to form an artificial epithelium while we cultured differentiated THP-1 cells at the bottom chamber to form a population of macrophages. Our findings reported that when the

<10KDa fractionate of the bacterial supernatant was added directly to the intestinal epithelium, it significantly inhibited macrophage production ofpro-inflammatory cytokine TNF-α or IL-1β secretion upon LPS or MSU crystal stimululation. Interestingly, the whole bacteria supernatant that contained both the <10KDa fractionate, and the >10KDa fractionate also were able to significantly inhibit LPS- or MSU crystal-stimulated macrophage to secrete TNF-α or IL-1β. In contrast, the >10KDa fractionate failed to suppress macrophage response in the presence of LPS or MSU crystal stimulation. Furthermore, the treatment of >10KDa fractionate, <10KDa fractionate or the whole bacteria supernatant directly on differentiated CACO-2 epithelium did not promote paracellular permeability. This suggested that <10KDa fractionates could suppress macrophage responses in the presence of epithelium cells. However, we did not know the mechanisms of <10KDa fractionates to suppress macrophage in the presence of epithelium. There are two possible ways that we suspected that <10KDa may suppress macrophages in the GI system. The first one is that <10KDa may act on intestinal epithelium to promote cytokine signaling to inhibit macrophage responses. The other one is that <10KDa fractionates may be transported into the epithelium cells via receptors on epithelium cells and then transported into the lamina propria to directly inhibit macrophage response. It is unknown how <10KDa can suppress macrophages in the GI system as well as the mechanisms that <10KDa can suppress macrophages with in the GI system.

On the other hand, we believed that the >10KDa fractionates could act as a stimulatory factor to induce macrophage activation. This stemmed from our preliminary

studies that >10KDa fractionates had the highest level of TNF-α secretion from macrophages compared to <10KDa fractionates or the whole, un-fractionated bacterial supernatants (Data not shown). Our findings reported that >10KDa fractionates failed to suppress macrophage responses in the presence of LPS or MSU crystal stimulation. Additionally, >10KDa fractionates did not promote paracellular transports between differentiated CACO-2 epithelium cells in the transwell system, and *in vitro* experiment, >10KDa fractionates required TLR2 instead of TLR4 to promote pro-inflammatory responses from macrophages. This suggested that during the steady state, >10KDa fractionates were excluded from the lamina propria due to the presence of intestinal epithelium, and hence our >10KDa fractionates could not assert their effects on macrophages directly. However, it is possible that intestinal macrophages may capture some of the >10KDa fractionates since they provide an important means of sampling luminal contents by extending TEDs toward the lumen to capture any antigenic stimuli or live bacteria [266], and subsequently, these samples are then transported to the neighboring DCs for T-cell priming process [266-267]. Collectively, though the >10KDa fractionates may be excluded from the lamina propria by the intestinal epithelium, we were not sure whether the >10KDa fractionates were accidentally taken by TED. However, it was unlikely to happen because only small group of macorphages which express CX_3CR1 [318] can promote transepithelial uptake from the lumen, and these macrophages may mainly function as a transporter to transfer the lumen samples to the DCs for T cell priming [266-267].

Commensal bacteria secrete <10KDa factors as well as >10KDa factors, and these factors play a different role on macrophage response. While <10KDa factors have an inhibitory effect on macrophage responses, >10KDa fractionates promote pro-inflammatory responses from macrophages. Together, both the >10KDa fractionates and the <10KDa fractionates provide a "yin-yang" of commensal bacteria in controlling the host macrophage immune responses. During inflamed state or pathogenic infection, the disruption of intestinal epithelium may allow substances represented in the >10KDa fractionates to enter into the lamina propria and cause a pro-inflammatory response from macrophages. Such pathogenic infection or inflammation disrupts the balance of "yin and yang" between commensal bacteria and host immunity. Overall, both the >10KDa fractionates and the <10KDa fractionates secreted from commensal bacteria are important to regulate macrophage responses in the host GI system.

Currently, the main issue now is that we do not know what is present in the <10KDa fractionates which are secreted from commensal bacteria. Since our preliminary experiments showed that there was no difference in the level of TNF-α secretion when the whole bacterial supernatants were treated either with DNAse, RNAse or proteinase, it suggested that the factors present in the supernatants might not be DNA, or RNA or proteins. Additionally, it is complicated to completely separate sugar groups from fatty acids since we suspect that the carbohydrate group might be conjugated with fatty acids. Thus, we believe what was present in our supernatants that contributed to macrophage function might be either fatty acids alone, or a carbohydrate group or the conjugation of

carbohydrates and fatty acids. We speculated that at least three components which might be present in the <10KDa fractionates are the following: 1) short-chain fatty acids (SCFAs), 2) polyunsaturated fatty acids (PUFAs), and 3) exopolysaccharides.

