Functional and Transcriptomic Changes in Synergy of Endotoxin and (1->3)-β-D-glucan in bone marrow derived macrophage from Fc gamma receptor IIb deficient mice

Miss Jiraphorn Issara-amphorn

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Medical Microbiology,Interdisciplinary Program
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หน้าที่และการเปลี่ยนแปลงการแสดงออกของยีนในเซลล์ Macrophage จากเซลล์ไขกระดูก ที่ถูกกระตุ้นด้วย Endotoxin และ (1->3)-β -D-glucan ของหนูที่ขาดยีน *Fcgr2b*

น.ส.จิราพร อิสระอัมพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Functional and Transcriptomic Changes in Synergy of Endotoxin and (1->3)-β -D-glucan in bone marrow derived macrophage from Fc gamma receptor IIb deficient mice
By	Miss Jiraphorn Issara-amphorn
Field of Study	Medical Microbiology
Thesis Advisor	NATTIYA HIRANKARN, M.D. Ph.D
Thesis Co Advisor	ASADA LEELAHAVANICHKUL, M.D. Ph.D

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the GRADUATE SCHOOL

(THUMNOON NHUJAK, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(RANGSIMA REANTRAGOON, M.D. Ph.D)

..... Thesis Advisor

(NATTIYA HIRANKARN, M.D. Ph.D)

..... Thesis Co-Advisor

(ASADA LEELAHAVANICHKUL, M.D. Ph.D)

..... Examiner

(TANAPAT PALAGA, Ph.D.)

..... Examiner

(PATCHAREE RITPRAJAK, Ph.D.)

..... External Examiner

(Prapaporn Pisitkun, M.D.)

จิราพร อิสระอัมพร : หน้าที่และการเปลี่ยนแปลงการแสดงออกของยีนในเซลล์ Macrophage จากเซลล์ไขกระดูก ที่ถูกกระตุ้นด้วย Endotoxin และ (1->3)-β -D-glucan ของหนูที่ขาดยีน *Fcgr2b*. (Functional and Transcriptomic Changes in Synergy of Endotoxin and (1->3)-β -D-glucan in bone marrow derived macrophage from Fc gamma receptor IIb deficient mice) อ.ที่ปรึกษาหลัก : ศ. ดร.ณัฏฐิยา หิรัญกาญจน์, อ.ที่ปรึกษาร่วม : ผศ. ดร.อัษฎาศ์ ลีฬหวนิชกุล

โรคเอสแอลอีเป็นโรคภูมิคุ้มกันบกพร่องเรื้อรัง และผู้ป่วยจะมีอาการทางคลินิคที่หลากหลาย สาเหตุของการเกิดโรคเอสแอลอีมีปัจจัยที่หลากหลาย รวมไปถึงปัจจัยจากสิ่งแวดล้อม ที่ทำให้เกิดการดำเนินไปของโรค ในงานวิจัยนี้ ได้ทำการศึกษาเกี่ยวกับผลกระทบสภาวะลำไส้รั่ว ต่อการติดเชื้อในกระแสเลือดของหนูที่เป็นโรคเอแอลอีโดยใช้โมเดลของหนู Fcgr2b-deficient โดยทำการศึกษาในหนูอายุ 8 สัปดาห์ และ 40 สัปดาห์ เพื่อเป็นตัวแทนของกลุ่มที่มีไม่มีอาการและมีอาการตามลำดับ การเกิดสภาวะลำไส้รั่วที่เกิดขึ้นเองสามารถทำการตรวจสอบได้ด้วยการวัดระดับของ FITC-dextran ในซีรุ่ม, การตรวจพบระดับของ LPS ในซีรุ่ม, และการตรวจพบระดับของ (1->3)-beta-D-glucan ในเซรุ่ม ซึ่งในกลุ่มของหนูที่มีอาการ (40 สัปดาห์) จะตรวจพบการแสดงออกของโมเลกุลเหล่านี้ในกระแสเลือด แต่ไม่พบการแสดงออกของโมเลกุลเหล่านี้ในกลุ่มของหนูที่ไม่มีอาการ นอกจากนี้ยังพบว่าการเพิ่มระดับของ (1->3)-beta-D-glucan ในคนไข้เอสแอลอีโดยไม่พบการติดเชื้อราในกระแสเลือด การจำลองทำให้เกิดสภาวะลำไส้รั่วในหนู โดยการให้หนูดื่มน้ำผสม Dextran sulfate solution หรือการฉีด LPS ร่วมกับ (1->3)-beta-D-glucan ในหนู หรือทำการฉีดทั้งสองโมเลกุลร่วมกัน พบว่า การเหนี่ยวนำทำให้เกิดสภาวะลำไส้รั่ว,การฉีด LPS ร่วมกับ (1->3)-beta-D-glucanในหนู และการฉีด LPS อย่างเดียวในหนู ทำให้อาการของการติดเชื้อในกระแสเลือดในหนูแย่ลงโดยใช้โมเดลการผ่าตัดที่เรียกว่า cecal ligation and puncture (CLP) และระดับความรุนแรงของการติดเชื้อในกระแสเลือดยังมีความรุ่นแรงมากขึ้นในกลุ่มของหนูเอสแอลอีอายุ 8 สัปดาห์ นอกจากนี้ การกระตุ้นเซลล์มาโครฟาจในหนูลูปัส ด้วย LPS ร่วมกับ (1->3)-beta-D-glucan สร้าง cytokine ที่เกี่ยวข้องกีบกระบวนการอักเสบเพิ่มมากขึ้น เมื่อเทียบกับเซลล์ มาโครฟาจจากหนูปกติ นอกจากนี้ยังพบการแสดงออกของยีน *FcgRs*, *NF-kB* และ *Syk* เพิ่มมากขึ้น เมื่อเปรียบเทียบกับมาโครฟาจในหนูปกติ จากการทดลองการใช้ ตัวยับยั้งสัญญาณต่างๆ และโปรตีนที่เกี่ยวข้องกับการส่งสัญญานใน มาโครฟาจ พบว่า การใช้ตัวยับยั้งตัวส่งสัญญาณ Dectin-1, Syk และ NF-kB สามารถลดระดับการอักเสบได้โดยการวัดจากระดับการแสดงออกของ TNF-alpha cytokine แต่การใช้ตัวยับยั้งโปรตีน Raf1 พบว่าไม่สามารถลดระดับการอักเสบได้ ดังนนั้น โปรตีนที่สำคัญในการส่งสัญญาณของมาโครฟาจที่ถูกกระตุ้นด้วย LPS ร่วมกับ (1->3)-beta-D-glucan นอกจากนี้ ระดับการแสดงออกของโปรตีน Syk ยังพบในหนูลูปัสมากกว่าหนูปกติ ดังนั้นโปรตีน Syk จึงเป็นโปรตีนที่มีบทบาทหน้าที่สำคัญในกระบวนการอักเสบ จากนั้นจึงได้ทำการทดสอบโดยการให้หนูกินตัวยับยั้งสัญญาณ Syk พบว่าหนูลูปัสมีอากการดีขึ้น และการให้ตัวยับยั้ง Syk ยังช่วยลดการอักเสบในหนูลูปัสที่ถูกทำให้ติดเชื้อในกระแสเลือดด้วยโมเดล CLP นอกจากนี้การใช้ตัวยับยั้งโปรตีน Syk ในเซลล์มาโครฟาจที่ถูกกระตุ้นด้วย LPS ร่วมกับ (1->3)-beta-D-glucan ผลการตรวจสอบระดับการแสดงออกของยีนโดยเทคนิค RNA sequencing พบว่ามาโครฟาจที่ถูกกระตุ้นด้วย LPS ร่วมกับ (1->3)-beta-D-glucan พบว่า Syk ลดระดับการแสดงออกของยีนที่เกี่ยวข้องกับการอักเสบ นอกจากนี้กลุ่มของยีนที่พบในมาโครฟาจที่ถูกกระตุ้นด้วย LPS ร่วมกับ (1->3)-beta-D-glucan ยังเกี่ยวข้องกับการแสดงออกของยีนที่พบในกลุ่มผู้ป่วยที่มีอาการรุนแรงจนถึงเสียชีวิต และพบว่าเมื่อใช้ตัวยับยั้ง Syk ในมาโครฟาจ การแสดงออกของยีนได้เปลี่ยนแปลงไปจากทิศทางเดิม ดังนั้น ตัวยับยั้ง Syk อาจจะเป็นยาทางเลือกสำหรับการรักษาเอสแอลอี

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Jiraphorn Issara-amphorn : Functional and Transcriptomic Changes in Synergy of Endotoxin and (1->3)-β-D-glucan in bone marrow derived macrophage from Fc gamma receptor IIb deficient mice . Advisor: NATTIYA HIRANKARN, M.D. Ph.D Co-advisor: ASADA LEELAHAVANICHKUL, M.D. Ph.D

Systemic lupus erythematosus (SLE) is an autoimmune disease with a diverse array of clinical symptoms. There are many factors including environmental factors may trigger disease progression. In this study, we investigated the influence of spontaneous gut leakage upon polymicrobial sepsis in Fc gamma receptor IIb deficient ($FcgRIIb^{-/-}$) mice aged 8 and 40 weeks, as representing asymptomatic and symptomatic lupus, respectively. The spontaneous gut leakage as determined by i) the level of serum FITC-dextran, ii) increased serum endotoxin (LPS), and iii) increased serum (1->3)-b-D-glucan (BG), were demonstrated in symptomatic $FcgRIIb^{-/-}$ mice, but not in asymptomatic group. Moreover, spontaneous gut leakage was detected by increased serum BG without fungal infection was also found in SLE patients with lupus nephritis. Gut leakage induced by dextran sulfate solution (DSS) or LPS injection with BG or LPS alone, but not BG alone, enhanced the severity of cecal ligation and puncture (CLP) sepsis more prominently in 8-week-old $FcgRIIb^{-/-}$ mice. In addition, BG+LPS activated $FcgRIIb^{-/-}$ macrophages produced higher level of proinflammatory cytokine when compared with wild type macrophage. Moreover, BG+LPS activated macrophages enhanced expression of activating FcgRs, NF-kB and Syk compared with wild type macrophage. The inhibitor against Dectin-1, Syk and NF-kB, but not Raf-1, reduced supernatant TNF-α in BG+LPS activated macrophages implying Syk dependent signaling. Additionally, Syk abundance in $FcgRIIb^{-/-}$ mice and in CLP surgery were higher than wild type mice possibly due to several Syk-activator (anti-dsDNA, LPS and BG), and Syk inhibitor attenuated proteinuria and serum cytokine only in $FcgRIIb^{-/-}$ mice. Moreover, administration of a Syk inhibitor prior CLP surgery in $FcgRIIb^{-/-}$ mice attenuated sepsis severity as evaluated by mortality, organ injury, Serum LPS and post serum cytokines. Furthermore, RNA sequencing analysis of BG+LPS activated macrophages with or without Syk inhibitor treatment, we found that Syk inhibitor downregulated several inflammatory pathways in macropahges, suggesting the potential anti-inflamatory impact of Syk inhibitor in lupus. The genes that found in BG+LPS activated macrophages associated with the prediction of genes involve in mortality in sepsis patients and Syk inhibitor treatment reversed almost direction of those genes.

In conclusion, spontaneous gut leakage and the induction of gut permeability worsened sepsis severity. Gut translocation of LPS and BG showed minor effect on wild type mice, but the synergistic effect of BG and LPS was prominent in $FcgRIIb^{-/-}$ mice, suggesting the therapeutic strategies addressing gut leakage may be interest in sepsis condition in patient with sepsis. In addition, Syk signaling is promising therapeutic target for SLE patients. Syk inhibitor appears to be an alternative drug for treatment of lupus with a counter on sepsis.

Field of Study:	Medical Microbiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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Jiraphorn Issara-amphorn

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LIST OF ABBREVIATIONS

BG	(1→3)-β-D-glucan
bp	Base pair
CLP	cecal ligation and puncture
DD	Death domain
DSS	dextran sulfate solution
EBV	Epstein–Barr virus
FcgRIIb-/-	Fc gamma receptor IIb deficient
FcgRs	Fc gamma receptors
GI	gastrointestinal
IFN	Interferon
IKK	IκB kinase
IL-10	Interleukin 10
IL17	Interleukin 17
IL-21	Interleukin 21
IL-6	Interleukin 6
IRAK1	IL-1 receptor-associated kinase-1
IRAK2	IL-1 receptor-associated kinase-2
IRAK4	IL-1 receptor-associated kinase-4
IRF5	Interferon regulatory factor 5
ITGAM	Integrin alpha M

LN	Lupus nephritis
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NF-κB	Nuclear factor-κB
PAMPs	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
STAT4	Signal transducer and activator of transcription 4
Syk	Spleen tyrosine kinase
T1D	Type 1 diabetes
TAK1	Transforming growth factor-β-activated kinase 1
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TJ	Tight junction
TLR-4	Toll like receptor 4
TLR7	Toll like receptor 7
TLR9	Toll like receptor 9
TNF-α	Tumor necrosis fctor alpha
TRAF3	TNF-receptor-associated factor 3
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF- related adaptor molecule

TRIF TIR domain-containing adaptor protein including IFN-β

CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by immunologic disorder involves innate and adaptive immune system. The hallmark of SLE is an autoantibody direct against anti-double stranded DNA resulting in presentation of the immune complexes in the blood circulation. These immune complexes caused illness when they are deposited in many organs including joint, skin, and kidney [1]. Moreover, gut dysbiosis can be considered a key factor for enhancing SLE progression. Although, the effect of gut dysbiosis on the SLE enhancement has been explored [2], the data about the effect of gastrointestinal (GI) leakage in the lupus are limited. Interestingly, increased circulating of endotoxin, an outer membrane of gram-negative bacterial cell wall was commonly found in active lupus patients [3]. A Previous study showed that oral antibiotics given during active disease progression attenuated disease progression in SLE prone mice [4]. Our gastrointestinal tract can be considered as an endogenous source of endotoxin, endotoxemia in active lupus may be caused by gut leakage. Furthermore, gut fungi are also considering as a common commensal organism in the human GI tract[5]. (1$\rightarrow$3)-β-D-glucan (BG) is a major component of a fungal cell wall, translocation of this molecule from the gut to the blood may also play a role in SLE. Of note, the synergistic effect of lipopolysaccharide (LPS) and BG on the immune activation and inflammation have been demonstrated [6]. The inflammation caused by the synergistic effect of these two molecules might be one of the precipitating-factor in SLE.

Fc gamma receptor IIb is an only inhibitory receptor among the FcgR family. The association between Fc gamma receptor IIb (FcgRIIb) dysfunction polymorphism is well known. Interestingly, FcgRIIb knockout mice (FcgRIIb$^{-/-}$) on the C57BL/6 background established an autoimmune disease with autoantibodies against double stranded DNA, elevated proteinuria, and immune complex deposition in the kidney. FcgRIIb deficient mice developed full-blown of lupus nephritis (LN) after 32-40-week-old [7, 8]. Moreover, FcgrIIb polymorphism is high in Asian population. Thus, the FcgRIIb$^{-/-}$ can be

consider as a good mouse model resembling SLE disease for study underlying mechanism of the disease.

Interestingly, spleen tyrosine kinase (Syk), a common downstream signaling between FcgR, TLR-4, and Dectin1 [9, 10], that might be activated by those molecules from the gut translocation (LPS, BG) and the circulating immune complexes in lupus. All of these receptors play an important role in pathogenesis of lupus including disease acceleration and worsen disease prognosis by inflammation [11-13] Thus, Syk may act as a potential target for SLE treatment. In addition, Fostamatinib is known as R788, the Syk inhibitor is a US Food and Drug Administration approved for chronic immune thrombocytopenia treatment [14] and is considered as an interesting candidate for other immune diseases. Moreover, the Syk inhibitor has been reported to attenuate inflammatory response in several lupus mouse models but has never been tested in FcgRIIb$^{-/-}$. Therefore, the Syk inhibitor might have the potential effects in FcgRIIb$^{-/-}$ mice by attenuating the inflammatory responses caused by FcgR inhibitory defect.

SLE patients were susceptible to the severe infection and remain a source of mortality [15, 16]. SLE patients showed a higher rate of serious infection more than normal populations. Although, the effect of the Syk inhibitor in the autoimmune disease has been demonstrated in many studies, its effect on sepsis and sepsis with SLE background is unclear. The cecal ligation and puncture (CLP) model is a polymicrobial sepsis mouse model similar to those of lupus patients. The CLP sepsis-model develop gut permeability defect-induce endotoxemia and glucanemia. In addition LPS and (1$\rightarrow$3)-β-D-glucan were also found in the sepsis patient and the level of (1$\rightarrow$3)-β-D-glucan is compatible with the patient with fungal infection and was associated with sepsis severity [17]. LPS and (1$\rightarrow$3)-β-D-glucan are pathogen associated molecular patterns (PAMPs) that can activate innate immunity especially macrophages [18-20] and induce FcgR expression to relate adaptive immunity [7, 21, 22]. Increased the PAMPs on the circulation may worsen lupus progression and sepsis severity. Therefore, the study of the therapeutic effect of Syk inhibitors on sepsis super-imposed on lupus is of interest before clinical translation in patients with lupus.

For the proof-of-concept study, we explore the spontaneous gut leakage in SLE patient and mouse model and determine the role of the synergy of serum endotoxin and (1→3)-β-D-glucan on the severity of sepsis. Moreover, we explore the Syk inhibitor therapeutic effect on the FcgRIIb$^{-/-}$ mice and a polymicrobial sepsis model in FcgRIIb$^{-/-}$ knockout mice.

CHAPTER II

OBJECTIVE

Hypothesis

1. The molecule derived from the gut translocation involved in pathogenesis of sepsis in SLE.
2. The activation of Syk leading to the development of SLE by increased inflammation on the innate immune cells especially macrophage.
3. Syk inhibitor treatment leading to blocking of the important signaling involved in the inflammatory responses in SLE and in sepsis with SLE background.

Research question

1. Do the gut leakage and molecules that derived from the gut translocation affect the pathogenesis of sepsis in SLE and, if yes, what are the mechanisms?
2. Dose Spleen tyrosine kinase (Syk) inhibitor attenuates SLE progression and sepsis in SLE, if yes, what are the genes that involved in this scenario?

Objective

1. To determine the gut leakage and explore the possible molecules derive from the gut translocation on the pathogenesis of sepsis in SLE.
2. To investigate the effect molecule from the gut translocation on the severity of sepsis.
3. To determine the signaling pathway and the important signaling proteins in the synergy of endotoxin and (1$\rightarrow$3)-β-D-glucan.
4. To investigate the effect of Syk inhibitor in SLE progression and poly microbial sepsis with SLE background in lupus mouse model of *Fcgr2b*-deficient mice.
5. To investigate gene expression profile in response to LPS, BG, and LPS+BG before and after treatment with Syk inhibitor.

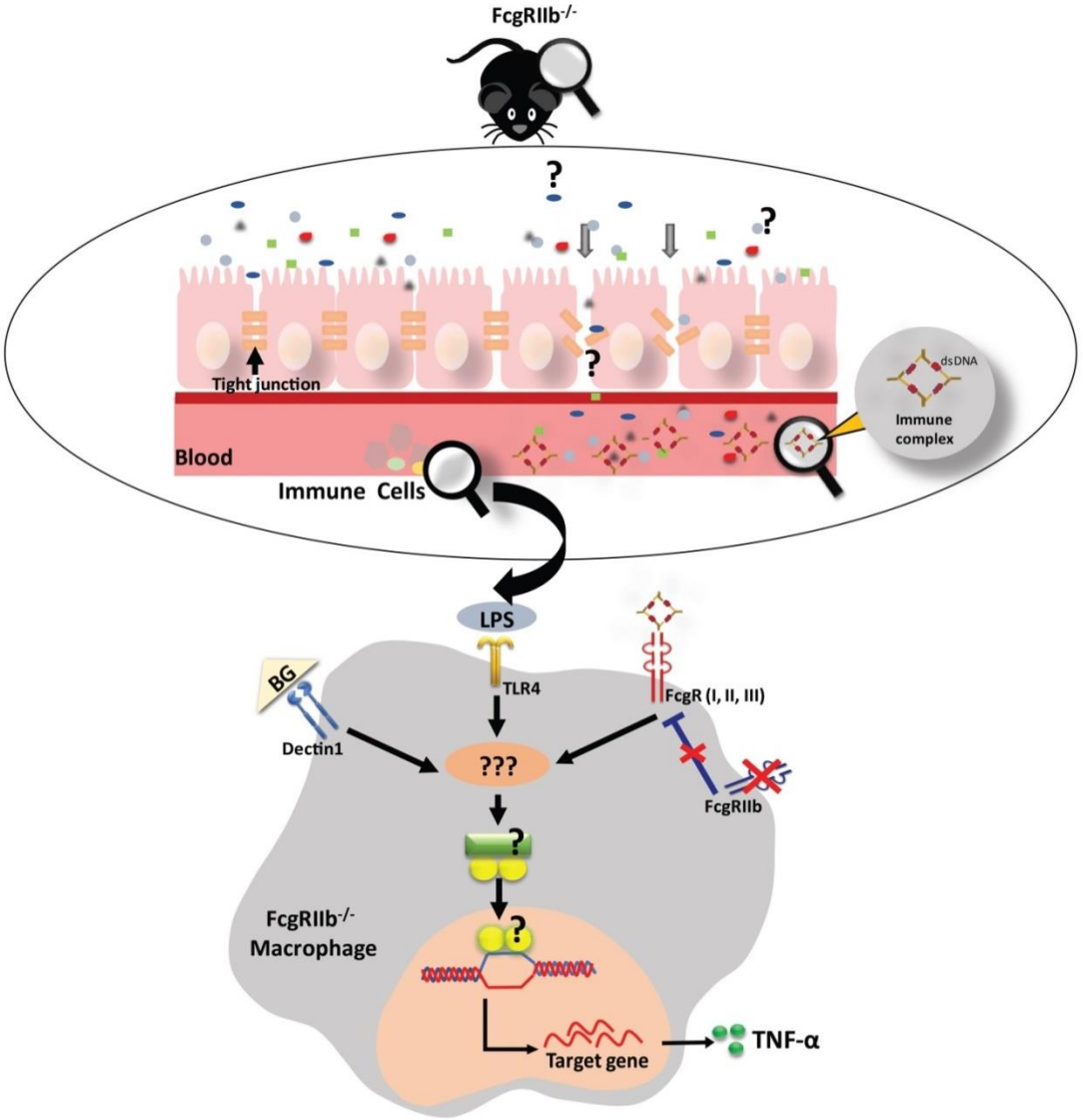
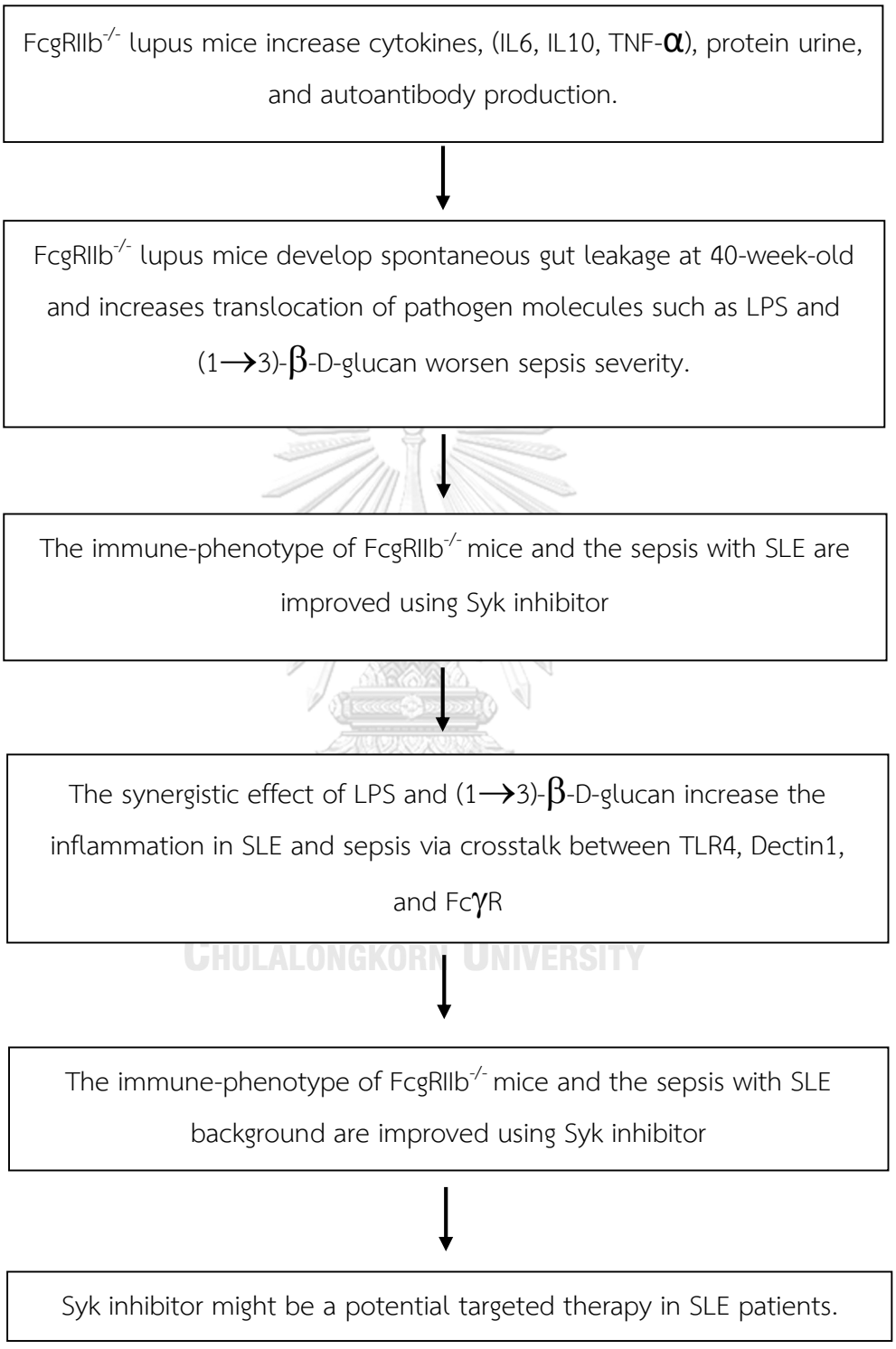


Figure 1 The overview of hypothesis, we hypothesized that if there is the gut leakage in the lupus and the possible molecule from the gut translocation might activate the immune system impact the pathogenesis of sepsis with SLE background.

Conceptual framework

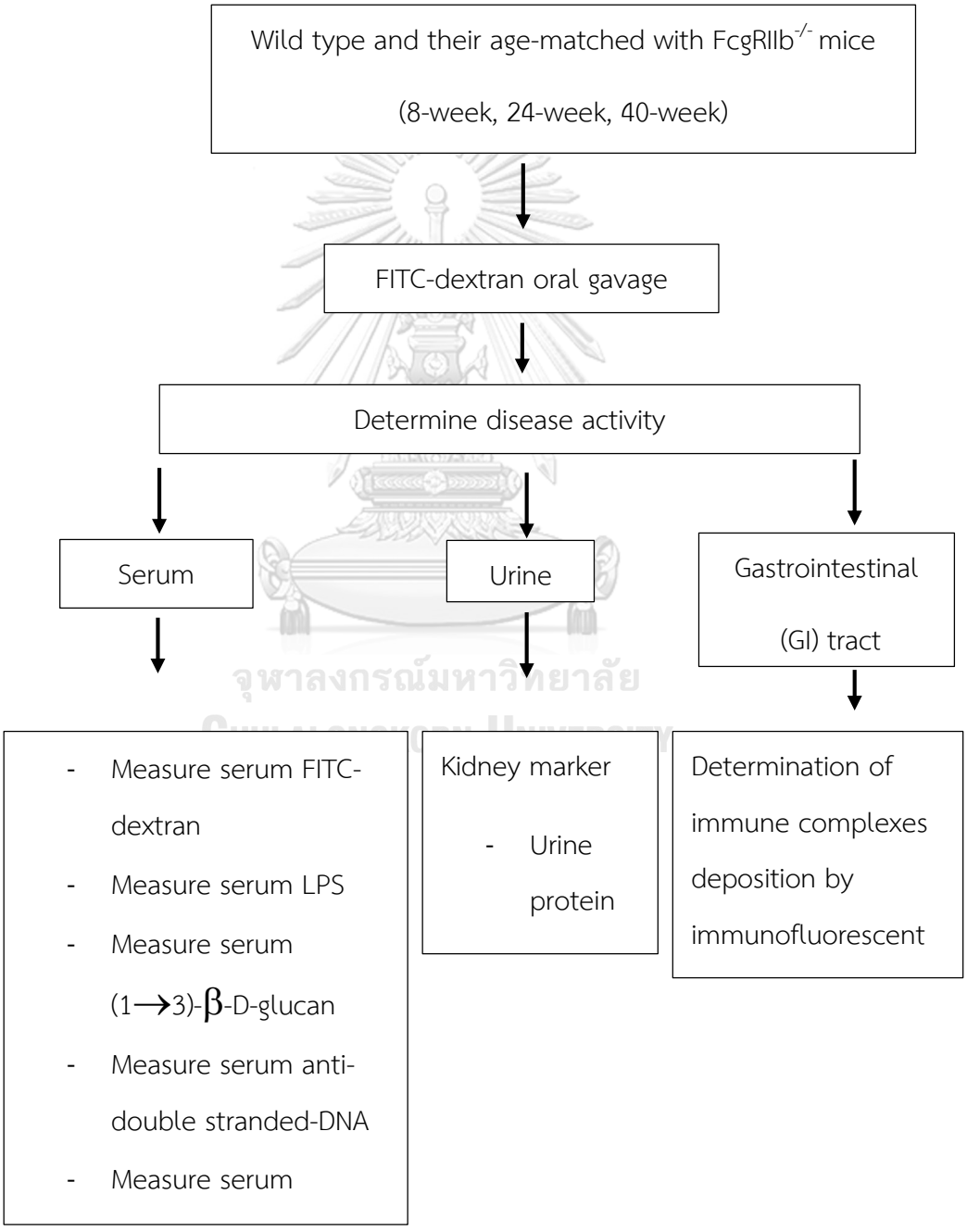


Research Design

This thesis can be divided into 3 parts as listed below

Part 1: Spontaneous gut leakage in *vivo*

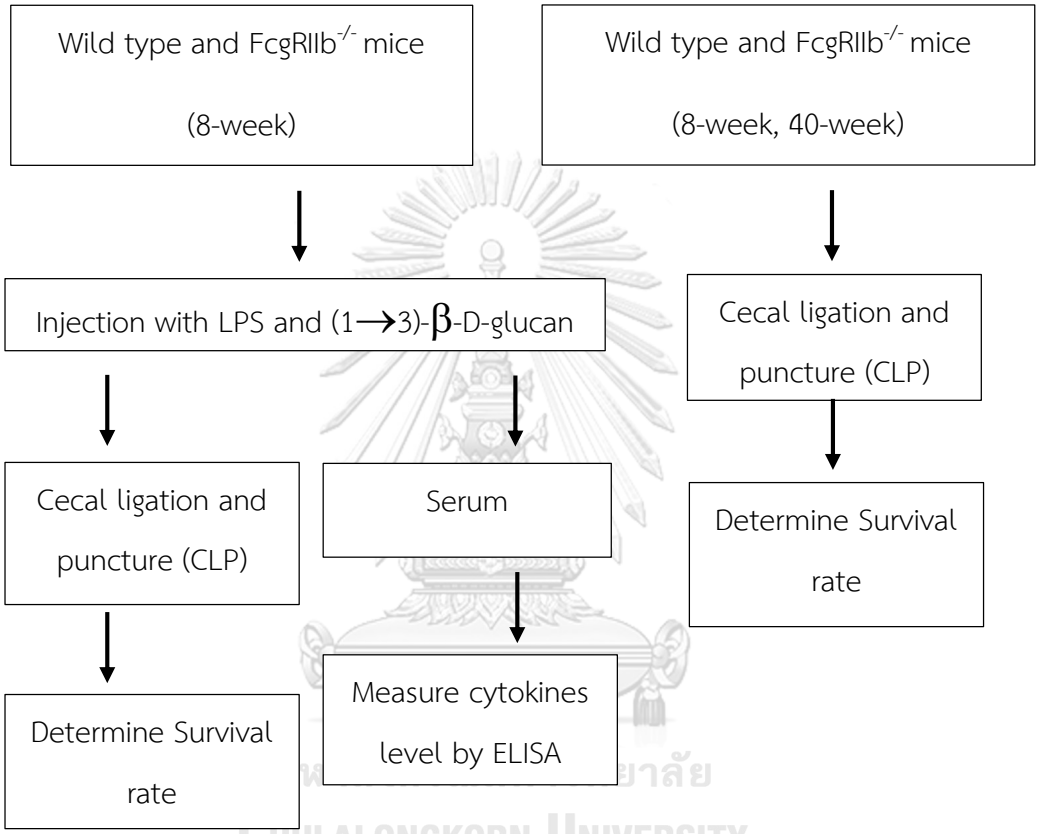
Objective: To determine the gut leakage and explore the possible molecules derive from the gut translocation on the pathogenesis of sepsis in SLE.



Part 2: The effect of the molecules derived from the gut translocation in pathogenesis of sepsis in SLE and possible mechanism.

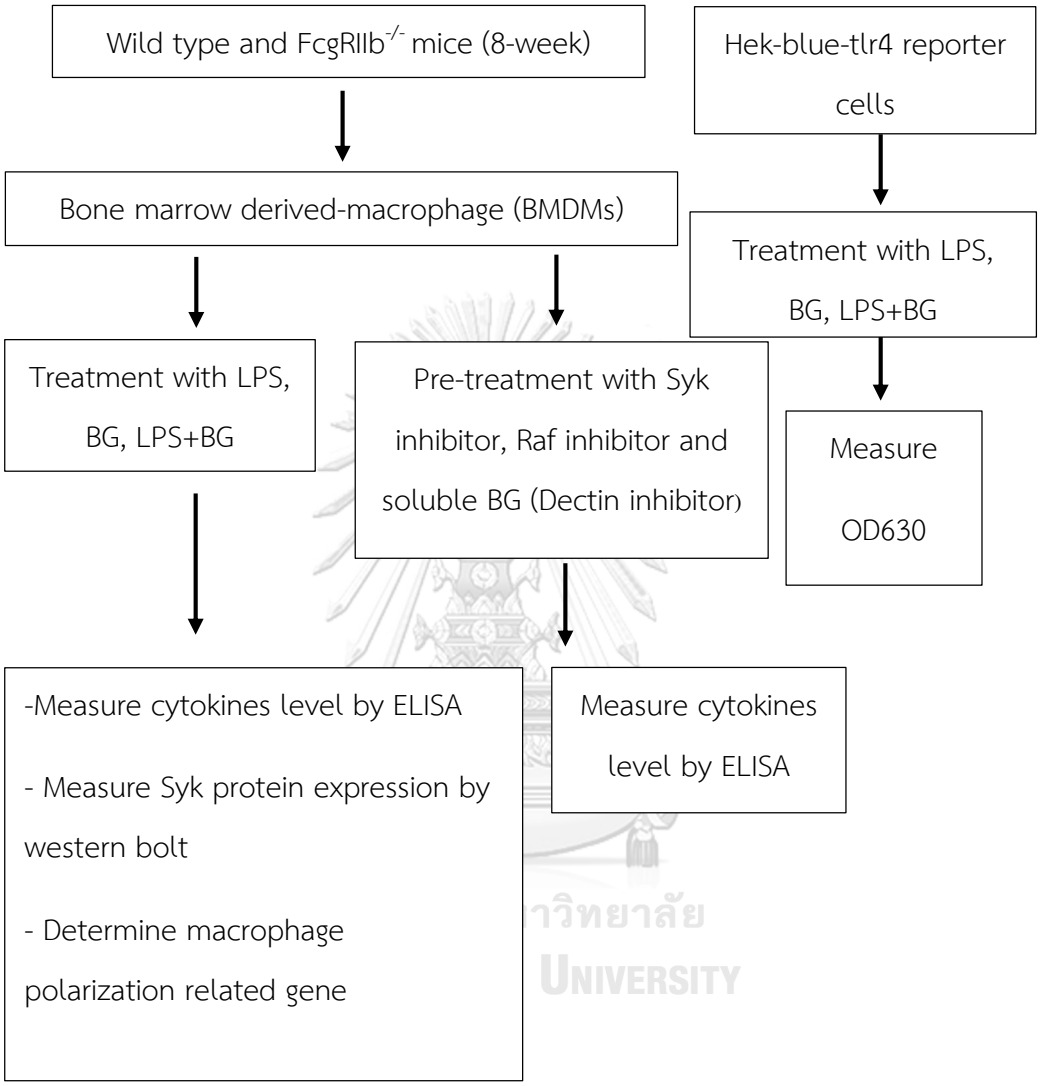
2.1 in *vivo* study

Objective: To investigate the effect of molecules from the gut translocation on the severity of sepsis.



2.2 in vitro study

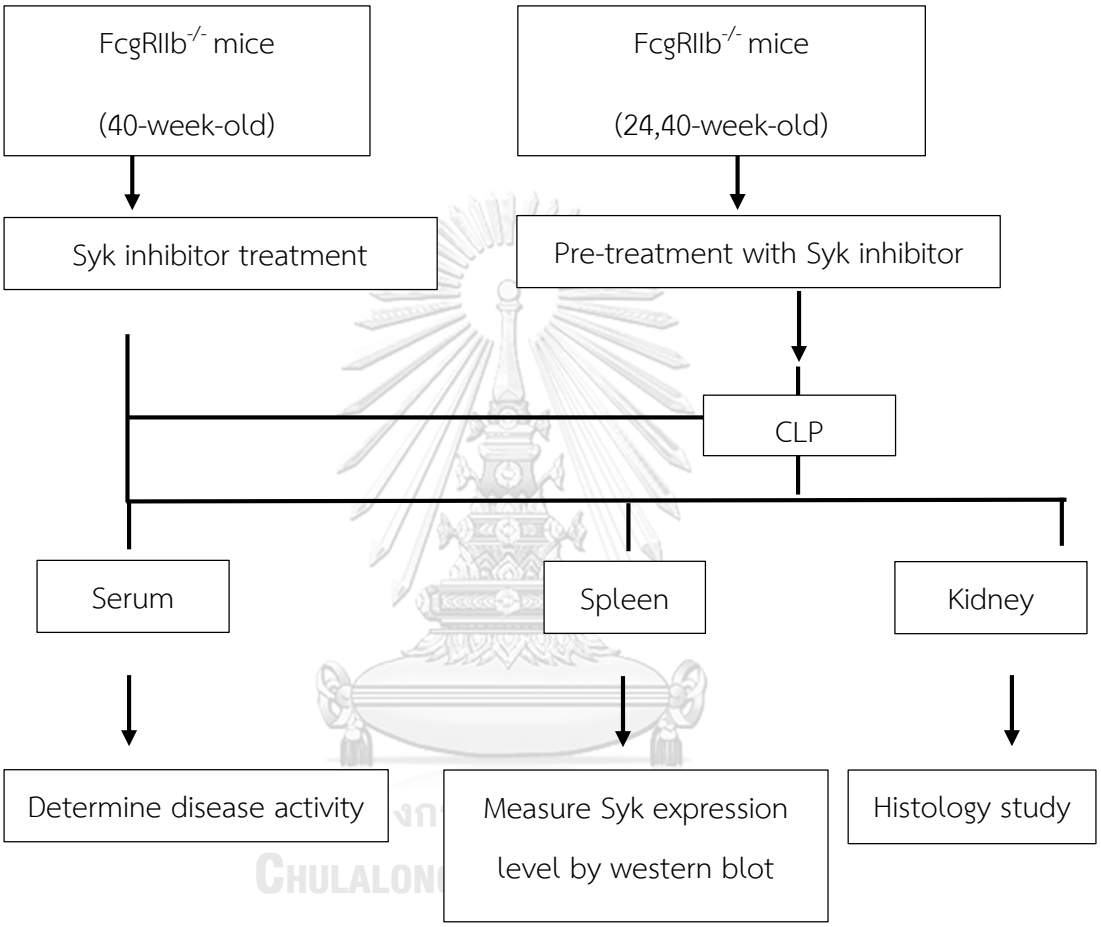
Objective: To determine the signaling pathway and the important protein in the synergy of endotoxin and (1→3)-β-D-glucan.



Part 3: Translational study

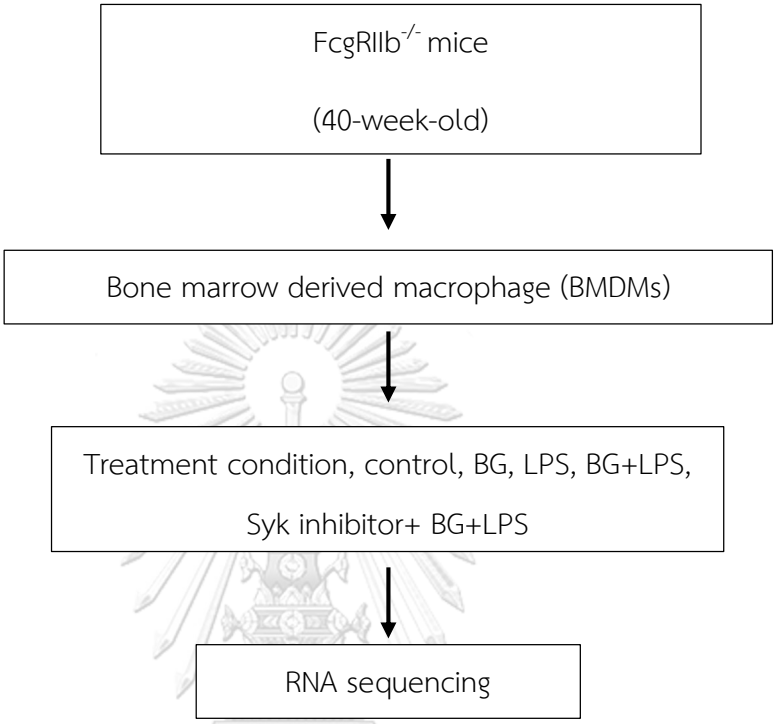
3.1 In *vivo* study

Objective: To investigate the effect of Syk inhibitor in SLE and sepsis with SLE background in lupus mouse model of *Fcgr2b*-deficient mice.



3.2 In *vitro* study

Objective: To investigate gene expression profile in response to LPS, BG, and BG+LPS before and after treatment with Syk inhibitor.



CHAPTER III

LITERATURE REVIEW

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease with multifactorial pathogenesis characterized by autoimmune reactions that involve innate and adaptive immunologic alteration [23]. Autoantibodies against anti-double-stranded nuclear are associated with a divert array of clinical manifestations [11]. SLE has been considered as a complex disease, according to the diverse array of clinical manifestation (including skin, joint, neurological, renal, cutaneous, and gastrointestinal manifestations) (Figure 2). Moreover, SLE can affect laboratory abnormalities which change in hematological and serology such as increased the level of anti-double stranded DNA and decreased the level of complement. Decreasing quality of life is commonly found in SLE patients because of the disease complication and pain-related treatment [24].

There are many causes of SLE development, genetic, and environmental factors have been involved. The various environmental factors can be considered as the risk factors for SLE contribution, including UV light exposure, Epstein–Barr virus (EBV) infection, endogenous retrovirus, and multiple drugs. The SLE predominant occurrence rate was found in women more than men, suggesting a endocrine factor may play an important role in SLE [25]. SLE is present in women more than men the average ratio 9:1 male/female in adolescence [26].

For genetic factors, there is about 25 % for the concordance rate of SLE in monozygotic twins but only 2 % in dizygotic twins, implying that only genetic factors do not describe the phenotype of SLE [27]. The well-known evident of genetic factors related SLE are the defection of complement component *C1Q* and *C4* single-gene defects [28, 29]. Genome-wide association studies reveal many importance loci that contributing to the risk factors for SLE development including Interferon(IFN) regulatory

factor 5 (IRF5), Integrin alpha M (ITGAM), *Signal transducer and activator of transcription 4* (STAT4) and some of the Fc gamma receptor and other loci are also important [29].

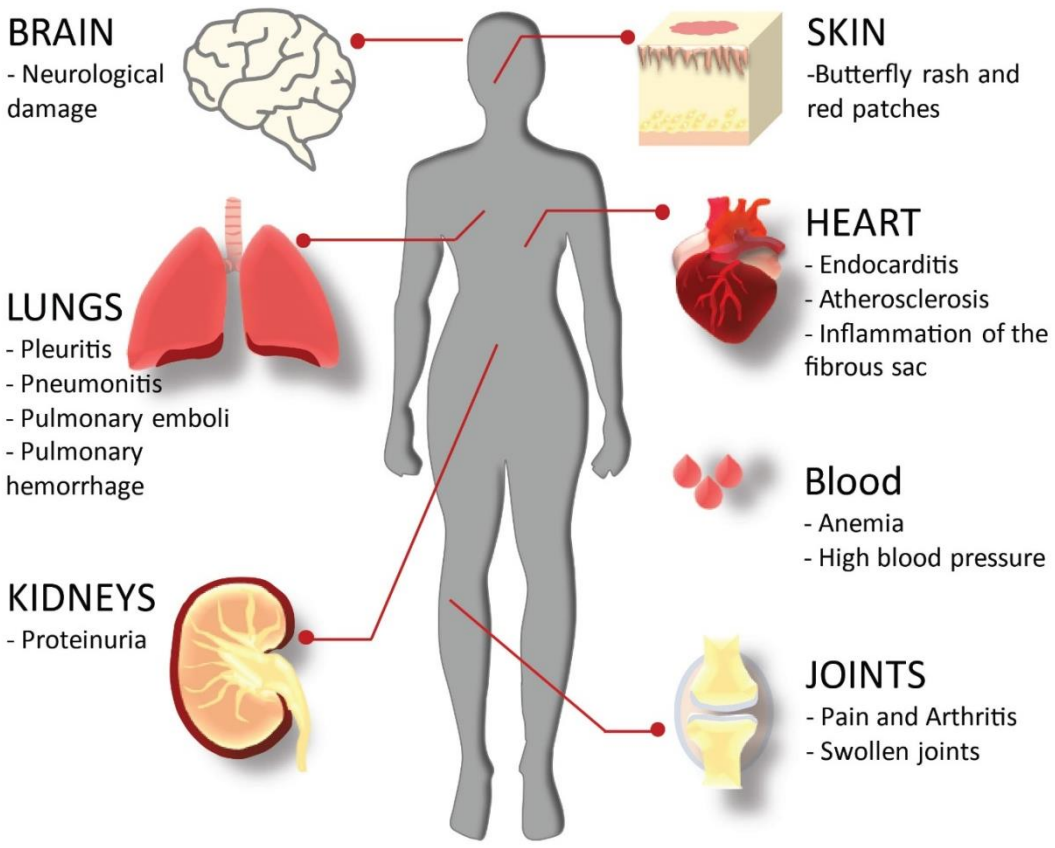


Figure 2 The illustration of multi-organ involvement in systemic lupus erythematosus.

Modified from: Arvind K. et al., Nature Review Disease Primer. 2016

Fc gamma receptor polymorphisms in systemic lupus erythematosus

Fc gamma receptors (FcγRs) are IgG receptors that play an important role for communication of humoral and cellular immune responses. Engagement of antibodies antigen complexes by the Fc receptor on macrophages or neutrophils give effective signaling for phagocytosis of antigen-antibodies complexes [30]. At present, there are four different classes of FcγRs also known as FcγRI, FcγRIIB, FcγRIII, and FcγRIV in mice. In human, there are six types of FcγRs that are FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, FcγRIIIB and FcγRIIIB. Mouse and humans express only single inhibitory receptor that is FcγRIIB [31] (Figure 3).

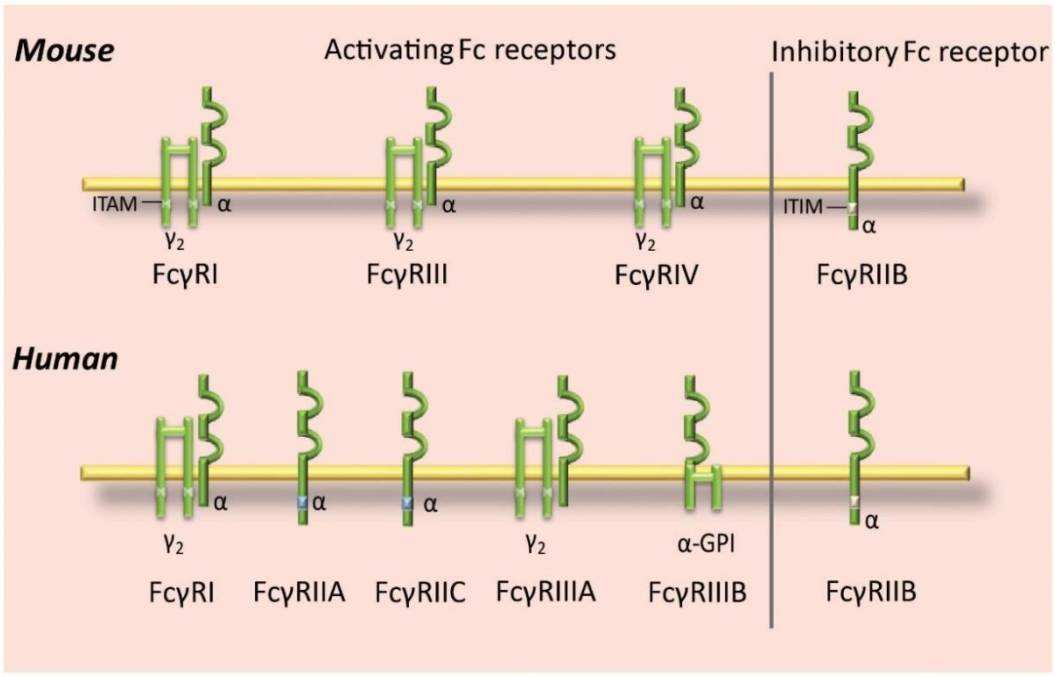


Figure 3 The representative of Fc gamma receptor family in mouse and human
Modified from: Falk N. et al., Nature Reviews Immunology. 2008

The inhibitory FcγRIIB is the most widely expressed FcγR, and present on almost leukocytes exception T cells and NK cells. FcγRIIB plays an essential role in regulation of autoantibodies production and their effector function including activation of innate immune cells and activation of adaptive immune system [32]. FcγRIIB acts as immune regulatory function by binding of the Fc domain of immunoglobulin G

(Figure 4). In order to investigate the function of the FcGRIIb inhibitory receptor, the FcGRIIb knockout mice have been generated by Dr. Silvia Boland in 2000. Interestingly, FcGRIIb deficient mice ($FcGRIIb^{-/-}$) on the C57BL/6 background are established lupus mouse model [33, 34]. At 24-wk-old, $FcGRIIb^{-/-}$ mice develop lupus nephritis as demonstrated by increased anti-dsDNA elevated proteinuria and lupus renal histopathology with immune complexes deposition. After 32 to 40-weeks age, mice develop the full-blown lupus nephritis [7, 8]. Moreover, FcGRIIB has been reported to cross-talk with TLR signaling pathway which is important for pathogenesis of SLE [35]. Previous study reported that dendritic cells with high expression of FcγRIIB inhibited pro-inflammatory cytokine secretion mediated by Toll like receptor 4 (TLR4) when stimulation with immune complexes and interaction between TLR4- FcGRIIb was indicated to dependent on PI3K and Akt pathway[36]. Suggesting that, FcGRIIb plays a pivotal role in autoimmune disease. However, the mechanism of how FcγRIIb regulates TLR signaling remained unclear [37].

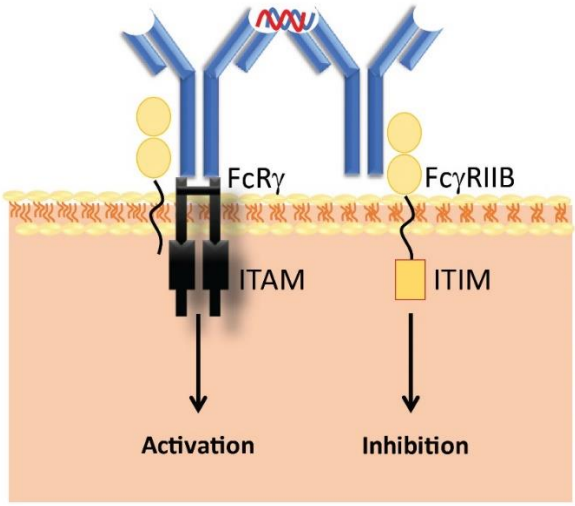


Figure 4 The illustration of Fc gamma receptor 2b

Modified from: Toshiyuki Takai. Nature Reviews Immunology 2, 580-592 (2002)

The single nucleotide polymorphisms (SNPs) in the human FcγR gene which changes nucleotide in the exon 5 of *FcγRIIb* gene from T>C at the position 695 which alters one amino acid from isoleucine (I) to threonine (T) at position 232 on the

transmembrane region (FcγRIIBT^{232}) [38]. Changing only one amino acid in transmembrane region causing FcγRIIBT^{232} is excluded from the sphingolipid rafts which impair signaling capacity (Figure 5) [39]. FcγRIIBT^{232} polymorphism frequency was significantly increased in patients with SLE in japan [40]. Moreover, the analysis of FcγRIIBT^{232} in Thai population showed an increasing of FcγRIIBT^{232} frequency in SLE [41]. These data suggested that FcγRIIBT^{232} is a high prevalence polymorphism in Asian population.