SCFAs are defined as 1–6 carbon volatile fatty acids existing in straight- and branched-chain conformations. They are formed during bacterial fermentation of carbohydrates in the colon. In the gut, the main SCFAs produced are acetate, propionate, and butyrate. These SCFAs are accountable for immune-suppression on host immune cells. For example, SCFAs can modulate inflammation and affect several leukocyte functions. They suppress NF-kB signaling pathway and inhibit the secretion of the pro-inflammatory cytokines (such as TNF-α and IL-6 cytokines) and ROS species (such as NO production) [276]. On the contrary, these SCFAs induce the secretion of the anti-inflammatory cytokines (such as IL-10) [276]. *In vitro*, the interaction of SCFAs down-regulates pro-inflammatory responses of the effect innate cells in the GI tract. Specifically, lactate and SCFA down-regulate pro-inflammatory cytokine secretion by LPS-activated macrophages, particularly IL-6, and IL-12 [277]. SCFAs significantly suppress the pro-inflammatory response of intestinal epithelium cells [277]. In humans, when the concentration of SCFAs (such as acetates, propionates, and butyrates) reaches 30 mM, these SCFAs suppress TNF-α secretion by LPS-stimulated neutrophils [278]. Similarly, in mice, propionates and butyrates can inhibit NF-κB activation signaling pathway by suppressing the production of pro-inflammatory cytokines (such as TNF-α, CINC-2αβ) and other ROS species (such as NO) secreted by neutrophils [279].

Additionally, the recruitment of neutrophils to the peritoneal cavity upon to the

exposure of inflammatory oyster glycogen solution was attenuated in mice that previously received tri-butyrin, a precursor form of butyrate. Additionally, oral administration of acetate in drinking water has been shown to suppress the chemically DSS -induced inflammatory colitis dependent on the G-protein-coupled Receptor 43 (GPR43) [16]. During adaptive immune responses, SCFAs are important for the differentiation of FoxP3+Tregs that are critical for anti-inflammatory response [9,222-223]. Mainly, butyrate is responsible for the differentiation of FoxP3+Tregs from naïve CD4+T cells *in vitro* [9,223] by inhibiting the activity of histone deacetylases [224]. Butyrates also induce the secretion of IL-10 and retinoic acids from DCs and macrophages, in which these molecules are essential for the proliferation and survival of IL-10–producing Tregs in the colon [225]. Unlike butyrate which has a function for Treg differentiation, the other two SCFAs, acetate and propionate, are important for the migration of Tregs into the colon by inducing the expression of homing molecules on Tregs [223]. Also, acetate has a role in enhancing gut epithelial barrier functions and suppressing colonic inflammation in the mouse model during *Escherichia coli* O157: H7 infection [176]. Since SCFAs have been well documented for inhibitory effects on inflammatory responses of both innate and adaptive immune responses, it is possible that SCFAs might be present in our <10KDa fractionates.

Besides SCFAs, poly unsaturated fatty acids (PUFAs) are believed to be present in the <10KDa fractionates. Polyunsaturated fatty acids are fatty acids that contains more than one carbon double bond. In humans, the most important metabolisms are ω-6 (n-6) along with ω -3 (n-3) poly unsaturated fatty acids (PUFAs) which are part of essential

nutrients for humans. Important ω -3 PUFAs are involved in human nutrition. These are α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). These n-3 PUFAs can be found in plant oil, vegetable oil, nuts as well as fish oils or other marine foods. As mentioned before, ω-6 fatty acids are also important fatty acids that are essential for humans. These fatty acids are mainly found in vegetable oil, nuts, and eggs. The major ω -6 fatty acids that are critical to humans are linoleic acids, γ- linolenic acid and arachidonic acids. While ω -3 fatty acids have an anti-inflammatory function that can suppress the immune responses, ω -6 fatty acids, on the other hand, can induce both host pro-inflammatory and anti-inflammatory responses. Interestingly, the probiotic *Lactobacilli* spp. adhere to intestinal surfaces and produce mostly ω -3 fatty acids such as EPA, DHA, and ALA and some ω -6 fatty acids, for instance, conjugated linoleic acid (CLA) isomers and γ-linolenic [280]. Previous studies found that ω -3 fatty acids have anti-inflammatory properties. Specifically, they can decrease chemotaxis of neutrophil and monocytes toward various chemical attractants [281-283]. They inhibit the secretion of pro-inflammatory IL-6 and IL-8 cytokines from human endothelial cells [284-285] as well as TNF-α from cultured monocytes [286-291]. They also inhibit inflammatory diseases and metabolic disorders through the suppression of NLRP3 inflammasome and further prevent the subsequent caspase-1 catalytic reaction and IL-1β secretion, where they are important to promote inflammatory responses in the host. *In vitro*, ω -3 fatty acids have been shown to suppress the T-cell proliferation as well as the production of IL-2 [292-298]. In contrast, ω -6 fatty acids display both pro and anti-inflammatory properties. Consuming enriched ω -6 fatty

acids can alleviate chemically-induced small intestinal damage via inhibition of leukocyte infiltration [299]. Furthermore, γ -linolenic acid and its intermediate mediators have an impact on many host gene expressions that are involved in the host immune functions as well as cell apoptotic pathways [300-302]. Collectively, it is possible that the <10KDa fractionates may contain both ω -3 and ω -6 fatty acids that may take part in the regulation of macrophage activation.