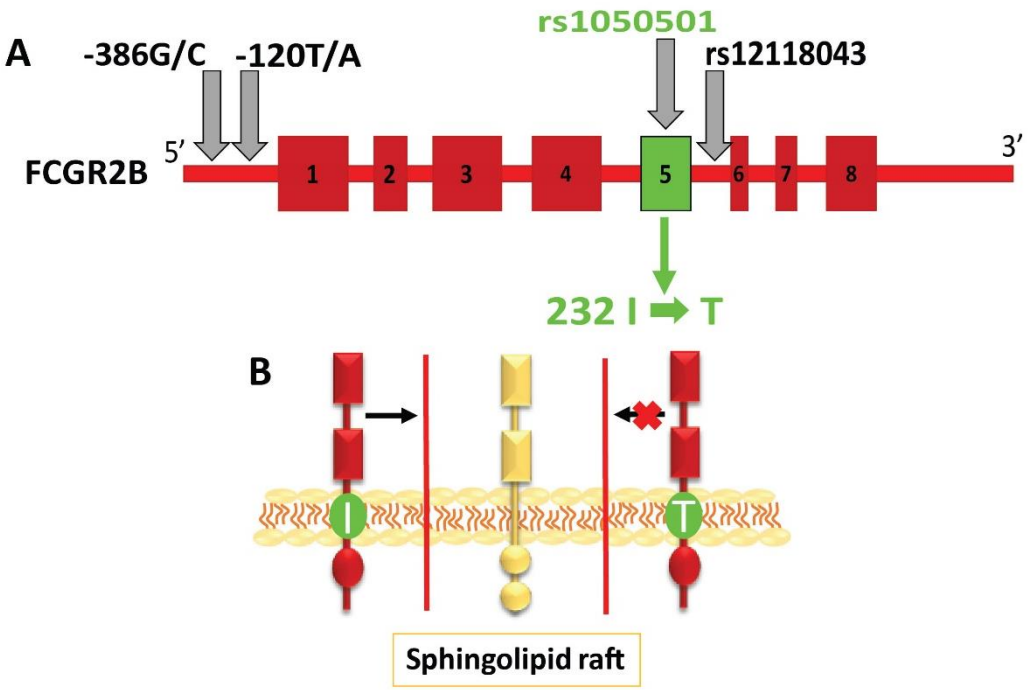


Figure 5 The illustration of genetic variation in FcGRIIB in human A) the representative of the single nucleotide polymorphisms (SNPs) on the exon 5 that leads to amino acid substitution (threonine for isoleucine) in the transmembrane receptor. B) The representative of threonine containing receptor which impacts the receptor function. Modified from: Msrion Espeli et al. Immunological Reviews 269(1) (2015)

Immunopathology of systemic lupus erythematosus

SLE is characterized by an autoimmune reaction that involved innate and adaptive immune system. The activation of the innate immune system initiates by the nucleic acid release from apoptotic cells which activate toll like receptor (TLRs) on the plasmacytoid dendritic cell, leading to type I interferons (IFNs) releasing. Type I interferon promote antigen presentation by dendritic cells using MHC molecules resulting in T cell activation and differentiation. Furthermore, T cell plays an important role in pathogenesis of SLE. For instance, T helper 17 (T_H17) releases IL17 cytokine for recruitment of the other inflammatory cells. In addition, T cells stimulate B cells in the germinal centers resulting in class switching, maturation and differentiation into plasma cells that can produce soluble autoantibodies against self-antigen. In order to produce autoantibodies, T_{FH} cells support this process by producing IL-21 for promoting B cell proliferation and differentiation. Next, the soluble autoantibodies from plasma cells bind to the antigen leading to Immune complexes formation. The depositions of immune complexes initiate local inflammation and complement activation which ultimately causes tissue damage in organs including joints, skin, and kidney [23, 42] (Figure 6). Moreover, apoptosis cells in the damaged tissues can act as novel antigens by antigen presenting cells, which supporting further activates T cells. In addition, the activation of TLRs receptors such as TLR7 or TLR9 [43] by the environmental factors including viral infection DNA damage, induce the IFN-I and other cytokines production, leading to the tissue damage.

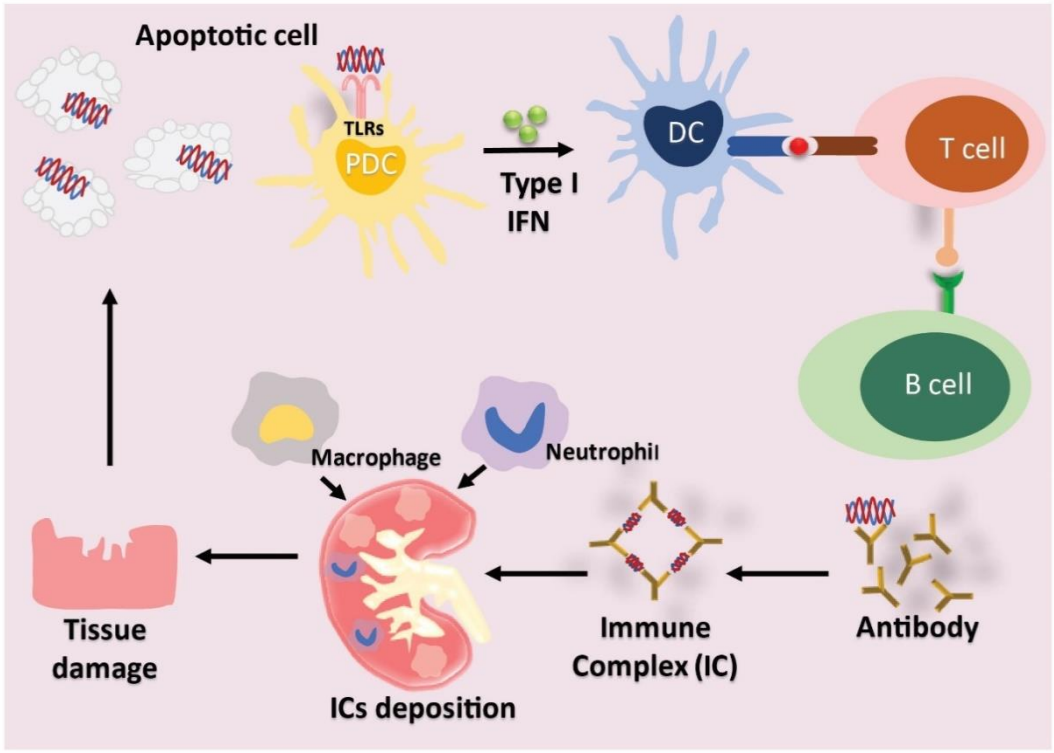


Figure 6 The illustration of immunopathogenesis in systemic lupus erythematosus.

Modified from: Arvind K. et al., Nature Review Disease Primer. 2016

Severity of SLE depends on clinical presentation to a specific organ. Kidney manifestation also known as lupus nephritis, presents up to 60% of the SLE population. The pathogenesis of lupus nephritis started with immune complexes of anti-dsDNA deposition in the kidney parenchyma follow by the complement activation, infiltration of immune cells. These Immune cells release cytokines, chemokines, proteolytic enzymes, and oxidative damage which induce kidney inflammation and organ damage [44]. Moreover, the gastrointestinal tract is commonly affected in SLE. On the other hand, the gastrointestinal manifestations may be underestimated clinically because patients may not obviously present the abdominal symptoms. Previous study showed that 60-70% of SLE patients had evidence of peritonitis, whereas only 10 % of all patients were recognized clinically [45]. The Enhancement of SLE progression influence by gut dysbiosis has been demonstrated [2]. However, there are limited data about gastrointestinal leakage in SLE.

Role of monocytes and macrophages in systemic lupus erythematosus

Monocytes/macrophages are innate immune cells, play an important role in inflammation, and induce immunity. The origin of monocyte comes from pluripotent stem cells in bone marrow stimulation by the specific growth factor. After the differentiation process, mature monocytes leave bone marrow and enter blood stream. Moreover, circulating monocyte can be differentiated into tissue-resident macrophages with specific functions depending on the local environment [46, 47]. The main function of monocytes/macrophages recognizes and removes pathogens, bacteria, senescent, dead cells. On the detection of invader cells, phagocytosis is a process of monocyte/macrophage engulfs bacteria or pathogens, which required the activation of receptors to initiate responses. After engagement of pathogen and receptors, monocyte/macrophage releases pro-inflammatory cytokines (i.e. IL6, IL10, TNF-α) as well as chemokines for the recruitment of other cells to the site of the infection. Then, the target cells were digested, monocyte/macrophage present target-derived antigen(s) to T cells, resulting in activation of adaptive immune responses.

So far, there are many studies that focus on understanding of the immune dysregulation in SLE; nevertheless, the role of monocytes/macrophages in the pathogenesis of SLE remains unclear. The first known of defective model for monocytes/macrophages in SLE was purpose in 1980s is the fact that monocytes/macrophages display defective in phagocytosis function, resulting in accumulation of apoptosis and debris cells. The consequence of defection in apoptotic cells clearance leading to an increased autoimmune reaction. Furthermore, the recent study showed that monocytes/macrophages play an important role in lupus nephritis by mediating tissue inflammation. Studies by Levine and colleges demonstrated that macrophage from several lupus mouse models have an identical abnormality in the expression patterns of the multiple cytokines after exposure with apoptosis cells. Those patterns from 15 different cytokines were reported in the similar expression pattern among many SLE-prone strains. [48-50]. For instance, monocytes/macrophages increased secretory factors (i.e. NO, IL10, IL6, IL12p70) to promote abnormal lymphocyte functions. The other well-known functional defects of macrophages are

list in Table 1. Moreover, monocyte from SLE patients has been reported to impaired phagocytosis function and this phenomenon is correlated with fcγRII and III expression level on the surface of monocyte [51]. Several studies confirmed that monocyte from SLE patient defect in clearance of apoptosis cells due to several reasons. For example, low expression of CD44 on the surface of monocyte leads to insufficient to provide "eat me" signal [52]. The abhorrent expression of activation marker (CD86, CD16, siglec-1, MRP-8 and COX-2) were commonly found infiltrating monocytes/macrophages in the kidney which impact local-inflammation and injury in lupus nephritis [53, 54]. Suggesting, the role "defective" function of monocytes/macrophages supports the idea that monocytes/macrophages from SLE can enhance the disease progression by their environmental factors or intrinsic defects.

Table 1 List of Monocyte/Macrophage abnormality and the clinical outcome in the autoimmune animal model.

Mice	Monocyte/Macrophages		Outcome	Ref.
	Molecular aberrations	Functional defect		
c-mer$^{-/-}$	Absence of c-Mer function only in Mφ	Decrease phagocytosis of apoptosis cell only	- Induction of autoantibodies - No polyclonal B-cell activation	[55]
Tyro/Axl /c-mer triple mutants	Inactive Tyro/Axl/ c-Mer kinase in Mφ	Activation of Mφ	- Production of autoantibodies - Lymphoproliferation - T cells and B cell activation - Arthritis, skin lesions, IgG deposits in glomerulus Thrombosis/hemorrhages	[56]
MRL/lpr MRL+NZ BWF1 LG BXSB	Reduced activation of the cytoplasmic protein Rho	"Misreading" apoptotic cells display as cytokine dysregulation, Increased adhesiveness, and DC-like morphology	-Lupus-like disease	[48, 49]

NZB/WF 1 FcR$\gamma^{-/-}$	Absence of γ chain	N/A	-Abundance of immune complex deposition in kidney	[57, 58]
TMPD model	N/A	↑ Type I Interferon production	-Lupus-like disease	[59]
Mo, monocyte(s); Mφ macrophage(s) TMPD, tretramethylpentadecane; N/A, not available				

Modified from Christina G. *et al*. Seminars in Arthritis and Rheumatism 39(6)

Gut leakage

The gastrointestinal (GI) system is important for digestion and absorption nutrients. It has been highly specialized and maintained by mucosal barrier [60]. Moreover, not only nutrient absorption, but our gastrointestinal tract also exposes to numerous exterior antigens, including food particles, pathogens, bacteria, fungi, and environmental toxins. Thus, the mucosal barrier function is required for blocking entry of external antigen from the gut lumen to host during nutrients absorption. This barrier is maintained by epithelial cells that are linked together by the special proteins also known as tight junction (TJ) proteins. Moreover, there are many factors that support this barrier including mucins, antimicrobial molecules, cytokines, and immunoglobulins. Sometimes, these factors may defect resulting in intestinal permeability increase, this phenomenon is called “leaky gut”. In the leaky gut, the tight junction proteins are loosened which allows the foreign molecules including undigested food particles, bacteria, fungus, or the other toxins can get through and stimulate the immune system causing inflammation (Figure 5). The gut barrier disruption is caused by many factors, including diet, infection, alcohol consumption, or stress. The Inflammation caused by the gut translocation of external antigens affect many diseases, Several studies showed that many diseases may worsen due to the gut leakage, including autoimmune diseases, Inflammatory bowel disease, celiac disease, autoimmune hepatitis, type 1 diabetes (T1D), multiple sclerosis, and systemic lupus erythematosus [61-65].

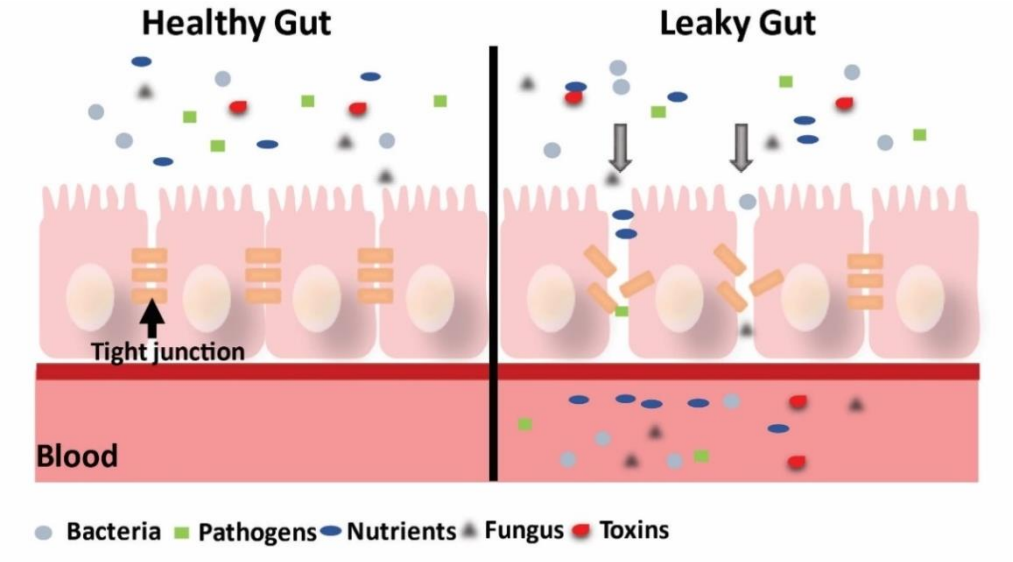


Figure 6 The illustration of healthy gut versus leaky gut.

Modified from: Vincent Pedre., Happy gut. 2015

Leaky gut and systemic lupus erythematosus

The molecule from the gut translocation might impact on lupus. As we know that our gastrointestinal tract is an endogenous source of the endotoxin. In the leaky gut, lipopolysaccharide (LPS), an outer membrane of Gram-negative bacterial cell wall, can enhance SLE development and progression by crossing the epithelium defect and enters to the bloodstream [13]. The specific receptor for LPS is Toll like receptor 4 (TLR4), activation of TLR4 receptor leading to pro-inflammatory cytokine production [66]. In SLE patients, monocyte released soluble CD14 marker in the blood when cells exposed to the LPS, suggesting the higher level of CD14 in the circulation indicate an increase in LPS exposure [67]. Furthermore, animal studies showed that increased TLR4 responsiveness during lupus development [68-70] and this phenotype significantly reduced by adding antibiotics to remove commensal gut flora [68]. It is clearly showed that TLR4 is hyperresponsive to the gut flora (including LPS) contributed pathogenesis of SLE.

However, gut fungi are important commensal organisms in our GI tract [5]. The major component of many fungal cell walls is polysaccharide beta (1□3)-D-glucan (BG) which activates Dectin-1 receptor resulting in cytokine production [71]. Moreover,

it has been reported that TLR4 and Dectin-1 pathway showed synergistic effect and synergized TNF-alpha expression [20]. The signaling mechanism cross talk between Dectin1 and TLR4 are not fully understood.

Toll like receptor 4 signaling pathway

TLR4 is a member of Toll like receptor family. There are many PAMPs that can stimulate TLR4. The well-known ligand for stimulation of this receptor is LPS. LPS stimulation involves with several proteins including LPS binding protein (LBP), CD14, MD-2 and TLR4 [72]. The classical activation of TLR4 signaling cascade has been divided into two pathways that are MyD88-dependent and MyD88-independent pathway (Figure 6).

1. MyD88-dependent pathway

During LPS recognition, TLR4 undergoes oligomerization and recruits downstream signaling protein by interacting with the TIR (Toll-interleukin-1 receptor) domain. This domain plays an important role in TLR signal transduction, because a single point mutation on the TLR domain can abrogate signaling during LPS stimulation [73]. TIR domain composed of 5 adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as MAL, MyD88 adaptor-like), TRIF (TIR domain-containing adaptor protein including IFN-β), TRAM (TRIF- related adaptor molecule) and SARM (sterile α and HEAT-Armadillo motifs-containing protein) [74]. Studies in the knockout mice showed the pivotal role of these adaptor proteins in TLR4 signaling. For example, MyD88 knockout macrophage fails to produce pro-inflammatory cytokine after LPS stimulation [35]. In addition, Type I interferon and interferon gene expression remained in MyD-88 knockout macrophages [75]. Furthermore, Study in MyD88-knockout mice demonstrated that mice have ability to resistant to LPS induce septic shock. Thus, the MyD88-dependent pathway is responsible for the proinflammatory cytokine expression.

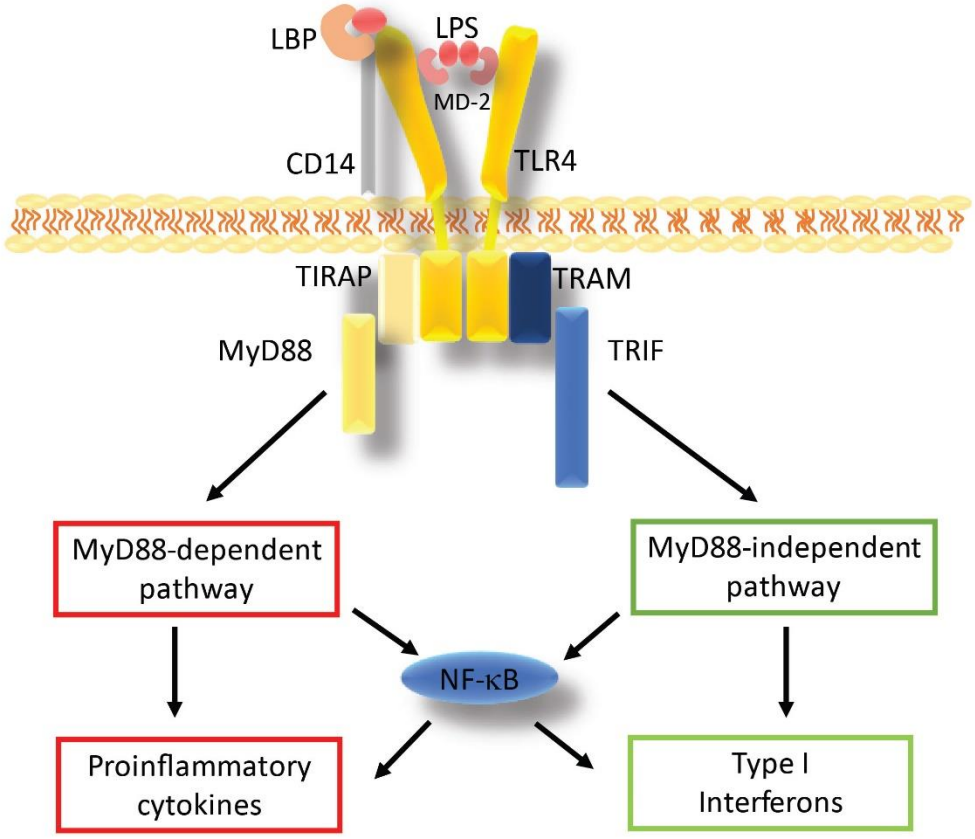


Figure 7 Overview of LPS/TLR4 signaling pathway.

Modified from: Yong-Chen Lu et al., Cytokine. 2008

Apart from TIR domain, MyD88 also contains a death domain (DD), this domain can recruit other adaptor proteins through homotypic interactions. During LPS stimulation, MyD88 activates and recruits a death domain-containing kinase, IL-1 receptor-associated kinase-4 (IRAK4). IRAK4 is a member of IRAK family composed of death domain and kinase domain [76]. The IRAK4 knockout macrophage showed a similar result to the MyD88 knockout macrophages, IRAK4 knockout macrophage failed to produce pro-inflammatory cytokine upon LPS stimulation and IRAK4 knockout mice also showed resistant to the septic shock induced by LPS [76]. Recent study demonstrated that IRAK4 also plays an important role in recruitment, activation, and

degradation of other proteins including IRAK1 and IRAK2 in the TLR4 signaling pathway [77, 78].

Another important adaptor protein for TLR4 signaling pathway is TRAF6 (TNF-receptor-associated factor 6), this protein is essential for MyD88-dependent pathway downstream of IRAK4 and IRAK1. TRAF6 interacts with UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant isoform A). Then, this complex activates TAK1 (transforming growth factor-β-activated kinase 1) [79, 80], and TAK1 activates downstream signaling pathway IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathway [81]. After that, the complex of IKKα, IKKβ, and IKKγ phosphorylate IκB (inhibitor of κ light chain enhancer B cells) protein. Subsequently, IκB degradation leading to translocation of NFκB transcription factor to the nuclease, this transcription factor controls proinflammatory cytokine expression. Moreover, the activation of MAPK pathways resulting in activation of the AP1 transcription factor, which also involved in the production of proinflammatory cytokine [82]. Of note, the activation of MAPK and NF-κB is remained in the MyD88 knockout macrophages even this activation a little bit delayed [83], this data suggested that MAPK and NF-κB can be activated by MyD88-independent pathway. Interestingly, MyD88 knockout macrophage failed to induce proinflammatory cytokine production. suggesting there are some other pathways, apart from NF-κB and MAPK, are essential for proinflammatory cytokine production (Figure 7).

2. MyD88-independent pathway

The adaptor protein important for MyD88-independent activation is TRIF protein. Knockout TRIF protein in macrophages showed the important function of TRIF on the activation of IRF3 transcription factor and delayed activation of MAPK and NF-κB. Double knockout of MyD88 and TRIF protein leads to abrogated MAPK and NF-κB activation[84, 85]. Many studies try to focus on how TRIF protein activates IRF3 protein, recent study reported that TRIF recruits the TRAF3 protein to activate IRF3 and this result was confirmed by the TRAF3-deficient cells fails to produce Type I interferon (IFN). Moreover, TRAF3 also involves with TANK(TRAF family member-associated NF-κB activator), TBK1 (TANK binding kinase 1) and IKKi to activate downstream signaling [86]. Dimerization and translocation of IRF3 belong to the function of TBK1 and IKKi protein [87]. IRF3 and NF-κB, acts as a transcription factor for the target genes including Type I interferons [88] (Figure 8).

On the other hand, Syk has been reported to mediate signaling via TLR4 responses. The activation of TLR4 resulting in the recruitment of Syk to form a complex has been reported in many studies. The pull-down assay using TLR4 and/or Syk specific antibodies demonstrated that Syk and TLR4 co-immunoprecipitated in monocytes, macrophages, and neutrophils in the absence of any stimuli [89-91]. During LPS stimulation, the levels of Syk binding to TLR4 along with phosphorylation were increased [89-92]. The phosphorylation of TLR4 and SYK is promoted by Lyn protein in neutrophils and macrophages [93]. Inhibition of Syk protein using pharmacologic inhibitor resulted in prevention of LPS-induced TLR4 phosphorylation [90]. These data suggested that Syk may promote tyrosine phosphorylation of TLR domain. In response to bacterial pathogens, Syk has been involved in the LPS stimulation, dephosphorylation of Syk in neutrophils leading to reduce inflammasome activity. In addition, the prominent function of Syk in response to LPS stimulation is activation of JNK, the pathway that regulates MCP-1 and TNF-α in neutrophil [89]. The Syk signaling in macrophage, activation of Syk leads to activation of the downstream signaling protein, including p85, AKT, IKK, PDK1 and NF-κB, leading to induction of

proinflammatory cytokine gene (TNF-α, COX-2, and iNOS) as well as proinflammatory mediator (NO and PGE_2) [94].

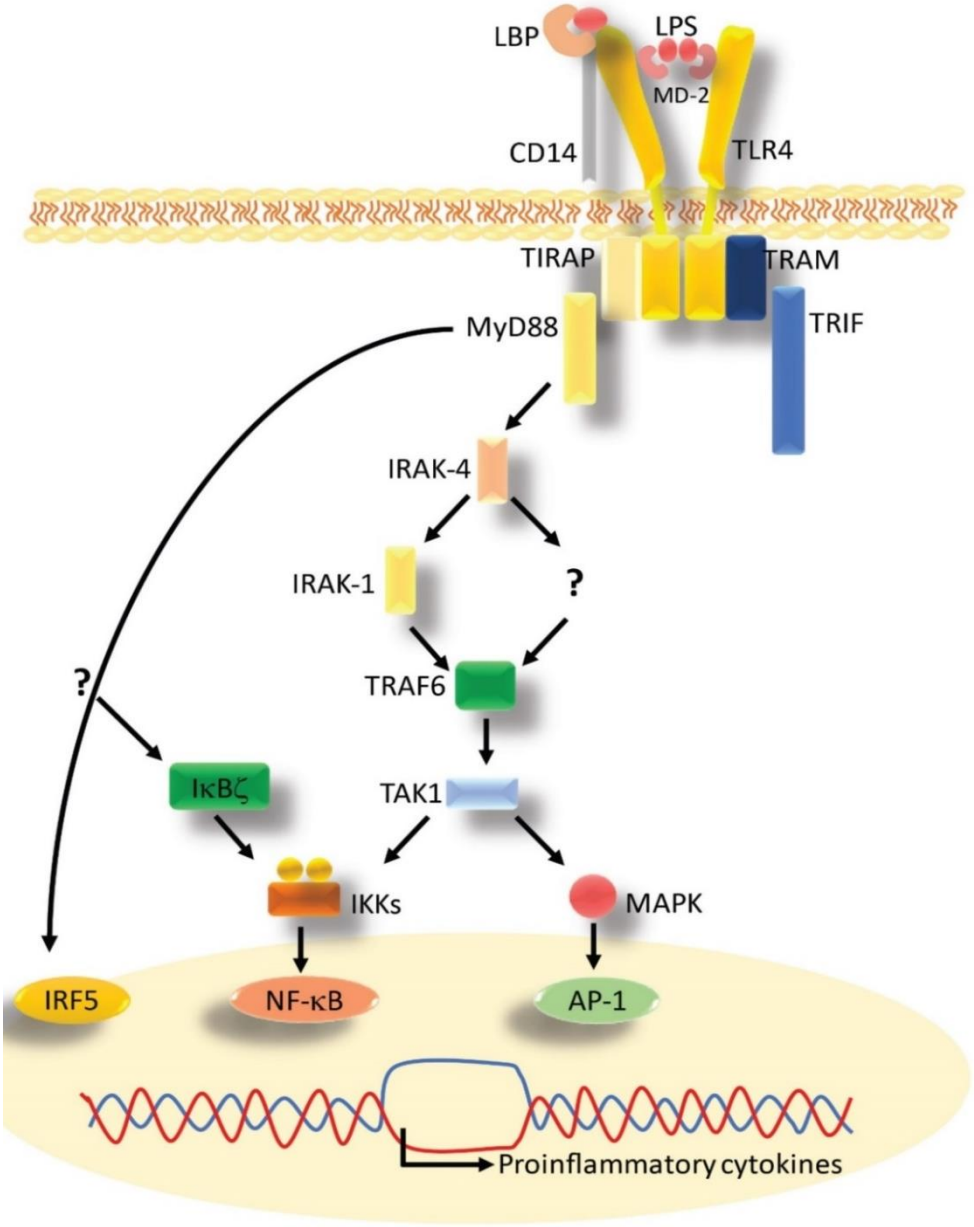


Figure 8 The illustration MyD88-dependent signaling pathway in response to LPS stimulation.