Lastly, exopolysaccharides (EPS) have a potential to limit inflammation. As mentioned previously EPS have a structure that comprises the carbohydrate polymer layer on the surface of many different microorganisms. The anti-inflammatory properties of EPS have been studied intensively in the past few years. For instance, a well-known EPS secreted by *B. fragilis*, known as polysaccharide A (PSA) can suppress the pro-inflammatory responses of IL-17 A by directly inducing IL-10 producing-Tregs and limit the resistance of the pathogenic infection via TLR-2 signaling pathway [91-94,227-232] and induce the expansion of the T-cell population and resolution of the aberrant TH1/TH2 imbalances [230] in GF mice. EPS secreted by *B. fragilis* also can induce a unique subset of M2 macrophages in which this subset of macrophages can promote the secretion of inhibitory cytokine IL-10 that can suppress the inflammatory responses via TLR-2 signaling pathway and protect the host from the chemically-induced colitis [233]. Sphingolipids, a particular type of EPS secreted by *B. fragilis*, can rescue mice from the chemically-induced colitis which is due to the accumulation of the invariant natural killer T-cells (iNKTs) [237]. In this case, the presence of sphingolipids can limit the the number of iNKT cells, thus, it protect host from such an inflammatory-mediated colitis. As

mentioned before, the nature and characteristics of EPS are dependent on the original sources that they come from. For example, EPS secreted by *B. breve* can inhibit antibody production [234] which reflects the nature of *B.breve* strains that participate in the host gastrointestinal microbiota. *B.breve* bacteria probably have developed several microbial products to restrict the host immune response to be able to reside in the host GI lumen. In contrast, EPS secreted by *L. kefiranofaciens* can promote the proliferation of plasma cells and induce the activation of the pro-inflammatory subset of macrophages [235-236]. Altogether, EPSs may play a role as an immune modulator. Since EPS possesses both anti-and pro-inflammatory properties, we do not know its effects on macrophage response in the intestinal immune system and whether EPS is present in our <10KDa fractionates.

Taken together, our recent findings demonstrate the immuno-regulation of commensal bacteria by secreting various factors that have different effects on macrophages and modulate macrophage function. The next step is to identify the characteristics of the molecules present in our bacterial supernatants. Further exploration may lead to protential drug discoveries for the future treatment of gastrointestinal disorders in humans.

CHAPTER FIVE

SUMMARY

In summary, the purpose of this project was to better understanded how commensal bacteria are able to immuno-regulate the activity of intestinal macrophages in the GI tract system. It was initially hypothesized that the secreted bacterial factors/metabolites present in the bacterial suppernantant can suppress macrophage activation. We discovered that different molecular weights of bacterial factors/ metabolites present in bacterial culture supernatants have different roles in macrophage activation. In this case, different fractionates of bacterial supernatants (either <10KDa or >10KDa fractionates) exerted different effects on macrophage activation. Specifically, <10KDa fractionates of bacterial factors/ metabolites had a suppressive function on macrophage activation after exposure toTLR or NLRP3 agonists *in vitro* as they significantly inhibitd the secretion of pro-inflammatory (TNF-α, IL-6 and IL-1β respectively) cytokines. *In vivo* these <10KDa fractionates of bacterial supernatants were able to suppres neutrophil recruitment in the peritoneal cavity of mice upon LPS stimulation. Furthermore, this suppression was also true in the presence of the artificial epithelial cells as we employed the transwell system to test whether the presence of <10KDa fractionates alone or in the combination with >10KDa fractionates could suppress macrophage activation. As a result, the presence of <10KDa fractionates of bacterial supernatants alone or in the combination with >10KDa fractionates resulted in

significantly decreased secretion of pro-inflammatory cytokines (TNF-α or IL-1β) upon the exposure of TLR or NLRP3 agonists respectively to macorphages. On the contrary, we found that >10KDa fractionates could act as stimulator for macrophage macrophage; and they required TLR2 to elicit pro-inflammatorr responses from macrophages.

These findings further supported the idea that commensal bacteria can communicate with the host immune system through the secretion of microbial factors/ metabolites. Particularly, our study demonstrated that different molecular weights of microbial factors/ metabolites exert different effects on the intestinal macrophages' functions. Further studies should be conducted to elucidate the actual identities of <10KDa probiotic factors present in our commensal supernatants. This identification might provide a potential therapeutic agent to treat gastrointestinal disorders in humans and a better understanding of the symbiotic relationship between host and mucosal microbiota.

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