Modified from: Yong-Chen Lu et al., Cytokine. 2008

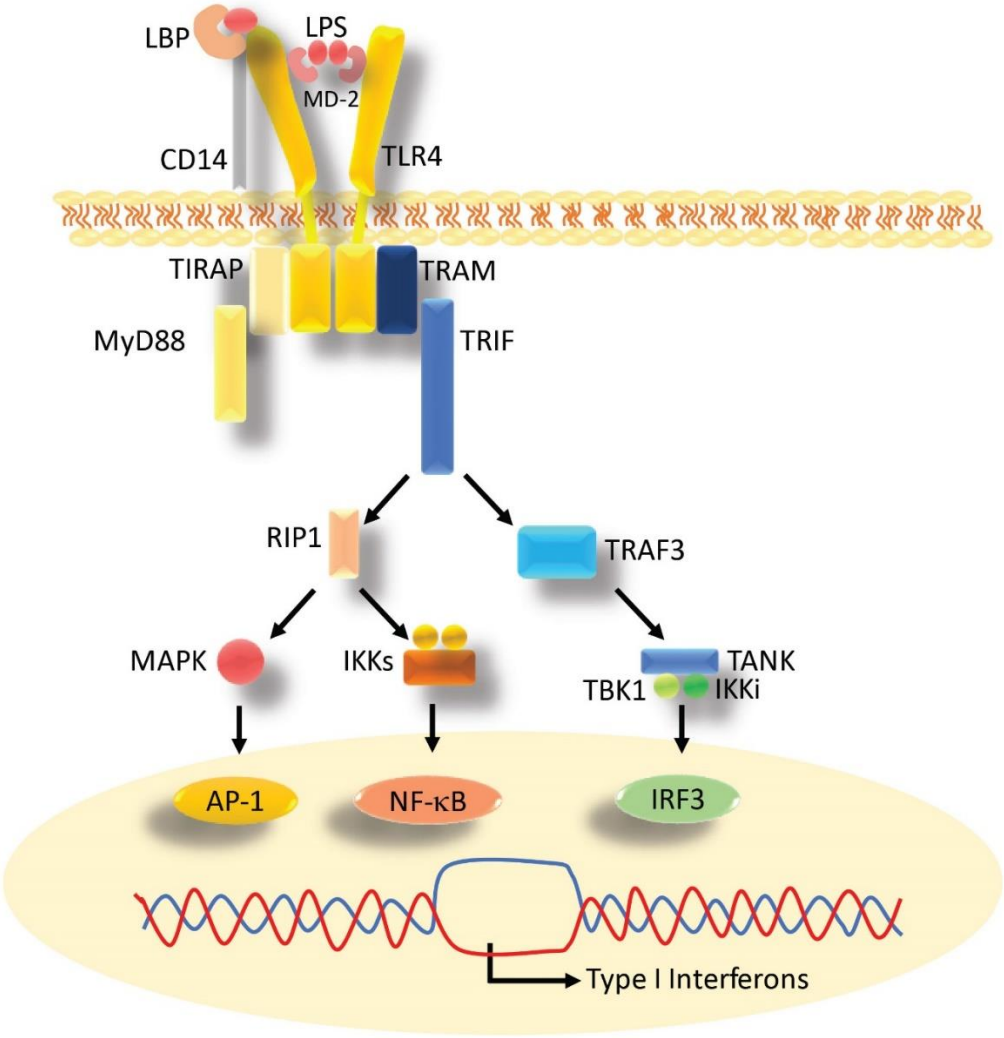


Figure 9 The signaling of MyD88-independent signaling pathway in response to LPS stimulation.

Modified from: Yong-Chen Lu et al., Cytokine. 2008

Dectin-1 signaling pathway

Dectin-1, a transmembrane receptor is a member of the C-type lectin receptor family plays a crucial role on antifungal immunity. Dectin-1 is the first known non TLRs signaling pathway in response to PRR, after binding specifically to (1→3)-β-D-glucan, induces the immune responses on its own signaling pathway [95, 96] and induction of various type of cytokines and chemokines (i.e. TNF, CXCL2, IL2, IL10 and IL12) [97].

Dectin-1 signaling pathway can be divided into Spleen tyrosine kinase (Syk) dependent signaling pathway and SYK independent signaling pathway. Dectin-1 signaling capacity depends on the ITAM-like motif, binding of ligand on the Dectin-1 receptor leading to the phosphorylation of tyrosine residues on ITAM-like motif. Then, Syk is recruited to phosphorylated receptor following by formation of CARD9, BCL-10, and MALT1 complex. This activated complex control NF-κB transcription factor activation and cytokines/chemokines expression including TNF-α, IL1, IL10, and IL6. Furthermore, activation of SYK also caused the activation of non-canonical NF-κB pathway, which mediated by NIK and IKK protein, resulting in translocation of RelB-p52 dimer to the nucleuse. Another SYK signaling pathway, SYK activates PLCγ2 leading to MAPKs-dependent signaling and Calcineurin signaling pathways. Activation of ERK leads to production of reactive oxygen species (ROS) which is essential for formation NLRP3 inflammasome, a process for production of IL-1β. For the activation of Calcineurin, this protein activates NAFT transcription factor and translocation of this protein to the nuclease leading to transcription of IL2, IL10, and COX-2 gene. In addition, on the SYK independent pathway, engagement of Dectin-1 ligand caused RAF1 phosphorylation promoted by RAS protein, and then phosphorylated RAF protein activates phosphorylation and acetylation. Then, binding of acetylated p65 protein to the IL10 enhancer resulting in increased the transcription of gene [98]. (Figure 9)

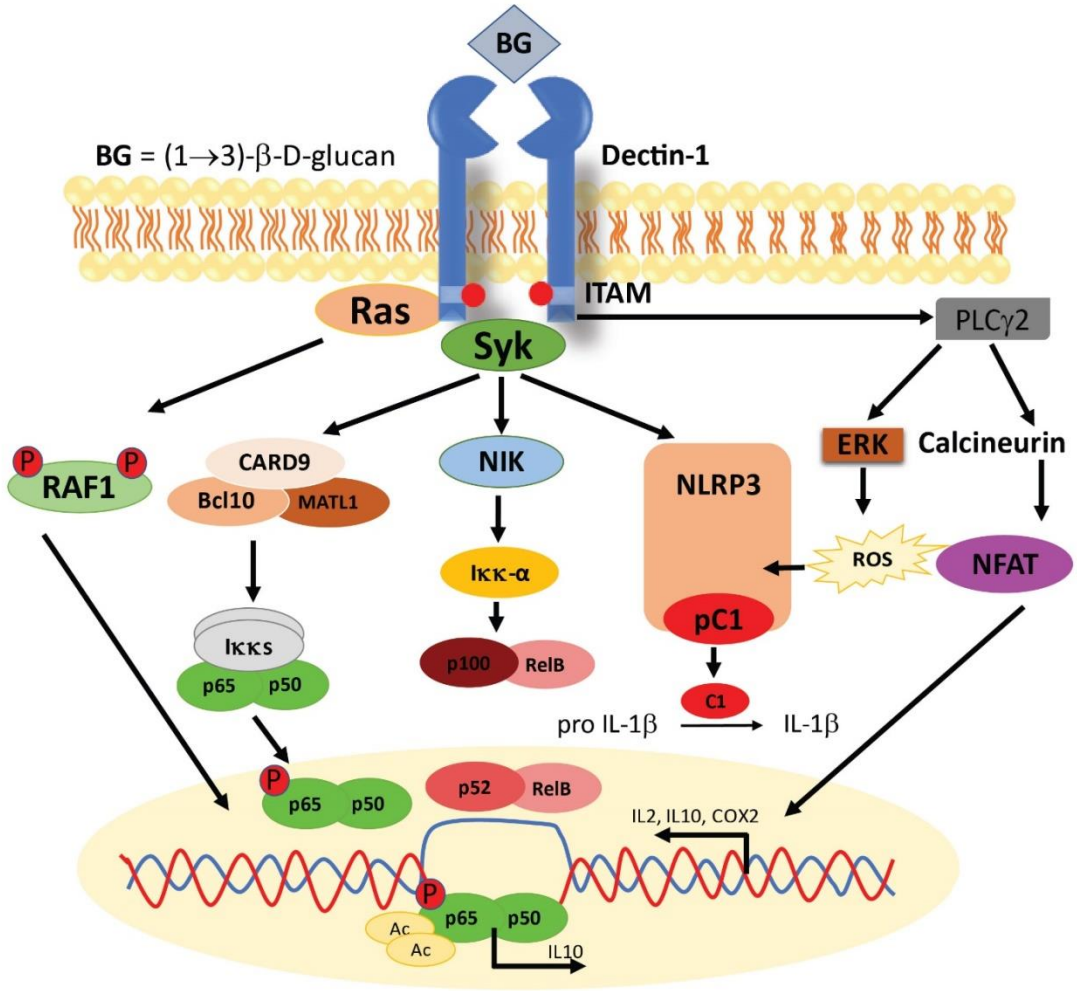


Figure 10 The representative of Dectin-1 signaling pathway.

Modified from: Bruno M. et al., Protein Kinase. 2012

Signaling innate immune crosstalk

Innate immune cells act as a first-line defense against pathogens by using a variety of cell surface receptors. These receptors are linked to the complicated signaling intracellular signal transduction network for an appropriate immune response. The intracellular signaling molecules that play a crucial role in innate immune response are tyrosine kinase. Tyrosine kinase can be divided into three major families, Src-family kinase, Syk family, and Tek family. These cytoplasmic tyrosine kinases play an essential role in activating and inhibiting pathway in innate immune responses. The signaling

initiate by Src-family followed by recruitment and activation of Syk family. Sharing of those downstream signaling targets leads to the signaling cross talk between pathways.

There are eight members of Scr family proteins and only four members found in innate immune cells that are Hck, Fgr, Lyn and Scr [99]. The Syk-ZAP70 contains 2 members of tyrosine kinase but only Syk is expressed in innate immune cells. Both of Scr and Syk play a key role in activating and inhibiting pathways. Considering the downstream signaling pathways of FcGR, TLR4, and Dectin1 pathway, Syk is a shared downstream protein of those receptors. Syk plays an important role in all innate immune cells involving ITAM or ITAM-like signaling adaptor. Loss of Syk activity showed a prominent blocking effect more than loss of a specific receptor [100]. Moreover, C-type lectin receptors are involved in the recognition of mycobacterial and viral pathogen molecules. Blocking of Syk signaling also affect all of these signaling in both of macrophage and dendritic cells [101]. In addition, the Syk signaling protein is linked to the downstream signaling trough CARD9, NF-κB, and involved in the formation of inflammasome leading to IL-1β production and pro-inflammatory cytokine production [98].

The crosstalk between TLR signaling and C-type lectin receptor are well describted in TLR2 and Dectin1. The collaborative signaling between TLR2 and Dectin-1 receptor are synergized in NF-κB activation, resulting in increased cytokines production (i.e. TNF-α, IL-23, and IL6) [97, 102]. SYK is a kinase protein that has been reported to play a key role in this phenomenon. Syk deficient macrophages abrogate the cytokine in response to the ligation of $Pam3CSK_4$ combination with β-glucan [103, 20]. Moreover, SYK has been reported to play an essential role in FcγR signaling, activation of FcγR in monocytes/macrophages required the Syk activity for downstream signaling and phagocytosis activity. Monocyte has been found to abrogate phagocytosis activity when treatment with *Syk* antisense oligonucleotides [104]. Syk-deficient macrophages demonstrated defective phagocytosis activity during FcγR ligation but showed the normal phagocytosis activity when induced by complement. Study from Syk-deficient Neutrophil failed to generate reactive oxygen intermediates

in response to the FcγR simulation [105]. Recent study reported that FcγR showed the synergistic activity with TLRs to increased proinflammatory cytokine production in dendritic cell. In addition, the synergistic effect has been reported to involve intracellular (TLR3, TLR7/8) and extracellular Toll like receptor (TLR2, TLR4, TLR5) [106, 107] suggesting the potential role of Syk on the collaborative signaling of extracellular receptor in response to the stimulus.

Spleen tyrosine kinase and Systemic lupus erythematosus

Spleen tyrosine kinase (Syk) is a cytoplasmic kinase protein, a member of the Src protein family. Syk is a well known to involve in tyrosine-based activation motifs (ITAMs) including signaling from B- and T-cells receptor, Fc receptors, and many C-type lectins receptor [108]. As mentioned earlier, Syk plays a key role in the signaling pathway that involves in the inflammation response. All of these signaling pathways have been reported to involve in the lupus progression. Increased Syk expression in T cells of SLE patient affects many aspects of protein expression including enzymes, cytokines, and receptors. In addition, IgG (ligand of FcγR) is involved the skin and kidney manifestation, this phenomenon was confirmed by the intradermal injection of IgG from lupus serum caused skin inflammation [109]. Syk level was found to upregulate in the skin lesion of lupus MRL/lpr mice. Thus, Syk appears to be the promising therapeutics target for the treatment of SLE [110, 111]. Previous study showed that Syk inhibitor (R788) can improve skin injury in MRL/lpr mice. Furthermore, Syk inhibitor administration showed the effect to improve kidney injury in the lupus-prone mice [112, 113]. However, as we know that SLE considers to a complex disease, using the Syk inhibitor in other models may provide benefits for the lupus treatment.

The Systemic lupus erythematosus and sepsis

Sepsis is a serious blood infection resulting in immune system massively release toxic molecules (i.e. Cytokines, chemokines) to fight infection. The imbalance production of those toxic molecules leads to tissue damage, organ failure, and death (Figure 12) [114].

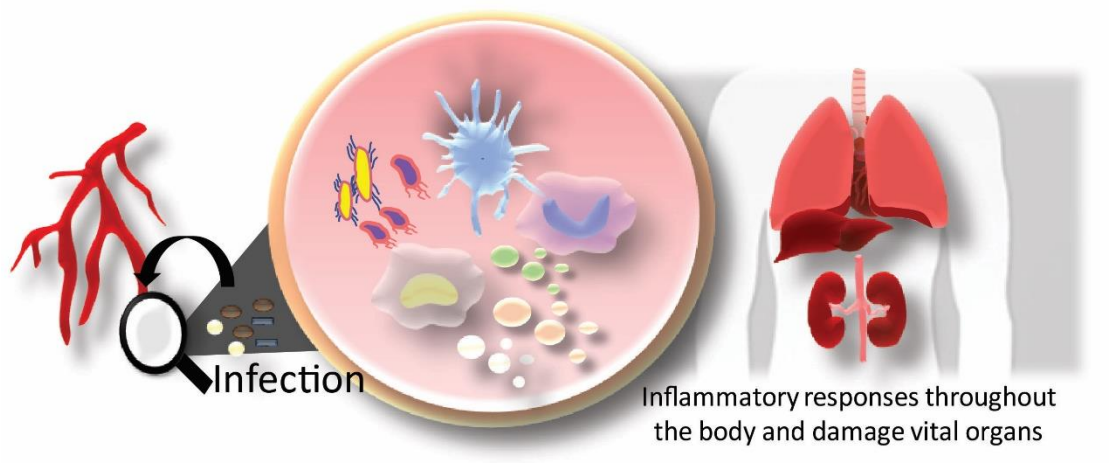


Figure 11 the illustration of the serious infection in the blood.

In SLE patients, serious infection is the main cause of Intensive Care Unit (ICU) admission[115]. During the course of the disease, studies have shown that nearly 50 % of SLE patients susceptible to severe infection [16] and it is a major cause of death in SLE patients [116]. The previous study, They recorded the number of sepsis with SLE compare to other patients, they found that the hospitalization rates for the serious infections in SLE increased over 12 times higher than patients without SLE and the reason for this phenomenon still remains unclear [117]. It is possible that immunosuppressive therapy may suppress immune function to cause opportunistic infection[118]. On the other hand, 25.9 % of SLE severe infection cases have been diagnosed without any immunosuppressive treatment [119] and the independent factors for the complication of infection in SLE are already demonstrated [119]. Taken together, increased susceptibility of infection in SLE patients may due to the immunologic disorder characterized by the disease or the external factors that may

affect the sepsis in SLE such as gut leakage and the molecules that is derived from the gut translocation may impact pathogenesis of sepsis in SLE.

In order to understand the pathogenesis of sepsis in SLE, FcGRIIb-deficient mice were used to explore the factors that affect sepsis severity and explain mechanism if there any target protein might be the potential target for the treatment of SLE and/or sepsis with SLE background in this mouse model.

CHAPTER IV

MATERIALS AND METHODS

Animal model

This study gained approval from the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, and followed the animal care and use protocol of the National Institutes of Health (NIH), USA. Only female mice were used in experiments. FcgRIIb deficient mice on a C57BL/6 background (FcgRIIb$^{-/-}$), a lupus mouse model, were provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA) and female wild-type (WT) mice were purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand). FcgRIIb$^{-/-}$ mice develop anti-dsDNA antibodies as early as 16–24 weeks and have increased serum creatinine levels at 40 weeks old, indicating lupus nephritis [120-122]. Therefore, FcgRIIb$^{-/-}$ mice at 24 and 40 weeks of age were used as representative models of asymptomatic and symptomatic lupus, respectively.

Patient samples

To explore endotoxemia and serum BG elevation in lupus, blood and spot urine were collected from patients with LN at the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The study protocol and sample accession process were approved by the Ethical Institutional Review Board, Faculty of Medicine, Chulalongkorn University, according to the Declaration of Helsinki, with written informed consent obtained from each individual patient. All patients had documented biopsy-proven class III or IV LN according to the 2003 International Society of Nephrology/Renal Pathology Society Classification [123].The inclusion criteria for active LN were: (1) a urine protein creatinine index of >1 g/day and (2) active urine sediments (red blood cells or white blood cells >5 cells/high-power field [HPF]). The inclusion criteria for inactive LN were: (1) a urine protein creatinine index of <0.5 g/day and (2) inactive urine sediments (red blood cells and white blood cells <5 cells/HPF). The exclusion criteria

were other causes that interfered with gut leakage determination by endotoxin and BG including (1) serum creatinine >1.5 mg/dL, (2) current infections or a history of infections within 2 weeks, (3) a history of invasive fungal infection, (4) pregnancy, (5) liver injury, (6) diarrhea or a history of diarrhea within 2 weeks. The SLE Disease Activity Index 2000 (SLEDAI-2K) scoring system was used to calculate disease activity at the time of sample collection [124]. To assess proteinuria, the urine protein creatinine index (UPCI) was calculated using the following equation: UPCI = spot urine protein (mg/dL)/urine creatinine (mg/dL). Samples from healthy volunteers were analyzed for controls. The demographic data are presented in Table 2.

Gut permeability test

Fluorescein isothiocyanate-dextran (FITC-dextran), a gut non-absorbable molecule, was orally administered to determine gut permeability as previously published [17]. Briefly, FITC-dextran (molecular weight 4.4 kDa; FD4; Sigma, St. Louis, MO, USA) at 0.5 mL (25 mg/mL) diluted in sterile phosphate buffer solution (PBS) was administered and serum FITC-dextran was measured by fluorospectrometry (microplate reader; Thermo Scientific, Wilmington, DE, USA) after 3 h. Spontaneous increases in (1→3)-β-D-glucan (BG) in serum, without systemic fungal infection, measured by Fungitell assay (Associates of Cape Cod), were used as an indicator of gut leakage. Values of BG <7.8 pg/mL were recorded as 0.

Sample analysis

Serum endotoxin and BG from human samples were measured as described above. Serum anti-dsDNA IgG in human samples was evaluated by ELISA assay (Euroimmun, Lübeck, Germany). In mouse samples, the detection of serum anti-dsDNA antibodies was performed following a previously published protocol [125]. Mouse urine was collected for 24 h using a metabolic cage (Hatteras Instrument, Cary, NC, USA) and urine protein was measured by Bradford protein assay. Kidney injury was determined

by serum creatinine (QuantiChrom creatinine assay, DICT-500, BioAssay, Hayward, CA, USA). Serum cytokines (IL-6, TNF-α, and IL-10) were measured by ELISA assay (ReproTech, Oldwick, NJ, USA).

Dextran sulphate sodium solution prior CLP

To examine the influence of GI barrier defect (gut leakage) upon bacterial sepsis, dextran sulfate solution (DSS) was administered before CLP. A short course and low dose of DSS lead to asymptomatic mice with only subtle histopathology changes as previously described (data not shown) [17]. Dextran sulfate (Sigma-Aldrich, St. Louis, MO, USA) was diluted into drinking water at concentrations of 1.5% (w/v) for 1 week before the CLP procedure.

CLP with LPS and/or BG Administration

To explore the effect of endotoxin and/or BG in polymicrobial sepsis, intra-peritoneal (i.p.) LPS and/or intravenous (i.v.) BG (into the tail vein) were administered after CLP surgery. LPS of *Escherichia coli* 026: B6 was purchased from Sigma-Aldrich. Pachyman *(*Associates of Cape Cod, Falmouth, MA, USA), was used for BG [19]. Subsequently, i.p. LPS (1 mg/kg) with i.v. NSS (LPS alone), i.v. BG (50 mg/kg) with i.p. NSS (BG alone), or i.p. LPS with i.v. BG (LPS and BG in combination) was administered at 3 and 6 h post-CLP surgery.

TLR-4 reporter cell

Because the synergy of WGP upon LPS response might due to the direct activation of WGP to TLR-4, WGP was incubated into Human Embryonic Kidney 293 cells with stable TLR-4 expression (HEK-Blue TLR4 reporter cell) (InvivoGen) in comparison with LPS as a positive control. Due to the stable expression of TLR4 and NF**K**B-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, HEK-Blue cell is used to test the response through TLR-4[126]. In short, HEK-Blue cell at $2.5x10^4$ cells/well were cultured overnight and incubated with WGP (at 100 and 500 µg/mL) or LPS (1 ng/mL) for 18 hours. Then, 20 µL of supernatant were transferred to tested with of QUANTI-Blue™ detection media (InvivoGen) (200 µL) and incubated at 37°C for 1 h before the quantitative analysis of the blue color at OD 630 nm with the microplate reader.

Bone marrow derived macrophages and *in vitro* experiments

Macrophages, derived from bone marrow following a published protocol [127, 128], were analyzed by flow cytometry analysis with anti-F4/80 and anti-CD11c antibody (BioLegend, San Diego, CA, USA) (fig 1C) before use. Macrophages ($1X10^5$ cells/well) were incubated with a representative of BG using whole glucan particle (WGP) purified from *Saccharomyces cerevisiae* (WGP® Dispersible, Biothera) at 100 or 500 µg/mL with or without LPS (*escherichia coli* 026:B6; Sigma-Aldrich) 100 ng/mL or DMEM (Dulbecco's Modified Eagle Medium supplemented 10 % Fetal bovine serum, 1 % sodium pyruvate, 1 % HEPES buffer and 1x of Penicillin-Streptomycin) a culture media control (control) for 6 h before the measurement of supernatant cytokine (ReproTech). In addition, to explore the mechanistic pathway of LPS with BG, in synergy, toward FcgRIIb-/- macrophages, Dectin-1 inhibitor (soluble glucan, a competitive Dectin-1 binding agent) (InvivoGen, San Diego, CA, USA) at 25-100 µg/mL, active form of Syk inhibitor (R788) (Selleckchem) at 1-10 µg/mL, inhibitor of RAF proto-oncogene serine/threonine-protein kinase (Raf-1 inhibitor; GW5074, Sigma, St. Louis, MO, USA) at 1-5 µg/mL, NF**K**B inhibitor (BAY11-7082; Sigma, St. Louis, MO, USA) at 1-5

μg/mL or DMEM complete alone (control) were incubated with FcgRIIb-/- macrophage for 1 h before the further 6 h incubation of WGP (500 μg/mL), with or without LPS (100 ng/mL), following by the measurement of supernatant cytokine.

Real-time PCR for macrophage polarization and Syk expression

Macrophage polarization is associated with pro- or anti-inflammatory effects, termed M1 or M2 polarization, respectively [129], and the proinflammatory properties of FcgRIIb$^{-/-}$ macrophages are prominent [7]. Therefore, the polarization of macrophages from WT and FcgRIIb$^{-/-}$ after induction might be different. Accordingly, macrophages at 2×10^6 cells per well were incubated with whole glucan particle (WGP), representative of (1→3)-β-D-glucan (BG), purified from *Saccharomyces cerevisiae* (WGP® Dispersible, Biothera), at 100 μg/mL with or without LPS (100 ng/mL) for 6 h. Then, total RNA was prepared using an RNA easy mini kit (Qiagen, Hilden, Germany) and the reverse transcription of 0.3 μg total RNA was performed using a high capacity reverse transcription assay (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem) with SYBR® Green PCR Master Mix (Applied Biosystems). The results were indicated in terms of relative quantitation using the comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta Ct}$). The expression of target genes in the sample, normalized to β-actin (an endogenous housekeeping gene) was demonstrated. A list of primers for PCR is shown in Table 1.

Western blot analysis

Isolated internal organs were maintained at -80°C until use for western blot analysis as previously described [128]. In brief, 20 µg of homogenized tissue as measured by Bicinchoninic acid assay (BCA) (Thermo Fisher Scientific), was used for SDS-PAGE following standard procedures before incubation with specific primary antibodies against Syk (Cell signaling Technology, Beverly, MA, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell signaling) overnight at 4°C. Then, a secondary antibody linked with horseradish peroxidase enzyme was used and visualized by ImageQuant™ LAS 500 (GE-Healthcare, Little Chalfont, Buckinghamshire, UK).

Syk inhibitor administration and histology

Because spleen tyrosine kinase (Syk) is a common downstream mediator among FcgR signaling, Dectin-1 (BG receptor), and TLR-4 (LPS receptor)[130, 131], Syk inhibitor is an interesting candidate for the anti-inflammation in a lupus model with endotoxemia and glucanemia. As such, Syk inhibitor (R788 disodium, Selleckchem, Houston, USA) in 0.1 M citrate buffer with pH 6.8 at 25 mg/ kg/ dose ,following a previous publication[132], was daily orally administered in 40-wk-old mice (FcgRIIb-/-, pristane, and WT) for 4 wk with serum and urine collection at before- and after-treatment. Of note, Syk inhibitor (R788) is a prodrug that is rapidly converted into the active form after the oral administration[132]. Urine collection was performed at 1 day prior to blood collection. Blood collection through tail vein was performed twice (50 µL/time) at 1 wk prior to the drug administration for the adequate serum sample for pre-treatment parameters and collected through cardiac puncture under isoflurane anesthesia with organ collection at sacrifice for the post-treatment parameters. Spleens were snap frozen in liquid nitrogen and kept in -80 ^{o}C until use. Kidneys were fixed in 10 % formalin, paraffin embedded, and stained with Hematoxylin and Eosin color (H&E) for the semi-quantitative evaluation modified from the previous publications[134 ,133] . In brief, glomerular injury was determined by the percentage

of moderate to severe glomerular injury (mesangial expansion >50%, crescentic formation and glomerulosclerosis) at 400x magnification. All glomeruli in the slide were examined. In parallel, the interstitial injury was estimated at 200x magnification in 25 randomly-selected fields by the score of damage-area (cell infiltration, interstitial edema and tubular injuries) by the estimation of the percentage of damage-area in each field as following: 0, <5%; 1, 5–10%; 2, 10–25%; 3, 25–50%; and 4, >50 %. To determine glomerular immune complex deposition by immunofluorescence, kidneys were fixed in Cryogel (Leica Biosystems, Richmond, IL, USA), processed into 4 mm thickness per slide and stained with goat anti-mouse IgG (Alexa Fluor 488, Abcam, Cambridge, MA, USA) and detected by ZEISS LSM 800 (Carl Zeiss, Germany). The intensity of immunofluorescence in each glomerulus from the score 0 to 3 under 200x magnification of 50 glomeruli in each mouse was used for the semi-quantitative analysis.

SYK inhibitor administration in lupus mouse model

Macrophage polarization is associated with pro- or anti-inflammatory effects, termed M1 or M2 polarization, respectively [129], and the proinflammatory properties of FcgRIIb$^{-/-}$ macrophages are prominent [7]. Therefore, the polarization of macrophages from WT and FcgRIIb$^{-/-}$ after induction might be different. Accordingly, macrophages at 2×10^6 cells per well were incubated with whole glucan particle (WGP), representative of (1→3)-β-D-glucan (BG), purified from *Saccharomyces cerevisiae* (WGP® Dispersible, Biothera), at 100 µg/mL with or without LPS (100 ng/mL) for 6 h. Then, total RNA was prepared using an RNA easy mini kit (Qiagen, Hilden, Germany) and the reverse transcription of 0.3 µg total RNA was performed using a high capacity reverse transcription assay (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem) with SYBR® Green PCR Master Mix (Applied Biosystems). The results were indicated in terms of relative quantitation using the comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta Ct}$). The expression of target genes in the sample, normalized to β-actin (an endogenous housekeeping gene) was demonstrated. A list of primers for PCR is shown in Table 1.

Induction of the cecal ligation and puncture sepsis model and Syk inhibitor administration

Cecal ligation and puncture (CLP) was induced following a previous publication [121] with some modifications to induce polymicrobial sepsis in asymptomatic and symptomatic lupus mice. In brief, cecal puncture with a 21-gauge needle was performed under isoflurane anesthesia. Tramadol, 20 mg/kg diluted in 0.5 mL normal saline (NSS) and antibiotic (imipenem/cilastatin) at 14 mg/kg in 0.5 ml NSS were administered subcutaneously after surgery and at 6 h post-CLP. Mice were sacrificed at 24 h after CLP under isoflurane anesthesia for tissue sample collection. The collected serum was kept at - 80°C until analysis. A Syk inhibitor (R788 disodium,

Selleckchem, Houston, USA) in 0.1 M citrate buffer (pH 6.8) at 25 mg/kg/dose was orally administered in two separate groups of experiments including i) daily oral administration for 14 days prior to CLP and at 6 h after CLP-surgery and ii) daily oral administration for 3 days prior to CLP and at 6 h after CLP-surgery. Blood collection through tail vein was performed 2 days prior to CLP and at sacrifice for pre- and post-CLP parameters, respectively. Then, blood was collected through tail vein or cardiac puncture to explore lupus characteristics including serum creatinine (Cr) (QuantiChrom Creatinine-Assay, DICT-500, BioAssay, Hayward, CA, USA), serum anti-dsDNA by a protocol using coated Calf DNA (Invitrogen, Carlsbad, CA, USA) [125] and serum cytokines by ELISA (PeproTech, Oldwick, NJ, USA). Symptomatic lupus was defined as increased serum anti-dsDNA antibodies and high serum Cr compared with age-matched control WT mice. In addition, endotoxin (LPS) was measured as a parameter for sepsis severity using the Limulus Amebocyte lysate test (Associates of Cape Cod, East Falmouth, MA, USA). Values of LPS <0.01 EU/mL were recorded as 0.

Histology analysis

Semi-quantitative evaluation of renal histology on paraffin-embedded slides was performed after 10% neutral buffered formalin fixation, followed by hematoxylin and eosin (H&E) staining [133, 134]. In brief, sepsis-induced renal injury (defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation) was performed at 200× magnification in 10 randomly-selected fields for each animal using the following scoring method: 0, area of damage <5%; 1, area of damage 5%–10%; 2, area of damage 10%–25%; 3, area of damage 25%–50%; and 4, area of damage >50%.

RNA sequencing

RNA-sequencing was performed to determine the influence of a Syk inhibitor on FcgRIIb$^{-/-}$ and WT macrophages after activation by WGP (500 µg/mL) plus LPS (100 ng/mL). FcgRIIb$^{-/-}$ and WT macrophages were treated with a combination of WGP and

LPS, with and without the active form of the Syk inhibitor, R788 (Selleckchem), at 10 μg/mL for 6 h. Then, cells were collected for RNA extraction using an RNA easy mini kit (Qiagen). RNA sequencing was performed by the BGI Company. Differential gene expression was determined using R-package. Biological process and pathway analysis was performed using GO analysis and gene ontology pathway analysis, respectively (Figure 12)

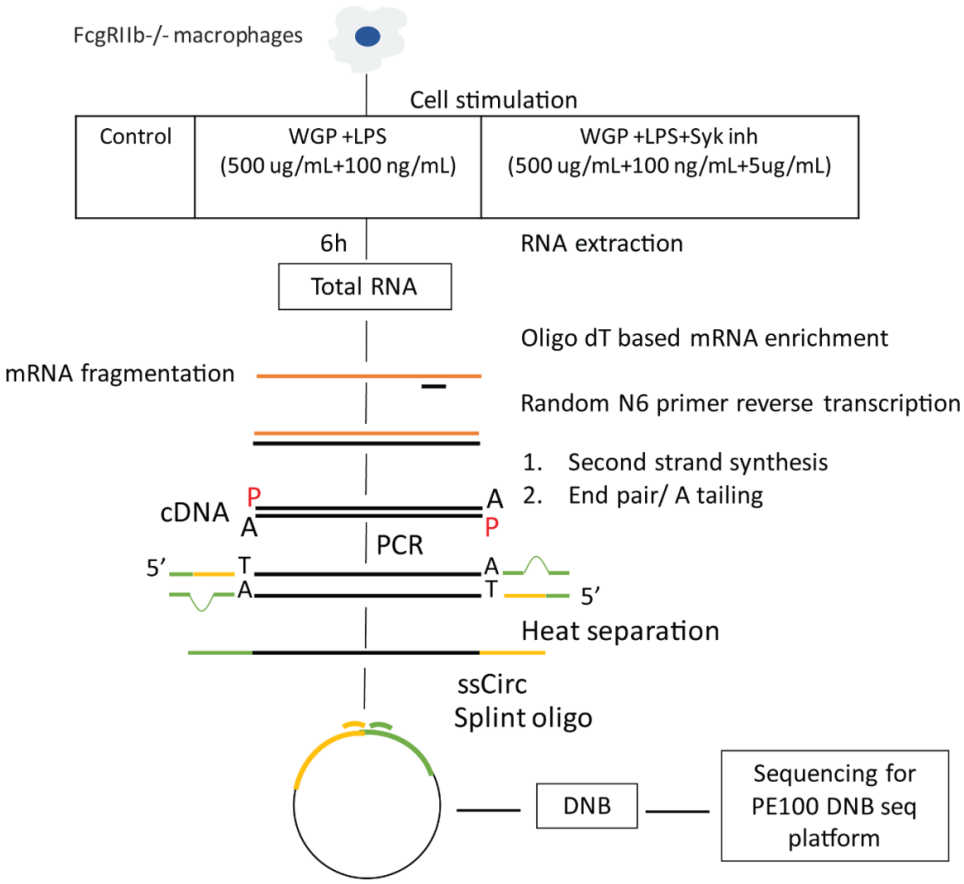


Figure 12 The illustration of overview of RNA sequencing workflow.

Statistical analysis

Statistical differences among groups were examined using the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with two groups or more than two groups, respectively, and are presented as the mean ± standard error (SE). Statistical comparisons of data

before and after treatment were conducted by paired Student's *t*-test. SPSS 11.5 software (SPSS, Chicago, IL, USA) was used for all statistical analyses.

CHAPTER V

RESULTS

Spontaneous gut leakage with elevated serum LPS and BG in patient with lupus nephritis

The spontaneous presentation of the foreign molecules such as LPS and BG in the blood, without any systemic infection, is suggestive of GI permeability barrier defect [135, 17]. In order to determine the spontaneous gut leakage, serum of the LN patients was obtained from King Chulalongkorn memorial hospital. Then, the clinical data was reviewed for dividing the serum of lupus LN into 2 groups, active lupus nephritis, inactive lupus nephritis compared with healthy volunteers. The SLE patient's clinical data indicate in Table 2. Furthermore, the exclusion criteria for exclude the patients that present the other symptoms that may affect gut permeability were discussed in the method session. We found that the spontaneous elevated serum LPS and elevated serum BG (>60 pg/mL) were predominantly observed in active LN (Figure 13). The serum LPS was observed in the group of active LN more than inactive LN (Figure 13A) Interestingly, the elevated of serum BG was found in 86% of active LN (12 of 14 patients) and 50 % of inactive lupus LN (Figure 13B). Moreover, both LPS and BG level were presented at very low level in serum of healthy volunteers. This data supported the hypothesis of gut leakage in lupus as previously reported [3]. Moreover, endotoxemia and β-glucanemia are also found in sepsis [17]. Thus, increased the level of LPS and BG in the blood of lupus patients, it might be possible that LPS and BG might physiological affect sepsis severity in the lupus context. Thus, we used lupus mouse model for further investigate this possibility.

Table 2 The clinical data of healthy volunteers and lupus patients

Patient characteristics	Heathy volunteers (n=10)	Inactive LN (n=14)	Active LN (n=14)
Female gender (%)	100	100	100
Age, years	31 ± 3	34 ± 2	31 ± 2
Serum creatinine, mg/dL	0.04 ± 0.06	1.01 ± 0.03	1.06 ± 0.05
Urine protein creatinine index	0.11 ± 0.02	0.52 ± 0.12	$1.11 \pm 0.27^{\#}$
Urine white blood cells/mm^3	0	2.77 ± 0.53	$41.46 \pm 15.71^{\#}$
Urine red blood cells/mm^3	0	10.23 ± 2.09	$30.69 \pm 6.74^{\#}$
Patient with positive anti-dsDNA	0	7(50)	11(79)
Patients with low CH50 values	0	0	6(48)
SLEDAI-2K	0	5.85 ± 0.59	$30.69 \pm 6.74^{\#}$
Steroid dosage, mg/day	0	5.85 ± 0.59	$5.85 \pm 0.59^{\#}$

Data are expressed as mean $\pm$ SE or n (%), unless otherwise indicated.

SLEDAI-2K, SLE Disease Activity Index 2000. $^{\#}$ $p < 0.05$ inactive versus active lupus.

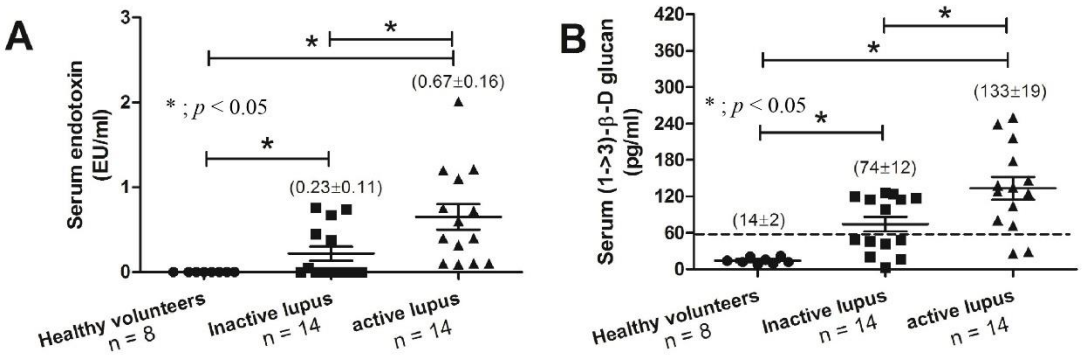


Figure 13 The spontaneous gut leakage in SLE patients (inactive SLE n=14, active SLE n=14) A) the representative of serum endotoxin level compare with healthy control (n=8). B) the representative of serum (1→3)-β-D-glucan in SLE patient and healthy control.

Spontaneous GI leakage with elevated serum LPS and BG in 40-week-old FcgRIIb$^{-/-}$ mice

To investigate the immunological evident and clinical manifestation of FcgRIIb$^{-/-}$ mice. Mice were divided into three different age groups, 8-week-old, 24-week-old, and 40-week-old respectively. Then, anti-dsDNA and proteinuria and serum creatinine were measured. As a result, we found that the biological characteristic was different in each age group. As such, at 8-week-old mice showed life span compatible with the wild type with no anti-dsDNA, urine protein, and serum creatinine. At 24-week-old mice developed anti-dsDNA and urine protein but not elevated serum creatinine. At 40-week-old mice developed anti-dsDNA, urine protein, and serum creatine (Figure 14 A-C parameter).

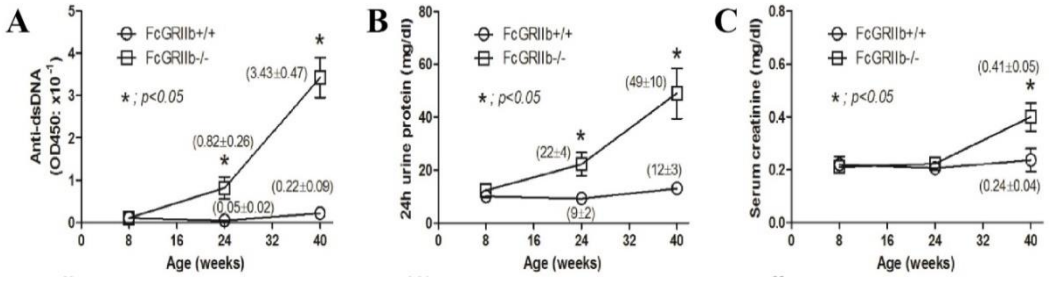


Figure 14 SLE disease activity parameter. FcgRIIb$^{-/-}$ mice from the different age groups (8, 24, and 40 weeks) were determined the disease activity. A) the level of anti-dsDNA, B) the level of urine protein and C) the level of serum creatinine (n=4-6/timepoint).

Moreover, we investigate serum cytokines level in mouse, we found that the proinflammatory cytokines (IL6 and IL10, but not TNF) significantly increased in the serum of 40-week-old mice (Figure 15 D-F). In order to see if there is gut leakage in the FcgRIIb$^{-/-}$ mice, the spontaneous gut leakage was determined by FITC-dextran assay, the elevation of LPS and BG level in the blood, was found in the FcgRIIb$^{-/-}$ at 40 weeks, but not in the other age group and wild type (Figure 15 A-C).

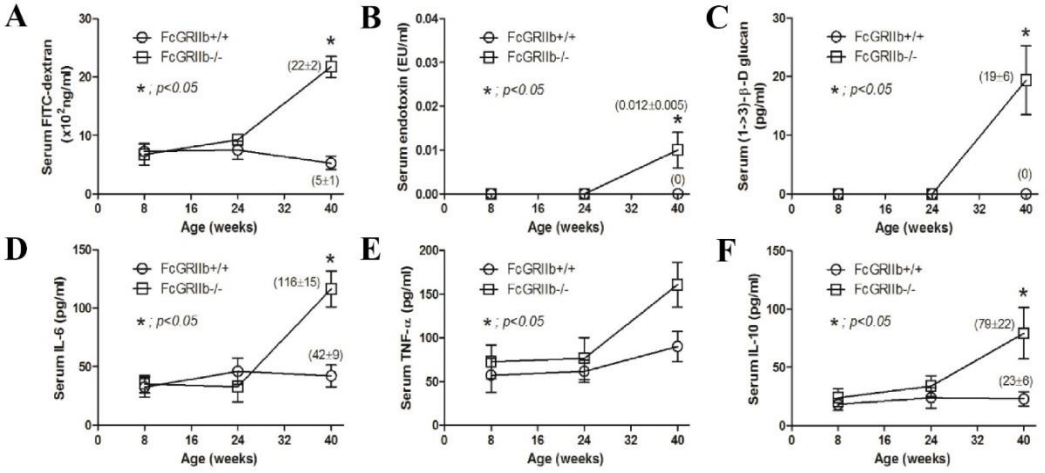


Figure 15 The biological characteristic of FcgRIIb$^{-/-}$ mice including A) the level of serum FITC-dextran, B) serum endotoxin, C) serum (1$\rightarrow$3)-β-D-glucan, D) serum IL6, E) serum TNF-α, F) serum IL10 respectively (n=4-6/timepoint).

Next, we observed whether if there was an age-dependent severity of sepsis in the different age groups. We performed cecal ligation and puncture surgery in both groups. We found that there was more severe sepsis than age-matched wild-type only in 40-week-old FcgRIIb$^{-/-}$ mice (Figure 16). The severity of sepsis in both age groups was compatible between 8 and 24-week-old mice.

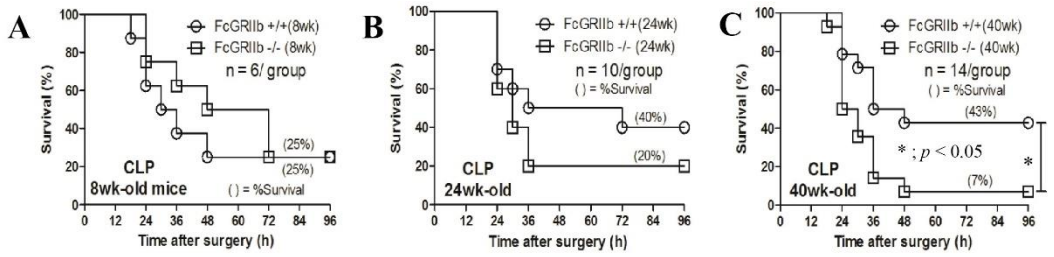


Figure 16 The mortality rate of sepsis in the different age group compared with age-matched with wild type A) the mortality rate of 8-week-old (n=6/group) B) the mortality rate of 24- week- old (n= 10/ group) C) the mortality rate of 40- week old mice (n=14/group)

According to the lupus manifestation, as age related characteristics of FcgRIIb$^{-/-}$ mice can be divided symptomatic and asymptomatic status in the different age groups. We selected 8- weeks- old as asymptomatic group and 40 weeks- old- mice as asymptomatic for the further experiment (Figure 17).

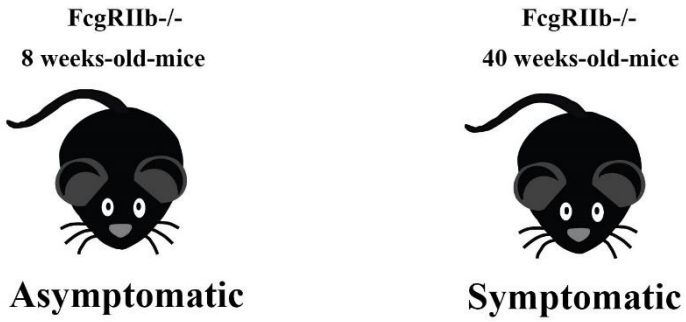


Figure 17 The representative of asymptomatic and symptomatic status in the different age group of FcgRIIb$^{-/-}$ mice.

To determine whether gut leakage in FcgRIIb$^{-/-}$ is caused by the immune complex deposition in GI tract. We determine the immune complex deposition in FcgRIIb$^{-/-}$ by immunofluorescent staining. The immune complex deposition in the GI tract of symptomatic mice was higher than age-matched wild-type, and the immune complex was found at the lamina propia of the mouse GI tract (Figure 18). This data suggested that immune induce gut injury in symptomatic FcgRIIb$^{-/-}$ mice.

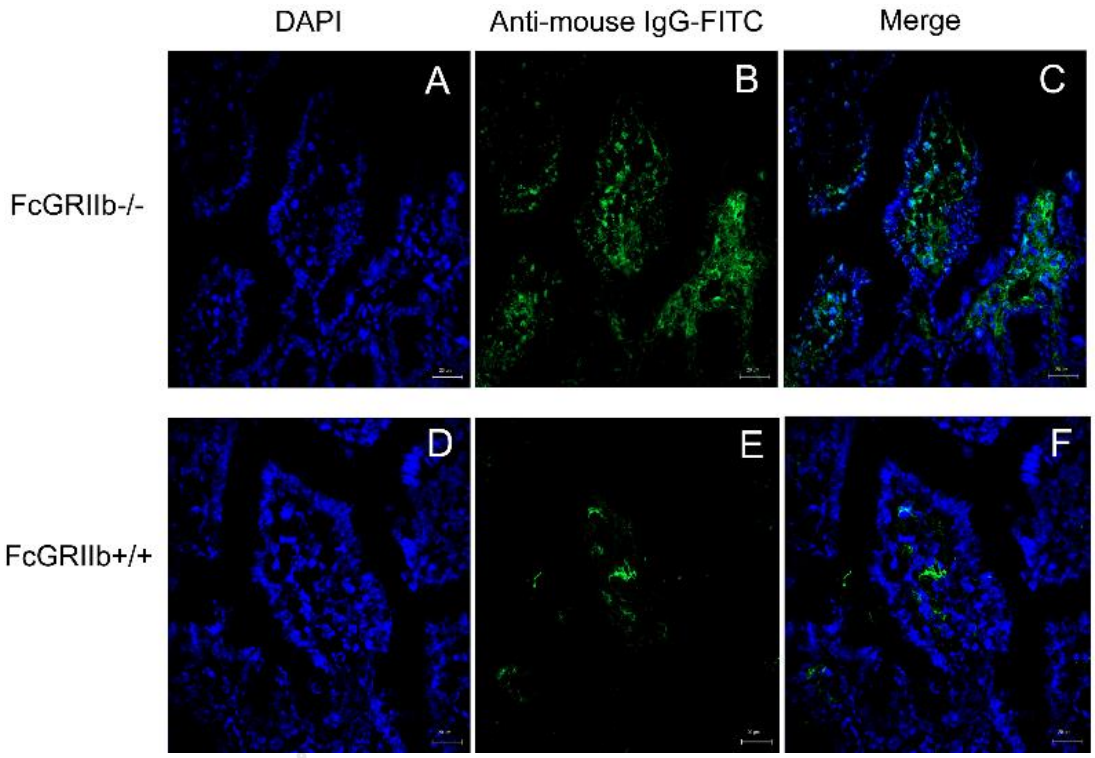


Figure 18 The illustration of immunofluorescent of jejunum FcgRIIb$^{-/-}$ (40-weeks-old) (A-C) and their age-matched with wild type.

Gut leakage enhanced sepsis severity in both symptomatic and asymptomatic FcgRIIb$^{-/-}$ mice

We hypothesized that gut leakage may affect the severity of sepsis in lupus context. To test this hypothesis, we induced gut leakage in both asymptomatic and symptomatic mice. The GI leakage was induced using Dextran Sulfate Sodium (DSS) mixing in drinking water for 1 week, to investigate the role of gut leakage in sepsis. At first, we confirmed the characteristic of gut leakage induced by DSS, serum FITC-

dextran, serum LPS, serum BG and were measured. Indeed, DSS induced mild gut leakage in both 8 weeks and 40 weeks of both strains of mice (Figure 19). Although, gut leakage has been found in the 40-week-old FcgRIIb$^{-/-}$, DSS increased the severity as demonstrated by those parameters (Figure 19). Interestingly, spontaneous polymicrobial bacteremia was presented only 40-week-old FcgRIIb$^{-/-}$ with DSS (Figure 19B). This implied that gut leakage is severe enough for translocation of viable bacteria after DSS administration. Of note, the level of proteinuria was not different after DSS insult (Figure 19D).

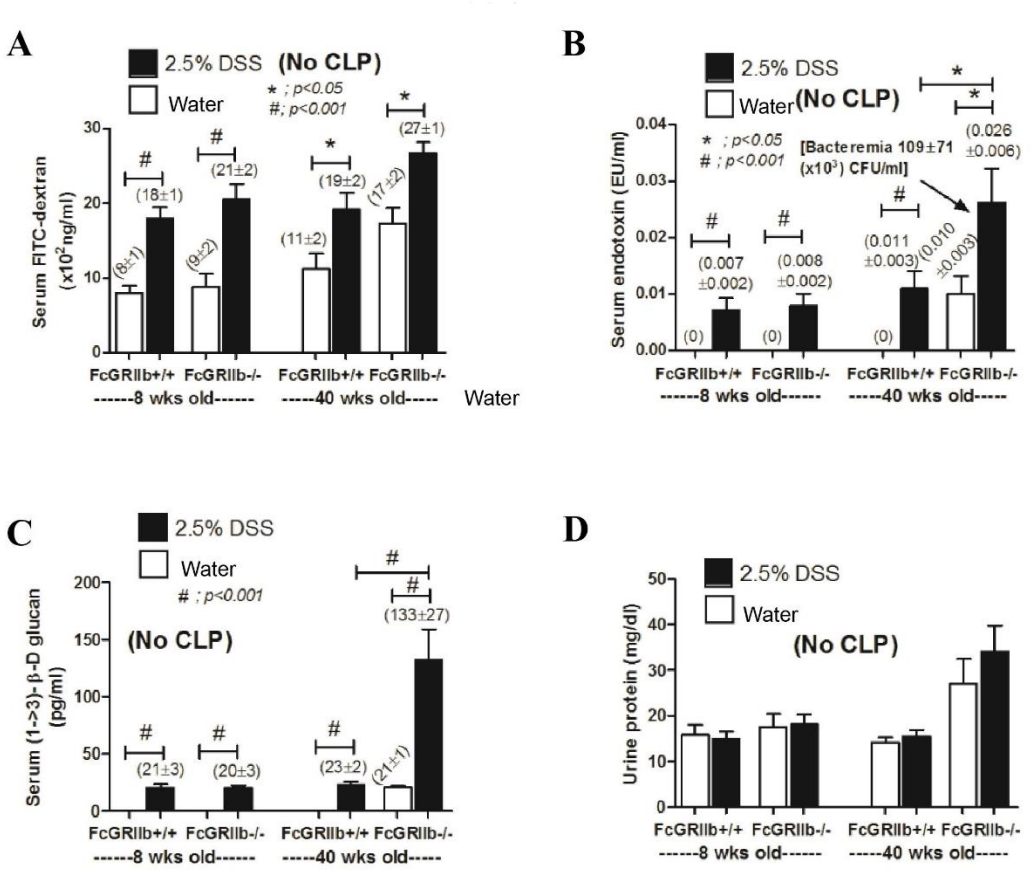


Figure 19 The characteristics of impaired gut permeability induced by 2.5% dextran sulphate solution (DSS), as measured by A) serum FITC-dextran, B) serum endotoxin, C) (1→3)-β-D-glucan, D) Urine protein in both strains were shown (n=5-6/group).

Consequently, CLP surgery was performed in both age groups, after 1-week administration, and the survival rate was analyzed. DSS administration, showed more

severe sepsis than wild type mice in both age group suggesting DSS administration enhanced sepsis severity in 40-week-old wild-type, but DSS administration enhanced sepsis severity in FcgRIIb$^{-/-}$ mice more than wild type from both age groups as determined by survival rate, bacteremia, serum IL-6 and serum creatinine (Figure 20 A-H). Moreover, we compare severity of sepsis after DSS compare with the control in both age groups of wild-type and FcgRIIb$^{-/-}$ mice. In the group of FcgRIIb$^{-/-}$ mice, all the mice die within 30 hours after CLP with DSS (Figure 20 A, E). Although, DSS enhanced sepsis severity in 40-weeks-old wild-type mice, we found that DSS enhanced sepsis severity was greater in FcgRIIb$^{-/-}$ more than wild type in both age groups (Figure 21).

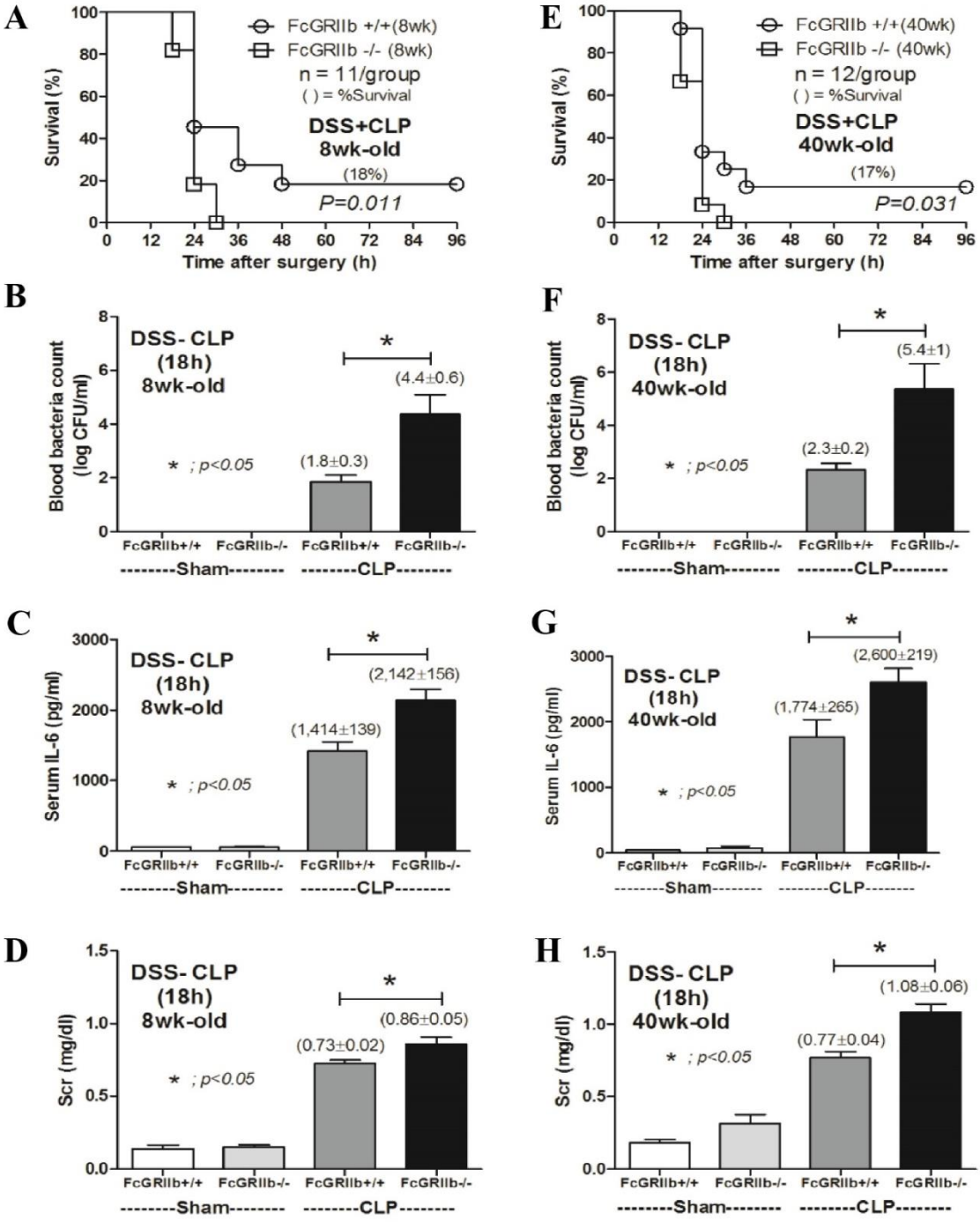


Figure 20 The survival analysis and biological parameter of FcgRIIb$^{-/-}$ and wild type mice after DSS administration (n=5-7/ group)

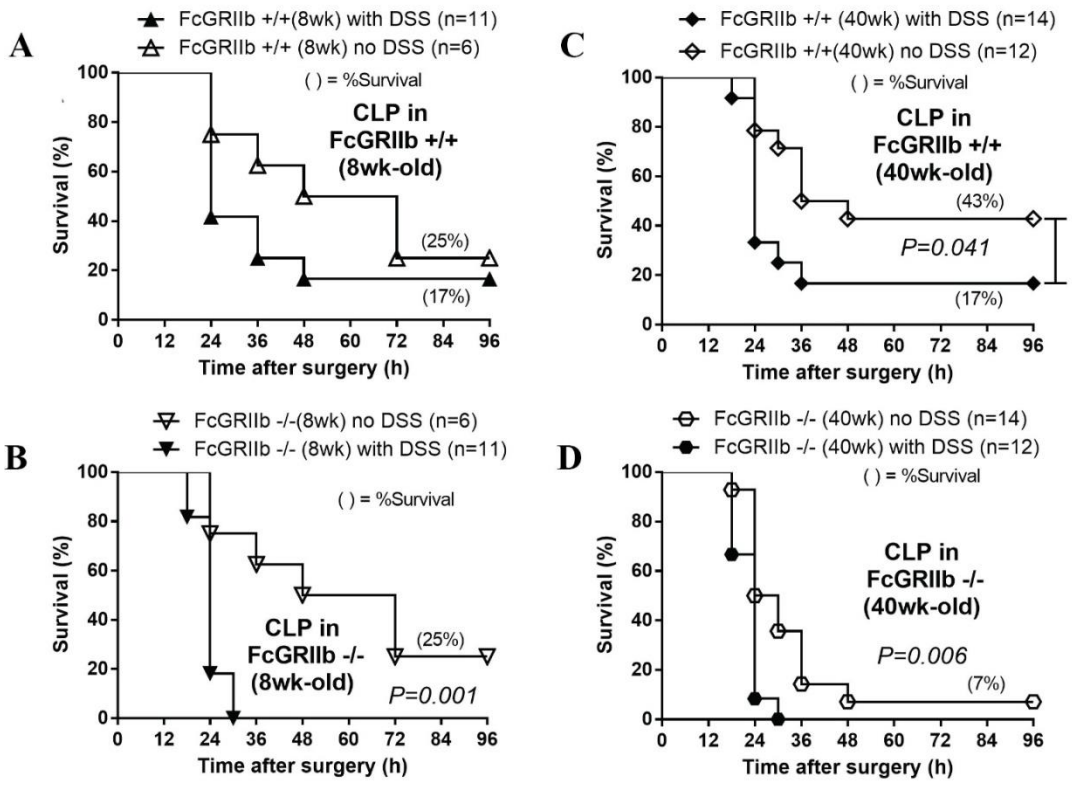


Figure 21 Survival analyses of cecal ligation and puncture (CLP) at 8-weeks-old and 40 weeks-old in wild-type (FcGRIIb+/+) and FcGRIIb-/- with or without 2.5% dextran sulfate solution (DSS) administration.

LPS alone or LPS+BG, but not BG alone, enhanced inflammatory responses and the sepsis severity in FcgRIIb$^{-/-}$ mice

LPS was observed together with BG in symptomatic FcgRIIb$^{-/-}$ mice. To investigate the physiological importance of these two molecules derived from the gut translocation, we injected LPS and BG alone or BG+LPS in 8-week-old (asymptomatic group). In the absence of sepsis induction, FcgRIIb$^{-/-}$ mice showed higher level of cytokine responses after the injection of LPS, BG, and BG+LPS (Figure 22, 23, 24). The prominent response occurred 1 hour after injection. The cytokine response in BG alone was lowest among three conditions (Figure 21B, 22B, 23B). of note, the concentration of BG is less than 5 mg/kg did not significantly induced cytokine responses in both

strains of mice (data not show). The synergistic effect of cytokine responses was found in the condition of LPS+BG, as determined by the AUC of the response of all cytokine in FcgRIIb$^{-/-}$ mice, but only IL6 in wild type mice (Figure 21D, 22D, 23D).

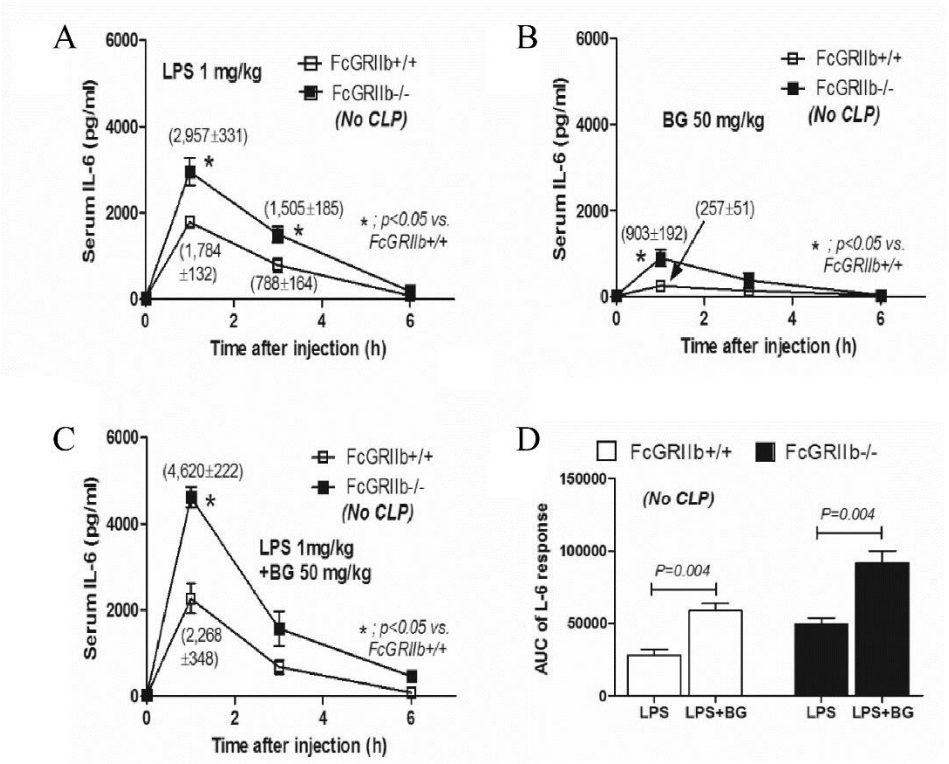


Figure 22 The representative of serum cytokine responses in FcgRIIb$^{-/-}$ and wild-type mice after intraperitoneal (ip) endotoxin (LPS) injection with intravenous (iv) normal saline (NSS) (LPS alone) or iv (1→3)-β-D-glucan (BG) with ip NSS (BG alone) and ip LPS with iv BG (LPS+BG), as determined by IL-6 (A-C) is shown.

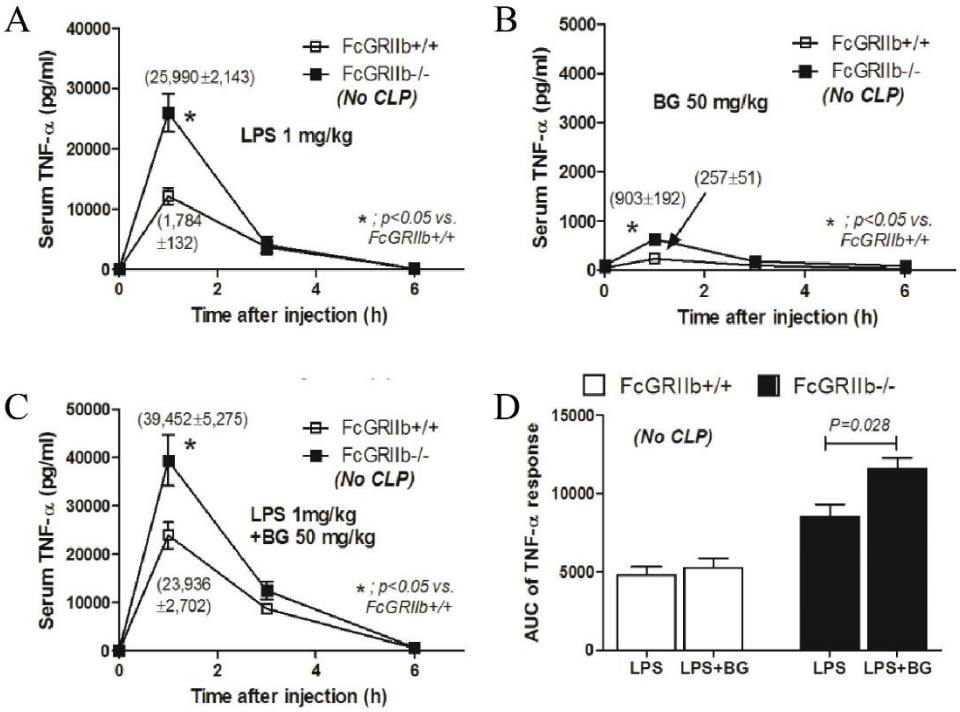


Figure 23 The representative of serum cytokine responses in FcgRIIb$^{-/-}$ and wild-type mice after intraperitoneal (ip) endotoxin (LPS) injection with intravenous (iv) normal saline (NSS) (LPS alone) or iv (1→3)-β-D-glucan (BG) with ip NSS (BG alone) and ip LPS with iv BG (LPS+BG), as determined by TNF-α (A-C) is shown.

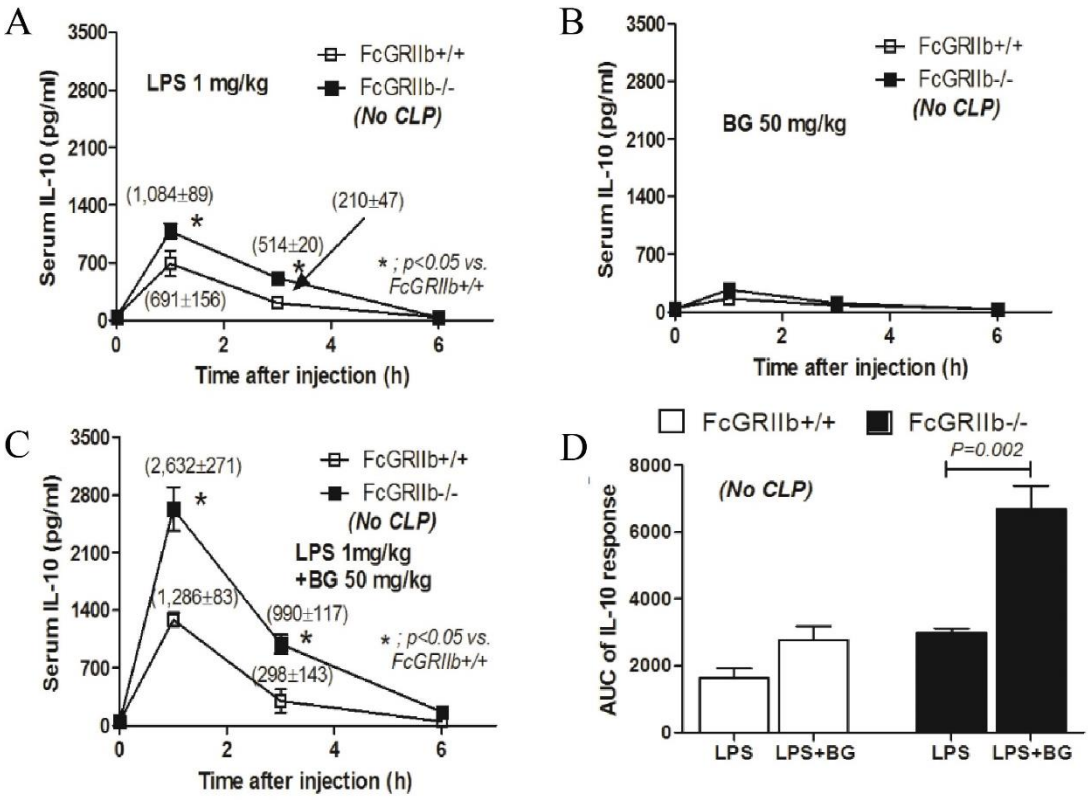


Figure 24 The representative of serum cytokine responses in FcgRIIb$^{-/-}$ and wild-type mice after intraperitoneal (ip) endotoxin (LPS) injection with intravenous (iv) normal saline (NSS) (LPS alone) or iv (1⟶3)-β-D-glucan (BG) with ip NSS (BG alone) and ip LPS with iv BG (LPS+BG), as determined by IL10 (A-C) is shown.

Next, we further examine the effect of BG, LPS, and LPS+BG in the severity of sepsis. We found that LPS injection, with or without BG (but not BG alone), enhanced sepsis severity as determined by survival rate and serum cytokine levels (Figure 25). FcgRIIb$^{-/-}$ mice showed higher severity of sepsis more than wild type (Figure 25 A, C, E). The synergy of serum cytokine after CLP induction in FcgRIIb$^{-/-}$ mice was shown only IL6 (Figure 25 B), but not with TNF-α and IL10 (Figure 25 D, F). In wild type group, the survival rate of control, BG injection, LPS injection, and BG+LPS injection was not different (Figure 26 A). In the FcgRIIb$^{-/-}$ mice, the sepsis the mortality rate of mice injection with BG+LPS were higher than sepsis with BG but there was no difference between sepsis with LPS injection (Figure 26 B).

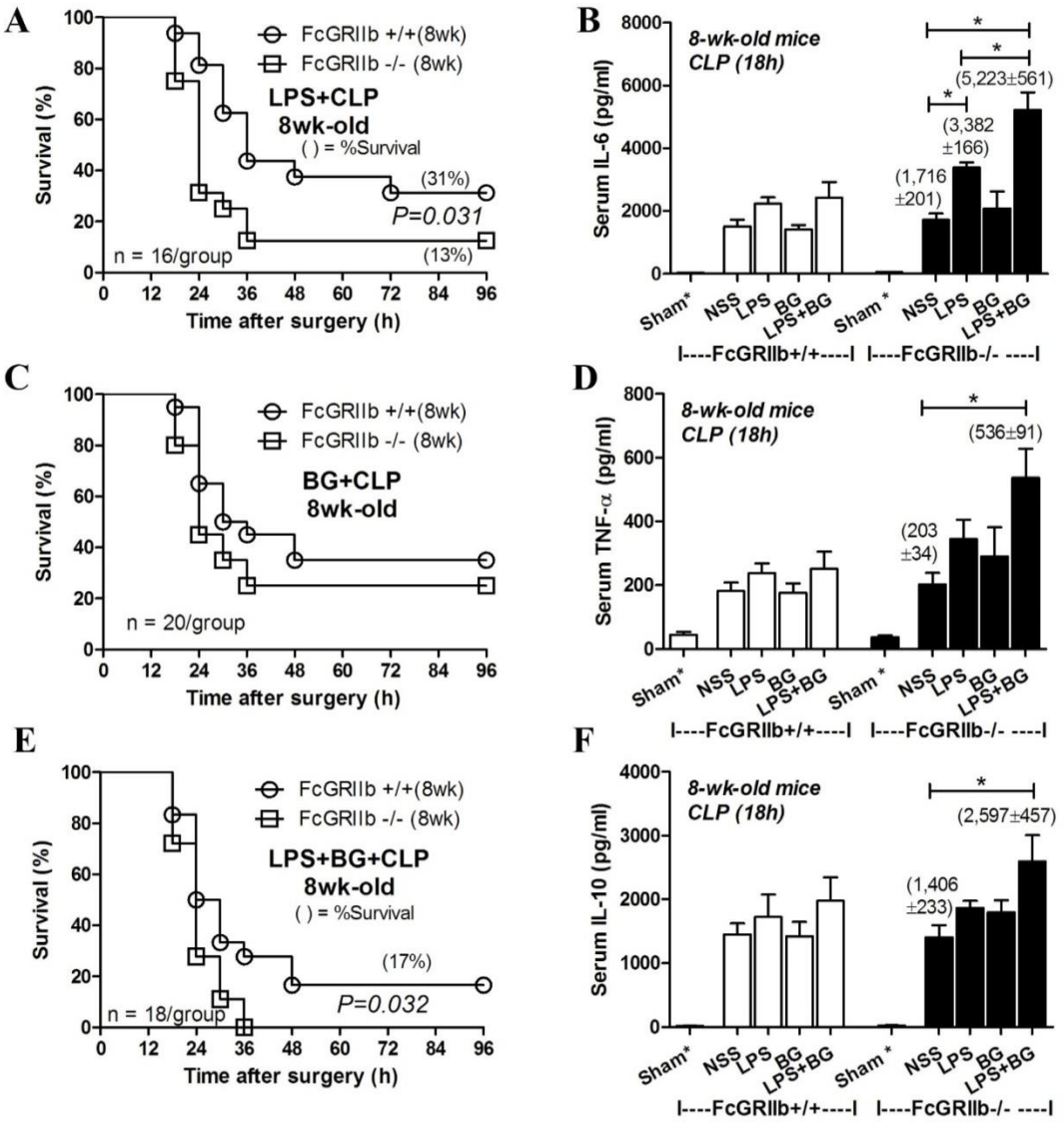


Figure 25 The illustration of survival analysis and serum cytokine with the CLP induction in FcgRIIb$^{-/-}$ mice and wild-type mice after intraperitoneal (ip) endotoxin (LPS) injection with intravenous (iv) normal saline (NSS) (LPS alone) or iv (1→3)-β-D-glucan (BG) with ip NSS (BG alone) and ip LPS with iv BG (LPS+BG).

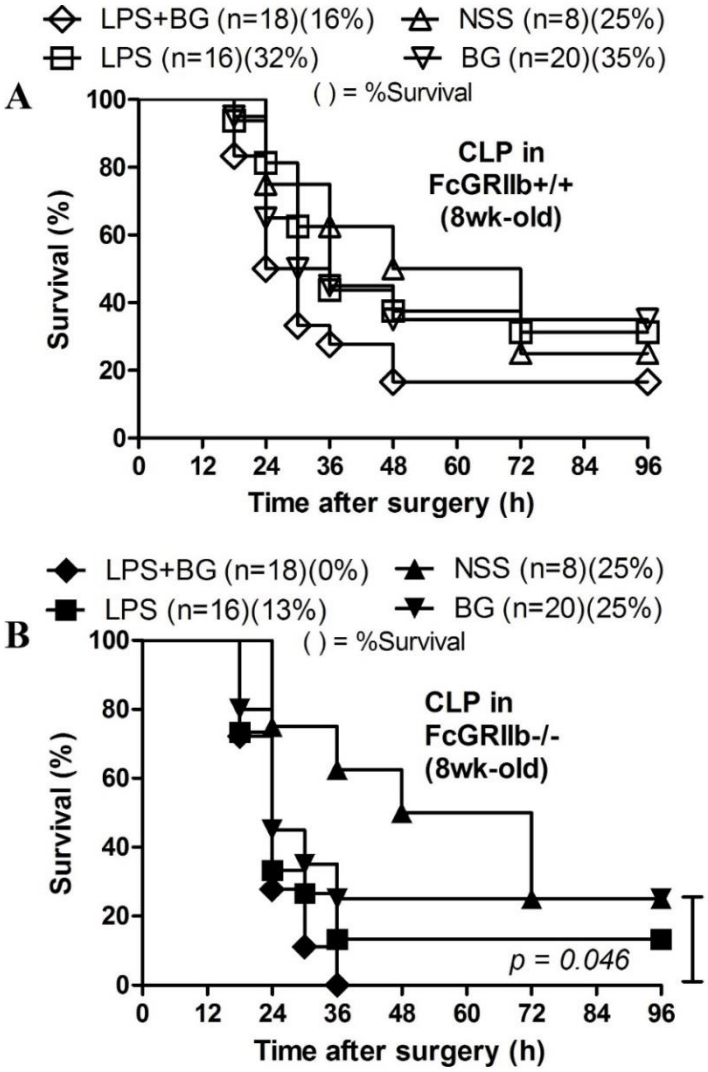


Figure 26 The survival analysis of FcgRIIb$^{-/-}$ mice and wild type mice after injection with normal saline (n=8), BG (n=20), LPS (n=16), or BG+LPS (n=18) are shown are shown.

According to the in *vivo* experiment, we cannot distinguish the difference between LPS sepsis and BG+LPS sepsis. In order to address this question, if there is a strain-dependent synergistic effect of LPS and BG on the immune-inflammatory responses. Bone marrow derived macrophage was generated and treatment with BG,

LPS, and BG+LPS compare with control. The result showed that LPS synergy with BG (IL10 and TNF-α) and the synergistic effect of these two molecules, was higher in FcgRIIb$^{-/-}$ mice (Figure 27). Thus, the translocation of these two molecules from the gut to the blood may play a crucial role in SLE and pathogenesis of sepsis in SLE. Considering the specific receptor for BG and LPS are Dectin-1 and TLR4 respectively. The synergistic activity has been demonstrated [20]. The molecular mechanism remained unclear.

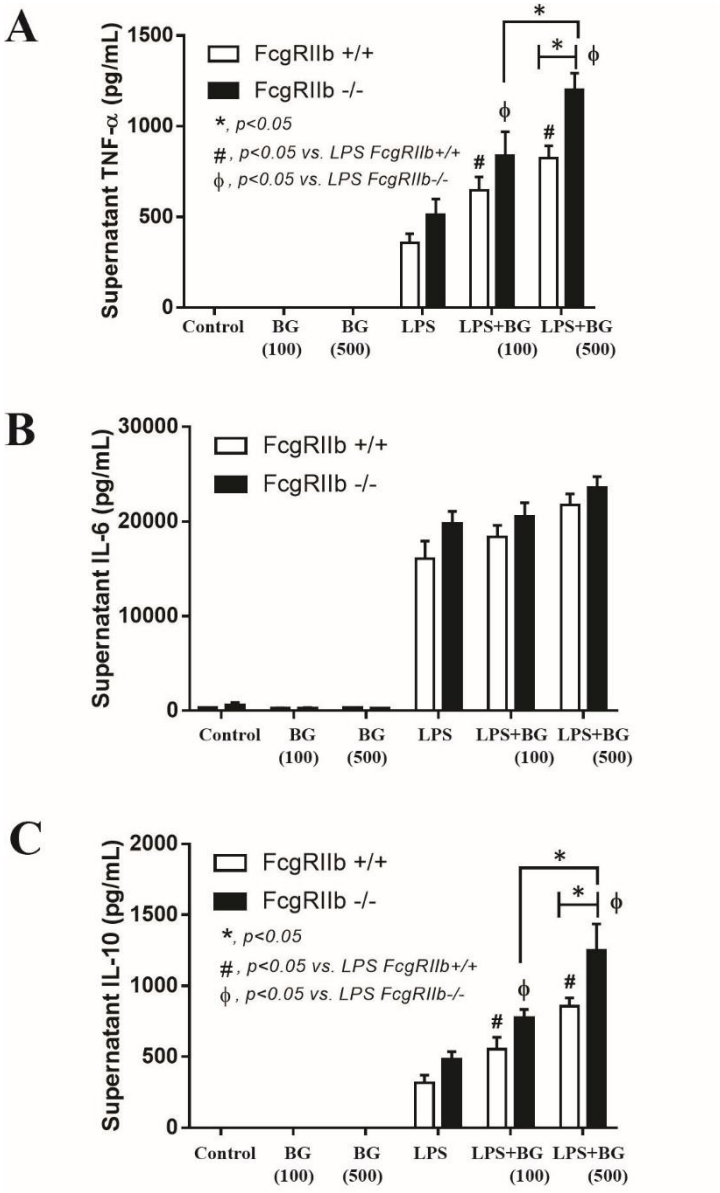


Figure 27 Supernatant cytokine from bone marrow derived macrophage from FcgRIIb$^{-/-}$ and wild-type mice after treatment with BG, LPS, or BG+LPS. Data are representative of 3 independent experiments.

Prominent responses against LPS and BG in FcgRIIb$^{-/-}$ macrophage compared with wild type macrophages.

Macrophages consider as an inflammatory cell plays a key role for inflammation in SLE, chronic inflammation leading to enhancement of SLE disease activity [136, 137].

The hallmark property of macrophages is changing polarization response to the different microenvironments. LPS and BG in the serum of symptomatic mice might activate macrophages and affects macrophage polarization. To examine gene expression related macrophage polarization bone marrow derived macrophages were treated with LPS, BG or BG+LPS compared with control. LPS, a potent proinflammatory inducer that induces M1 polarization, enhanced the expression of proinflammatory genes, *TNF-*α and *iNOS* and increase anti-inflammatory gene expression, *IL-10*, the expression level is similar in Wild type and FcgRIIb$^{-/-}$ macrophages (Figure 28 A-C). The addition of BG enhanced LPS induce *TNF-*α, but not iNOS expression and decrease *IL-10* expression in FcgRIIb$^{-/-}$ macrophages (Figure 28 A-C), this data suggested that BG showed the synergistic effect with LPS. Meanwhile, activation with LPS, with or without BG, showed the minor effect on the expression of other anti-inflammatory genes including *Fizz-1*, *Arginase-1,* and *TGF-*β, the expression of these genes lower than the control group (Figure 28 D-F), except the activation by BG alone in FcgRIIb$^{-/-}$ macrophages (Figure 28F). Thus, the activation of BG+LPS enhanced the proinflammatory characteristic FcgRIIb$^{-/-}$ of macrophage.

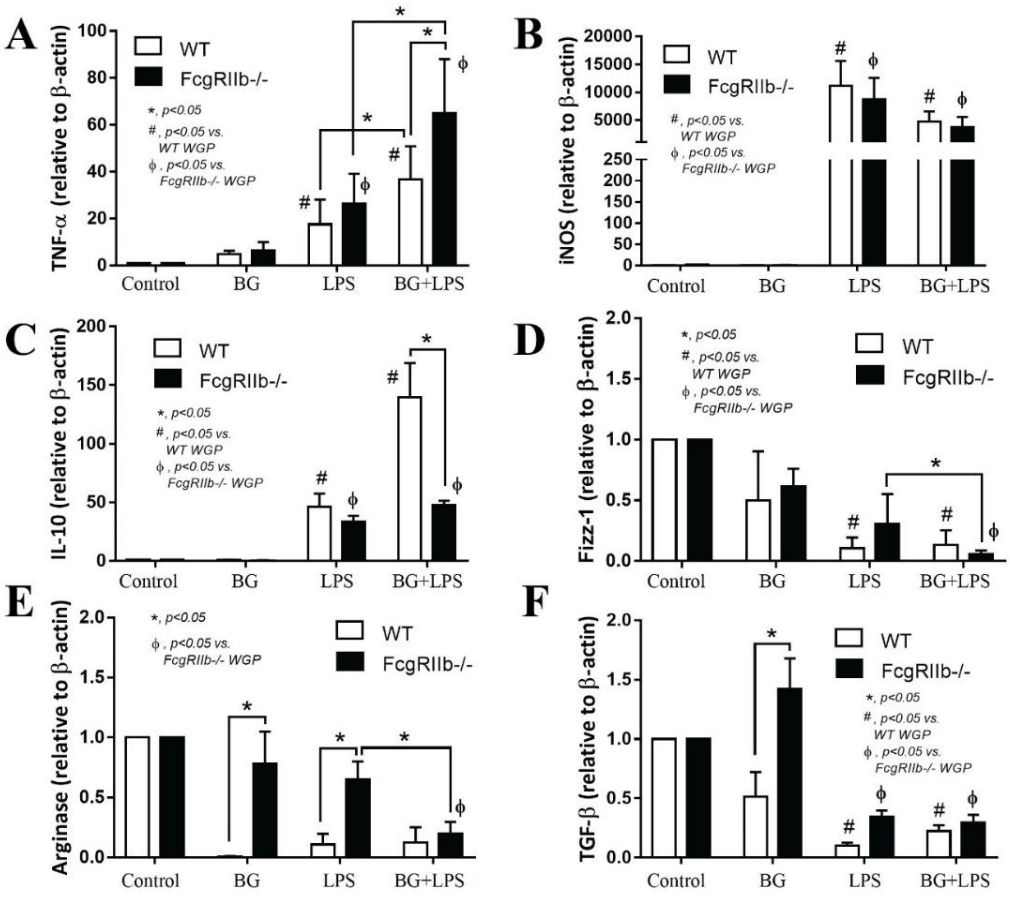


Figure 28 The representative of relative gene expression in wild type and the FcgRIIb$^{-/-}$ after treated with BG, LPS or BG+LPS for 6 hours. Data are representative of 3 independent experiments.

The synergy of LPS plus BG through Syk dependent Dectin-1 pathway toward pro-inflammation in FcgRIIb$^{-/-}$ macrophage.

We used bone marrow derived macrophages isolated from wild type and FcgRIIb$^{-/-}$ mice for study mechanism. As such, BG alone could not induce proinflamatory cytokine in macrophages from both wild-type and FcgRIIb$^{-/-}$ macrophages. But stimulation with BG+LPS compared with LPS alone, induced higher level of pro-inflammatory cytokine (TNF-α and IL10, but not IL6 in both strains of mice), the level of cytokine in FcgRIIb$^{-/-}$ macrophages are higher when compared with wild type (Figure 27). Furthermore, we investigate whether the additive effect of BG, upon LPS, was depending on the activation of BG on the TLR4. We found that the effect of BG+LPS was not due to the direct activation of BG on TLR4 as demonstrated by HEK-blue TLR-4 reporter cells (Figure 29).

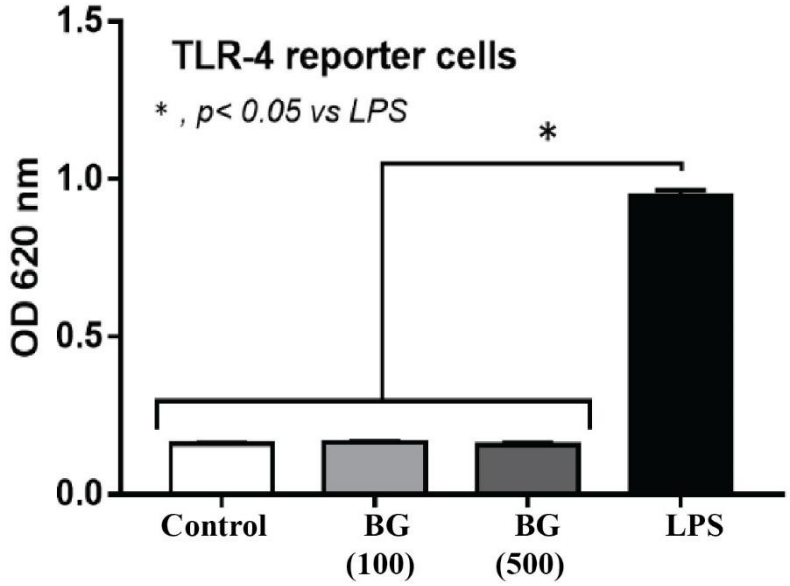


Figure 29 HEK-Blue reporter cells treated with BG, LPS, and LPS+ BG. Data are representative of 3 independent experiments.

It is interesting that the recognition of the foreign molecules from the innate immune responses provides the activation of the adaptive immune signaling, for example, the cross-talk between TLR and FcgR, sharing of these receptors at least in

part of shared- downstream target molecules are frequently mentioned [138-140]. We examined the expression level of Fcgrs and other downstream target genes, we found that the activation of macrophages by BG, LPS or BG+ LPS, we found that LPS or BG+LPS, but not BG alone, induce Fcgrs expression in both strains of macrophages but predominant expression of *Fcgrs* was higher in FcgRIIb$^{-/-}$ macrophages (Figure 30 A-E). Interestingly, LPS+ BG, compared with LPS alone, enhanced the expression of *FcgRIII, NF-κB,* and *Syk* in both strains of macrophages (Figure 30 C, E, F). In addition, the abundance of spleen tyrosine kinase (Syk) in these cells with the representative of Western blot (WB) analysis (Figure 30 G) was also demonstrated

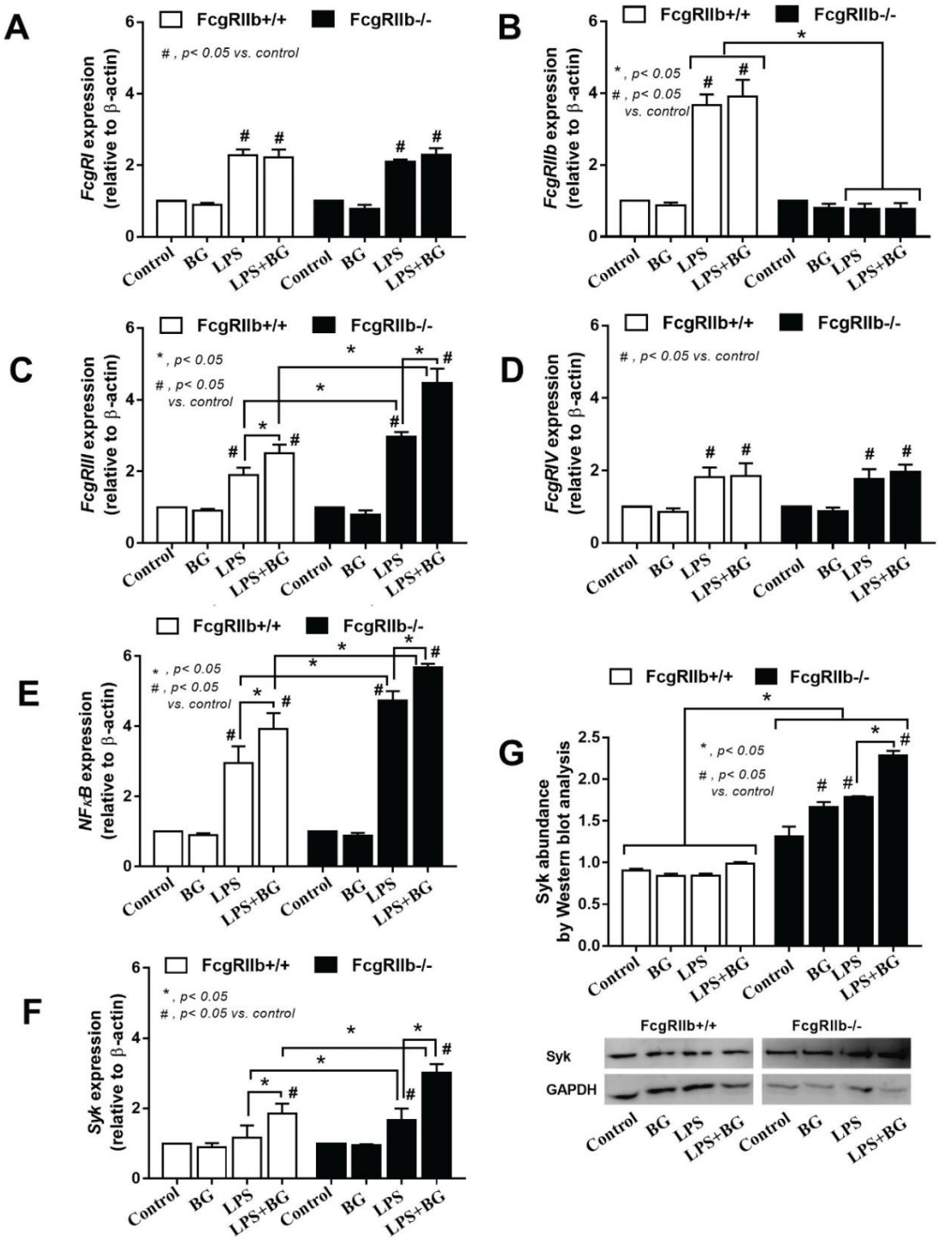


Figure 30 The gene expression by qPCR of several molecules in wild-type (FcgRIIb+/+) and FcgRIIb-/- macrophages after the 6 h stimulation by BG at 500 μg/mL with or without lipopolysaccharide (LPS) at 100 ng/mL were demonstrated (A-F). Data are representative of 3 independent experiments.

Moreover, the expression of those genes and Syk protein abundance in FcgRIIb$^{-/-}$ macrophages stimulated with LPS+BG were higher than WT (Figure 30G). While LPS or BG+LPS activated macrophages, when compared with control, the enhanced expression level of *FcgRIII,* the member of activating *FcgRs*, and *FcgRIIb*, the inhibitory *FcgR*, in WT macrophages and FcgRIIb$^{-/-}$ could not express inhibitory *FcgRIIb* (Figure 30A-D). This phenomenon is possibly responsible for the hyper-responsiveness of FcgRIIb$^{-/-}$ cells and our results support the previous study for collaboration of TLR4, and FcgR. The increased expression of *Syk* and Syk abundance after stimulation with BG+LPS, especially in FcgRIIb$^{-/-}$ macrophage, suggesting that Syk is a downstream signaling target of this stimulation. To support this finding, we used the inhibitor against Dectin-1 receptor on the synergy effect of BG and LPS by using soluble beta-glucan (Dectin inhibitor). The proinflammatory cytokine was decreased after pre-incubated with soluble glucan Dectin-1 inhibitor suggesting the effect of BG was due to the direct activation of Dectin-1 receptor (Figure 31 A). In addition, we further investigate the downstream signaling of Dectin-1 and TLR4 we found that the level of TNF-α cytokine was significantly decreased after pretreatment with Syk, NF-κB inhibitor, but not Raf-1 inhibitor, in both strains of macrophage (Figure 31 B-D). This data suggested that the similar signaling between wild type and FcgRIIb$^{-/-}$ macrophages through Dectin-1/Syk-dependent pathway following by NF-κB activation, but not via Raf-1 protein. This finding supported a previous publication[141].

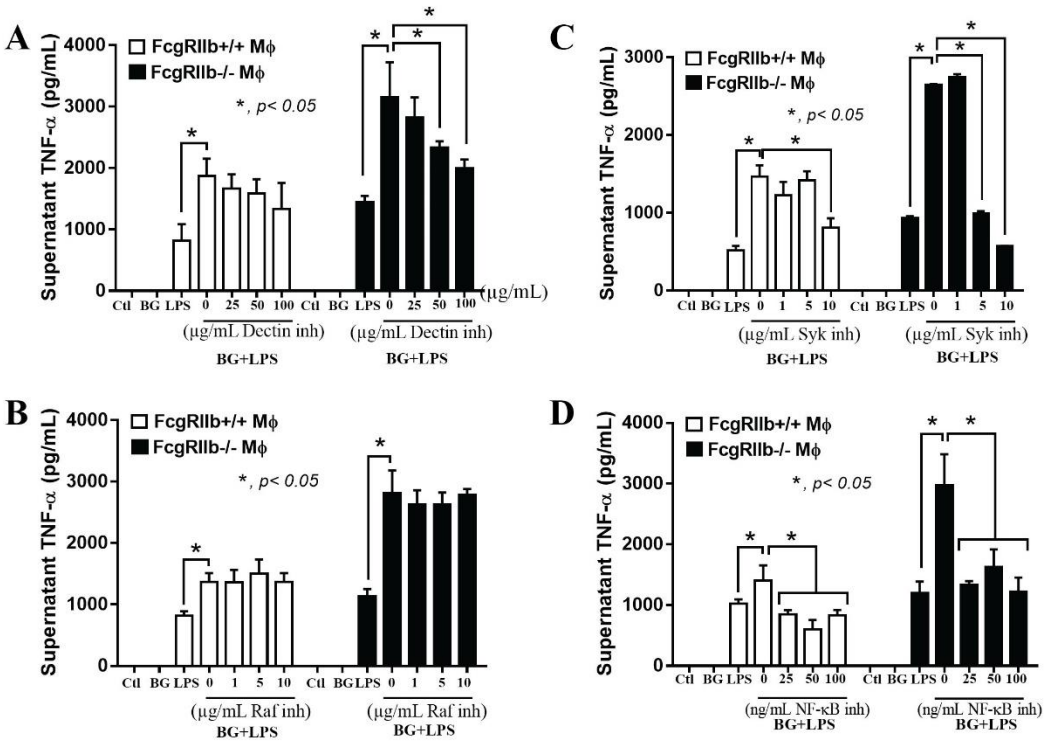


Figure 31 The representative of TNF-α cytokine level after treatment with Dectin-1 (A), Raf1 (B), Syk inhibitor (C) , and NF-ΚB inhibitor (D). Data are representative of 3 independent experiments.

The effect of Syk inhibitor in FcgRIIb$^{-/-}$ mice, the impact of pathogenesis in the treatment outcome.

Interestingly, the elevation of anti-dsDNA, LPS, and BG in the serum of symptomatic FcgRIIb$^{-/-}$ mice might activate their receptor and Syk as shared-downstream signaling [130, 131]. This activation supporting by the highest activation Syk abundance in spleen 40-wk-old FcgRIIb$^{-/-}$ mice comparing to another group (Figure 32).

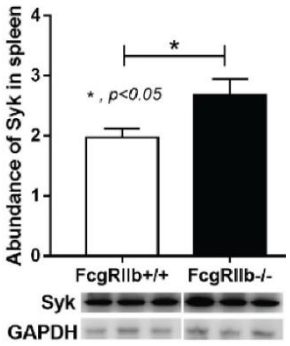


Figure 32 The Syk protein levels in the spleen of 40 week-old FcgRIIb$^{-/-}$ and their age-matched with wild type mice (n=6/group). Data are representative of 2 independent experiments.

To investigate the effect of Syk inhibitor for the treatment of lupus. We used Syk inhibitor R788 oral administration to the FcgRIIb$^{-/-}$ mice compared with wild type mice. After Syk inhibitor administration, we observed the leakage marker by determining the level of serum endotoxin and serum BG. We found that Syk inhibitor had no effect on the leakage parameters as determined by serum endotoxin and serum BG (Figure 33).

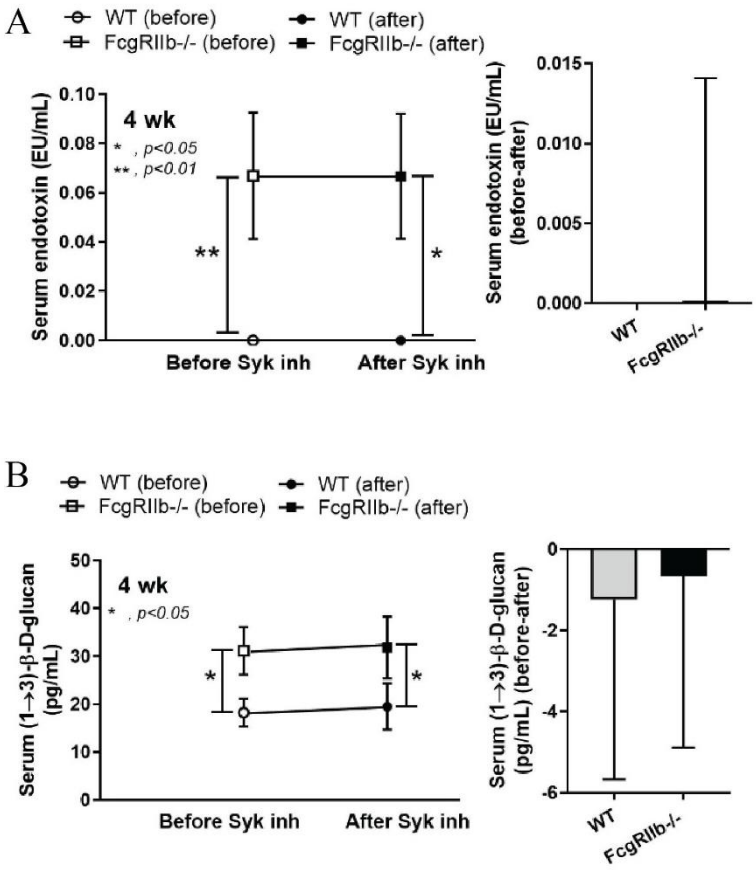


Figure 33 The representative of serum endotoxin (A) and serum BG (B) at before and after the 4 wks Syk inhibitor administration were demonstrated (n=5-7/ group).

The Syk inhibitor significantly decreased proteinuria and serum TNF-α (Figure 34 B and Figure 35 A) when compared with baseline value. Although, Syk inhibitor was not significantly reduced anti-dsDNA, IL6, and IL10 in FcgRIIb$^{-/-}$ mice in comparison to baseline, but there was a statistically significant difference on these parameters as determined by delta change between before-versus-after-administration (Figure 34, 35). Moreover, Syk inhibitor administration attenuated glomerular immune complex deposition, but not renal histopathology as determine by renal histology (Figure 36) in FcgRIIb$^{-/-}$ mice.

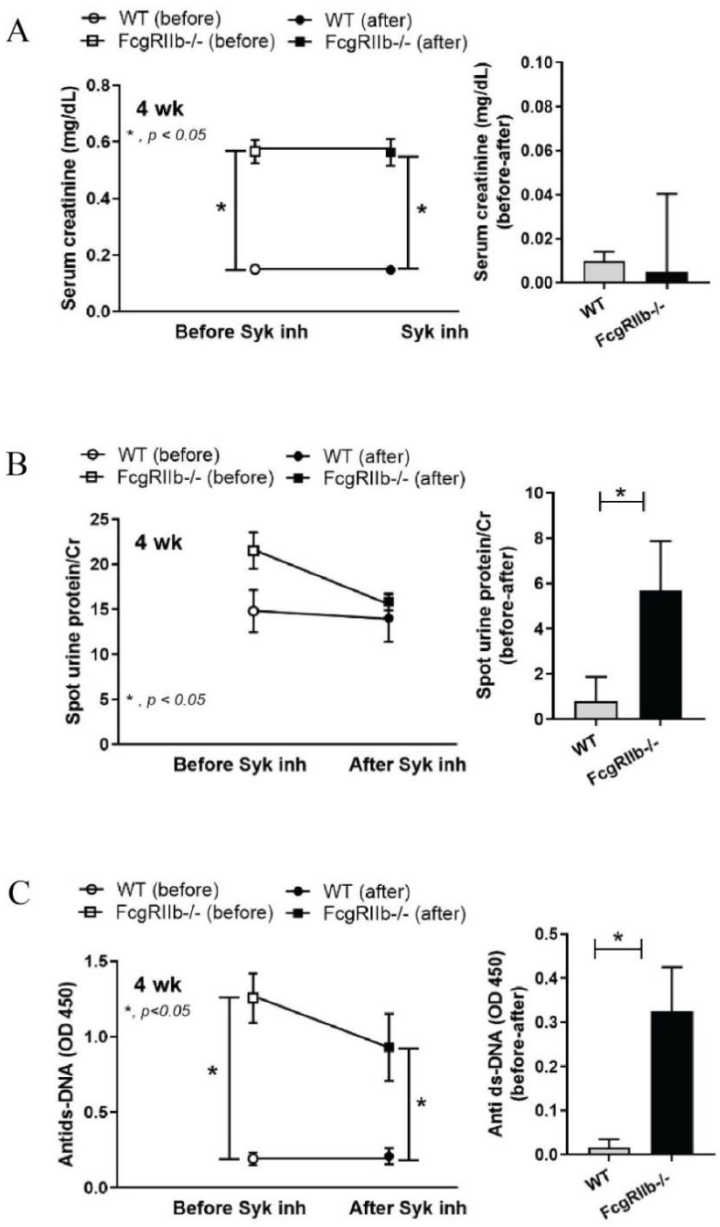


Figure 34 The biological parameters of FcgRIIb$^{-/-}$ mice and wild type mice before and after 4 wks treatment of Syk inhibitor (n= 5-7/group). A) the representative of urine protein, B) the representative of Anti-double stranded DNA.

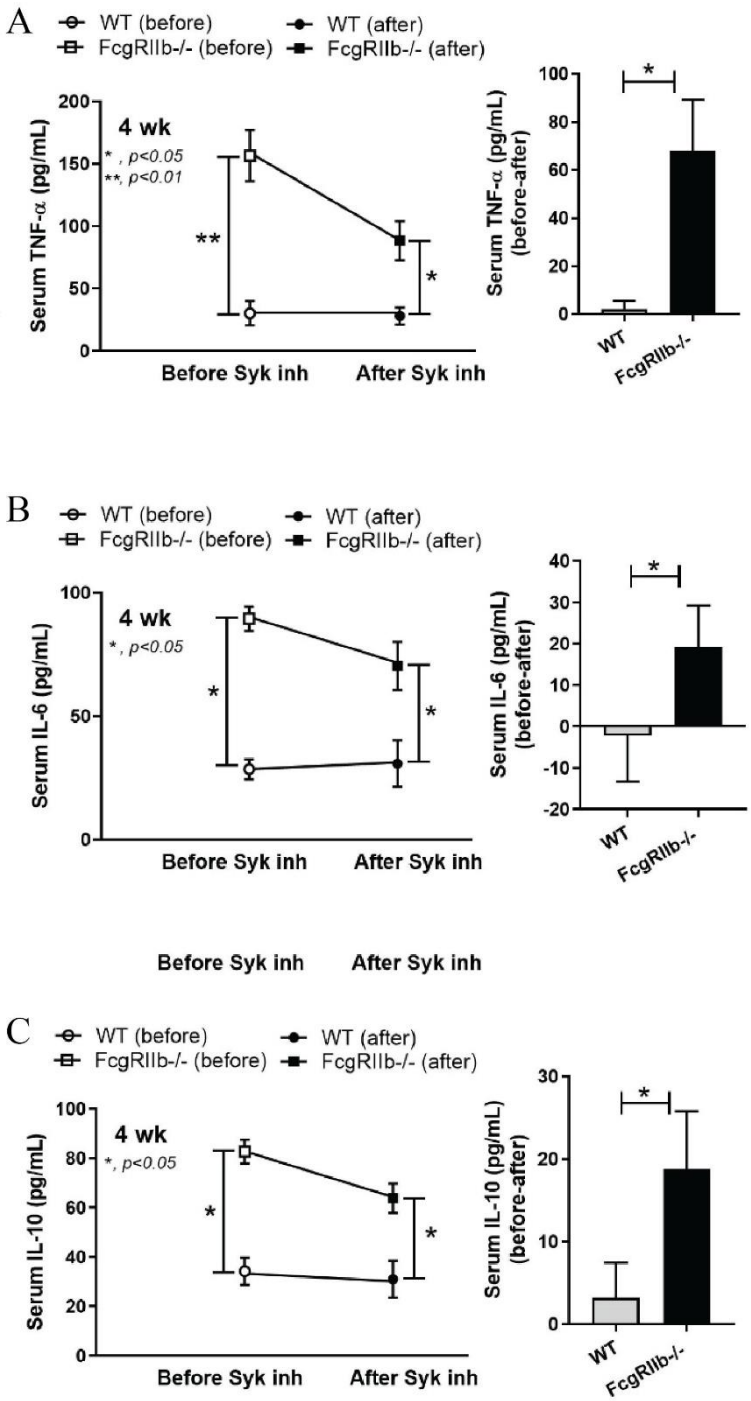


Figure 35 The representative of proinflammatory cytokine level, A) serum TNF-α, B) serum IL-6, C) serum IL-10 before and after 4 wks Syk inhibitor administration (n= 5-7/group).

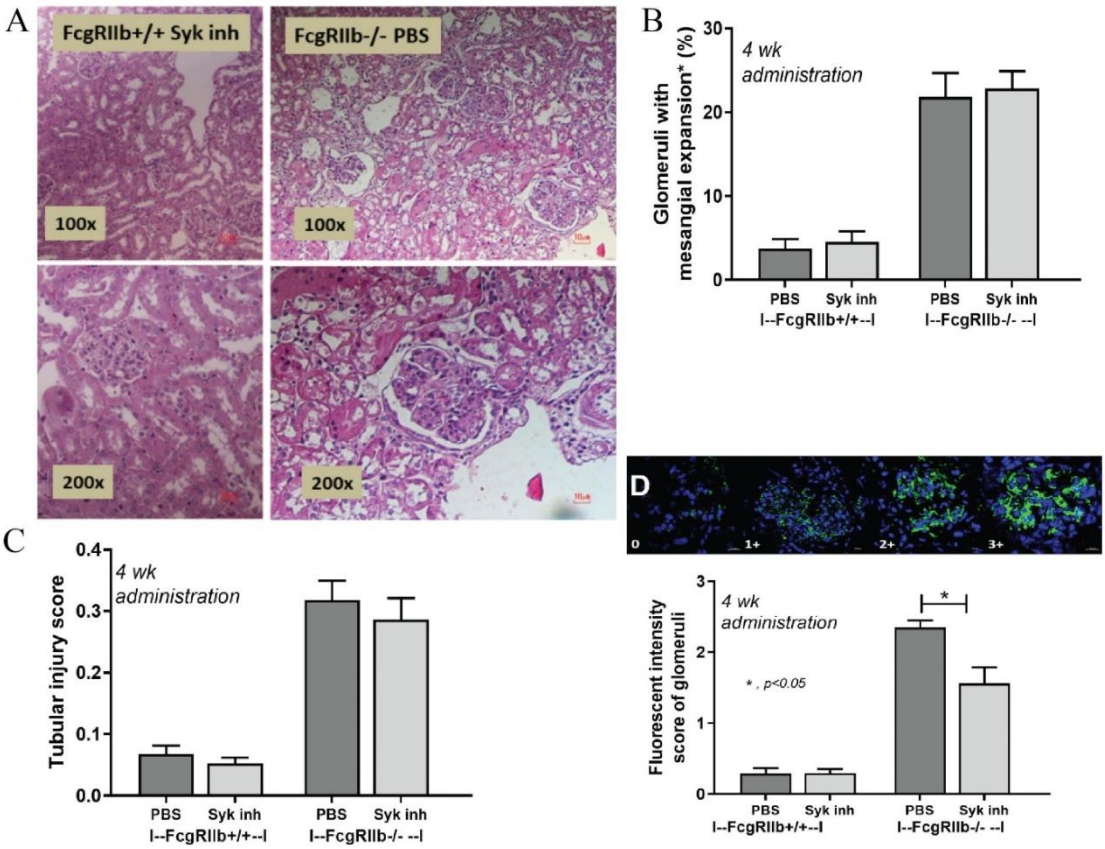


Figure 36 The representative figures of kidney histology of wild type and FcgRIIb$^{-/-}$ mice after treament with Syk inhibitor. A) the histology staining with Hematoxylin and Eosin color from mice with or without Syk inhibitor administration, B) the glomerular injury (percentage of glomeruli with more than 50% of mesangial expansion), C) the tubular injury score, D) the fluorescent intensity score of immune complex deposition (score 0-3+) were demonstrated (n=4/group).

High level of Syk in FcgRIIb-/- mice and sepsis attenuation by Syk inhibition.

BG and LPS are also found in sepsis patients. The effects of these two molecules also impact sepsis severity. As we know that the downstream signaling collaboration between BG and LPS are Syk. We hypothesized that Syk signaling protein may play an important role in sepsis in SLE context. To test this hypothesis, we

examined the level of Syk expression in FcgRIIb$^{-/-}$ and wild type mice after CLP induction. As a result, Syk expression level were higher in the spleen compared with other organs, in FcgRIIb$^{-/-}$ mice at 24 and 40 weeks of age, and in wild type mice at 40 weeks, but not in 24 weeks, of age (Figure 37). Syk levels in the spleens of 40-weeks-old WT mice were similar to those in 24 weeks FcgRIIb$^{-/-}$ mice, and Syk levels in 40-week-old FcgRIIb$^{-/-}$ mice were highest among all groups (Fig.37A). In addition, Syk levels in spleens were enhanced by CLP sepsis in both mouse strains but levels were highest in FcgRIIb$^{-/-}$ mice with sepsis (Figure 37B). Because Syk levels were increased in the spleens of CLP sepsis mice, we investigated the effect of the Syk inhibitor *in vivo*.

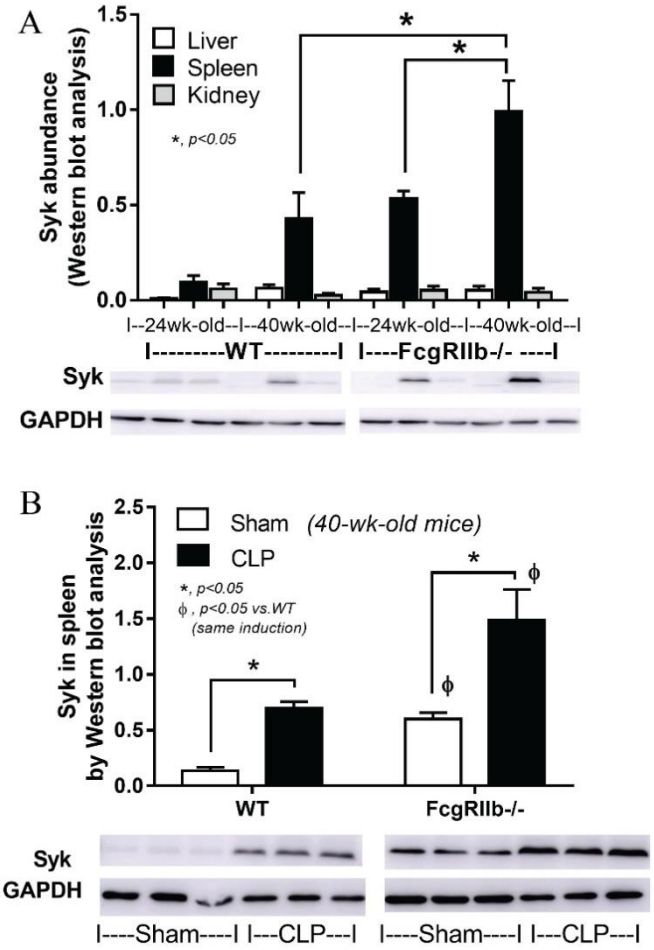


Figure 37 Syk protein expression level in organs. Syk levels in organs from wild-type (WT) and FcgRIIb$^{-/-}$ mice aged 24 and 40 weeks. Western blot analysis without cecal

ligation and puncture (CLP) (A) (n = 5/group). Syk levels in spleens of 40-week-old WT and FcgRIIb$^{-/-}$ mice 24 h after CLP or sham surgery (Sham) (B).

We found that the mortality rate of CLP sepsis in 40-week-old FcgRIIb$^{-/-}$ mice was higher than in age-matched WT sepsis mice but a similar mortality rate was observed for 24-week-old mice of either strain (Figure 38 A, B). Furthermore, the mortality rate of sepsis in FcgRIIb$^{-/-}$ mice aged 40-weeks was higher than in 24-week-old mice (Figure 38 C). Organ injury determined by serum creatinine (Scr), renal histology, liver enzyme (ALT) (Figure 38 D-I, Figure 39), gut leakage (FITC-dextran, serum LPS, and serum BG) (Figure 40), and inflammatory cytokines (Figure 41) at 24 h post-CLP was more severe in 40-week-old FcgRIIb$^{-/-}$ mice compared with 24-week-old FcgRIIb$^{-/-}$ mice or WT mice. levels of anti-dsDNA antibodies were higher in 40-week-old FcgRIIb$^{-/-}$ mice than in 24-week-old FcgRIIb$^{-/-}$ mice and sepsis did not alter anti-dsDNA antibody levels (Figure 34J-L). In addition, there was pre-conditioning damage in 40-week-old FcgRIIb$^{-/-}$ mice as indicated by the higher baseline levels of Scr (Figure 38 F), gut leakage (Figure 40 C, F, I), and inflammatory cytokines (Figure 41 C, F, I), but not ALT or anti-dsDNA antibodies (Figure 38 I, L), in 40-week-old FcgRIIb$^{-/-}$ mice (pre-CLP) compared with 24-week-old FcgRIIb$^{-/-}$ mice, which might explain the higher sepsis mortality rate of 40-week-old FcgRIIb$^{-/-}$ mice compared with 24-week-old FcgRIIb$^{-/-}$ mice (Figure 38C). In parallel, there was no pre-conditioning injury in 24-week-old FcgRIIb$^{-/-}$ mice compared with age-matched WT mice by these pre-CLP parameters (Figure 38-41), except for anti-dsDNA antibodies (Figure 38J), resulting in a similar mortality rate of CLP sepsis between 24-week-old FcgRIIb$^{-/-}$ mice versus age-matched WT mice (Figure 38A).

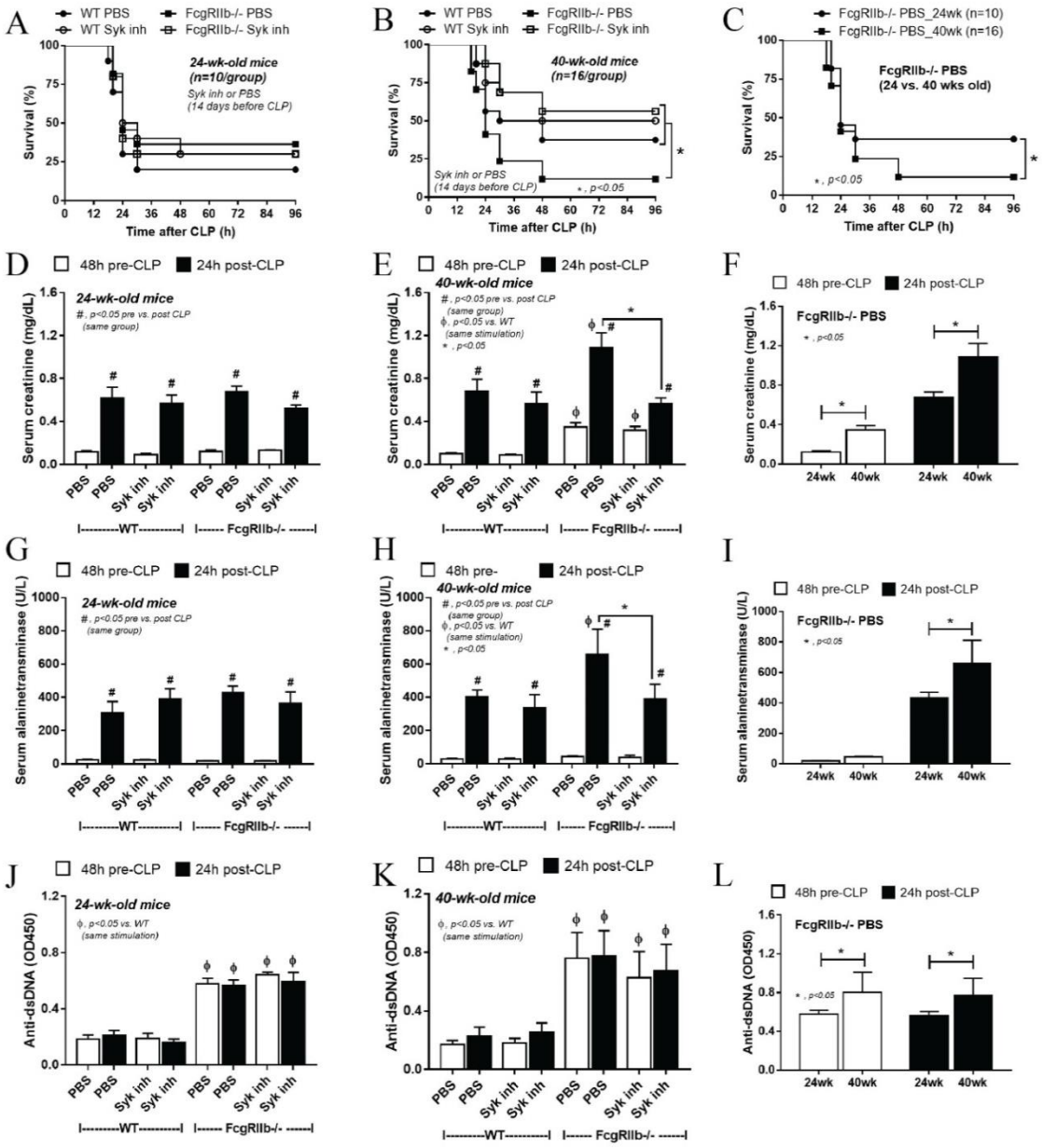


Figure 38 Characteristics of mice before and after cecal ligation and puncture (CLP) surgery in FcgRIIb$^{-/-}$ or wild-type (WT) group after 14 days of Syk inhibitor administration (Syk inh) or phosphate buffer solution (PBS) control in 24-wk-old and 40-wk-old mice as determined by survival analysis (A-C) (n =10 and 16/ group for A and B, respectively), serum creatinine (D-F), serum alanine transaminase (G-I) and serum anti-dsDNA (J-L) were demonstrated (n = 5-7/ group for D-L). Figure C, F, I and L are demonstrated for better visualization of the difference between sepsis of FcgRIIb$^{-/-}$ mice at 24 wks old versus 40 wks old.

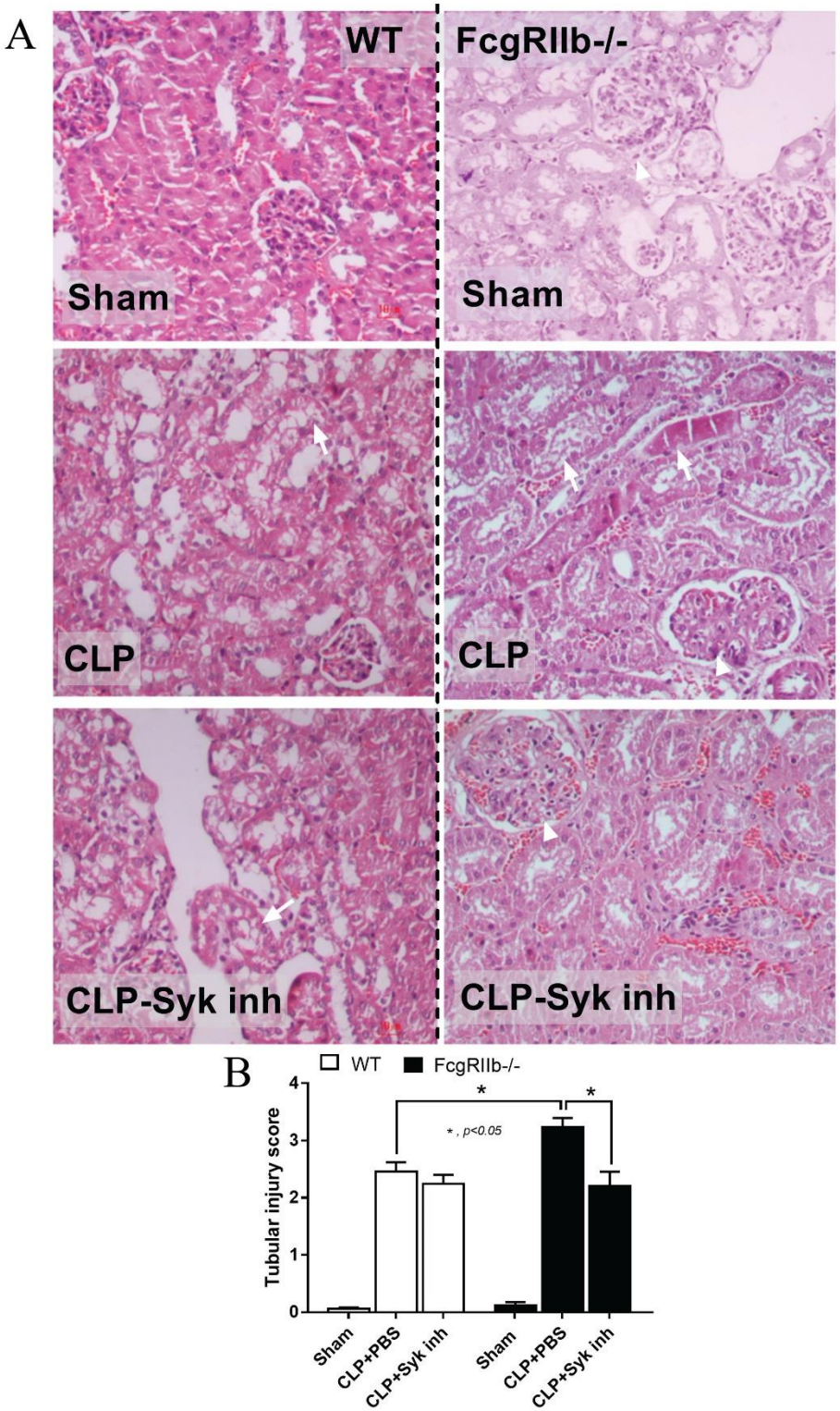


Figure 39 The histological effect of kidney before and after treatment with Syk inhibitor. A) The representative of kidney histology before and after Syk inhibitor

administration before CLP induction. B) The tubular injury score before and after Syk inhibitor administration.

Compared with the PBS control group, after 14-day administration of a Syk inhibitor prior to CLP surgery, the severity of sepsis was lower in 40-week-old FcgRIIb$^{-/-}$ mice, but not 24-week-old FcgRIIb$^{-/-}$ mice and WT mice, as determined by mortality rate and several post-CLP parameters including serum creatinine (Scr), renal histology, liver damage (ALT) (Figure 38, 39), and inflammatory cytokines (Figure 41). Furthermore, the Syk inhibitor did not decrease the severity of sepsis-induced gut leakage as indicated by FITC-dextran and serum BG (Figure 40 B, H) but did attenuate serum LPS levels (Figure 40E), which might explain the attenuation of sepsis severity. However, 14-day treatment with the Syk inhibitor reduced baseline proinflammation as indicated by reduced levels of pre-CLP serum cytokines (Figure 41 B, E, H) despite no effect on Scr, anti-dsDNA antibodies (Figure 38 E, K), or gut leakage (Figure 39B, E, F). These data indicate the importance of pre-conditioning injury upon sepsis severity [142, 134]. Indeed, the 3-day administration of a Syk inhibitor prior to CLP did not decrease pre-CLP serum cytokine levels or sepsis severity in lupus mice of both ages (Figure 42).

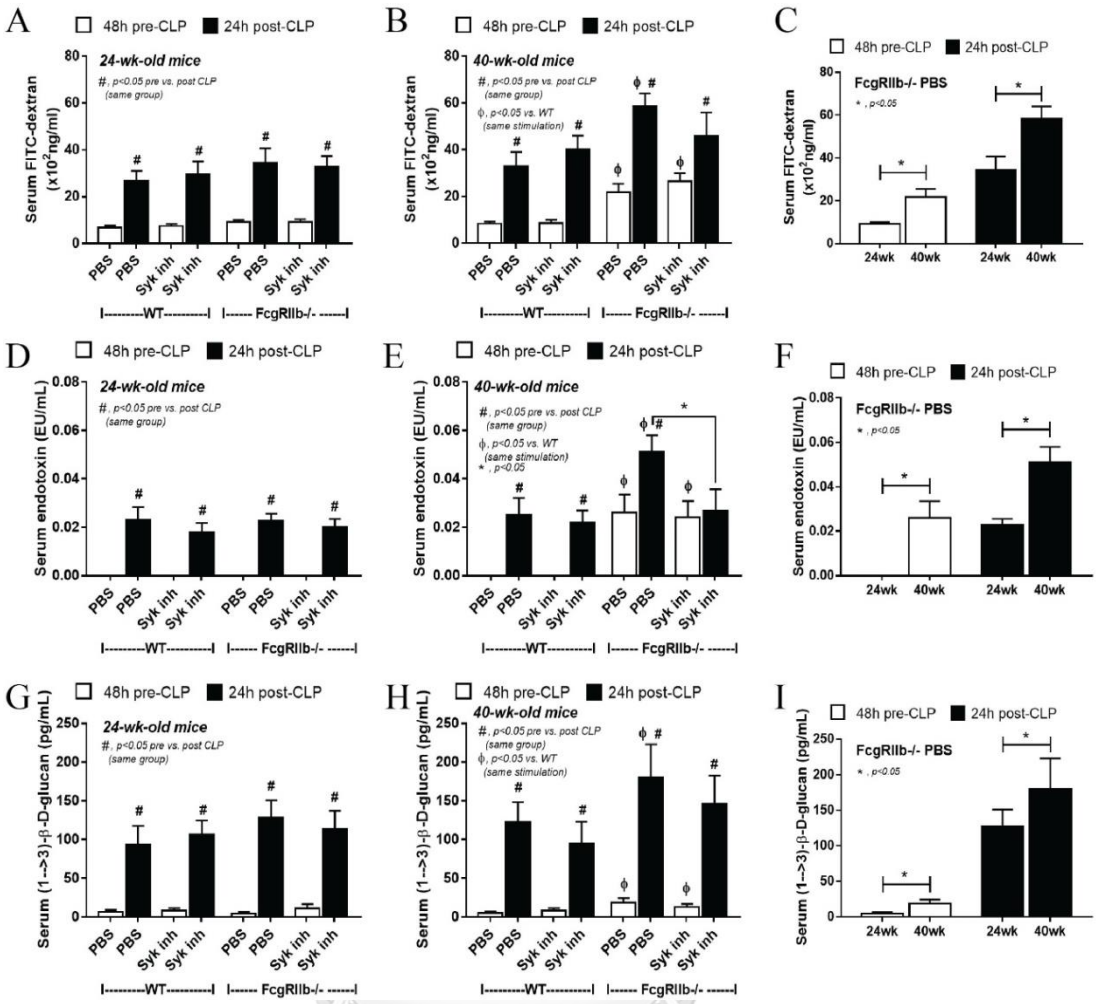


Figure 40 Characteristics of mice at before and after cecal ligation and puncture (CLP) surgery in FcgRIIb$^{-/-}$ or wild-type (WT) group after 14 days of Syk inhibitor administration (Syk inh) or phosphate buffer solution (PBS) control in 24-wk-old and 40-wk-old mice as determined by serum FITC-dextran (A-C), serum endotoxin (D-F) and (1→3)-β-D-glucan (BG) (G-I) are demonstrated (n = 5-7/ group). Figure C, F, I and L are demonstrated for the better visualization of the difference between sepsis of FcgRIIb$^{-/-}$ mice at 24 wks old versus 40 wks old.

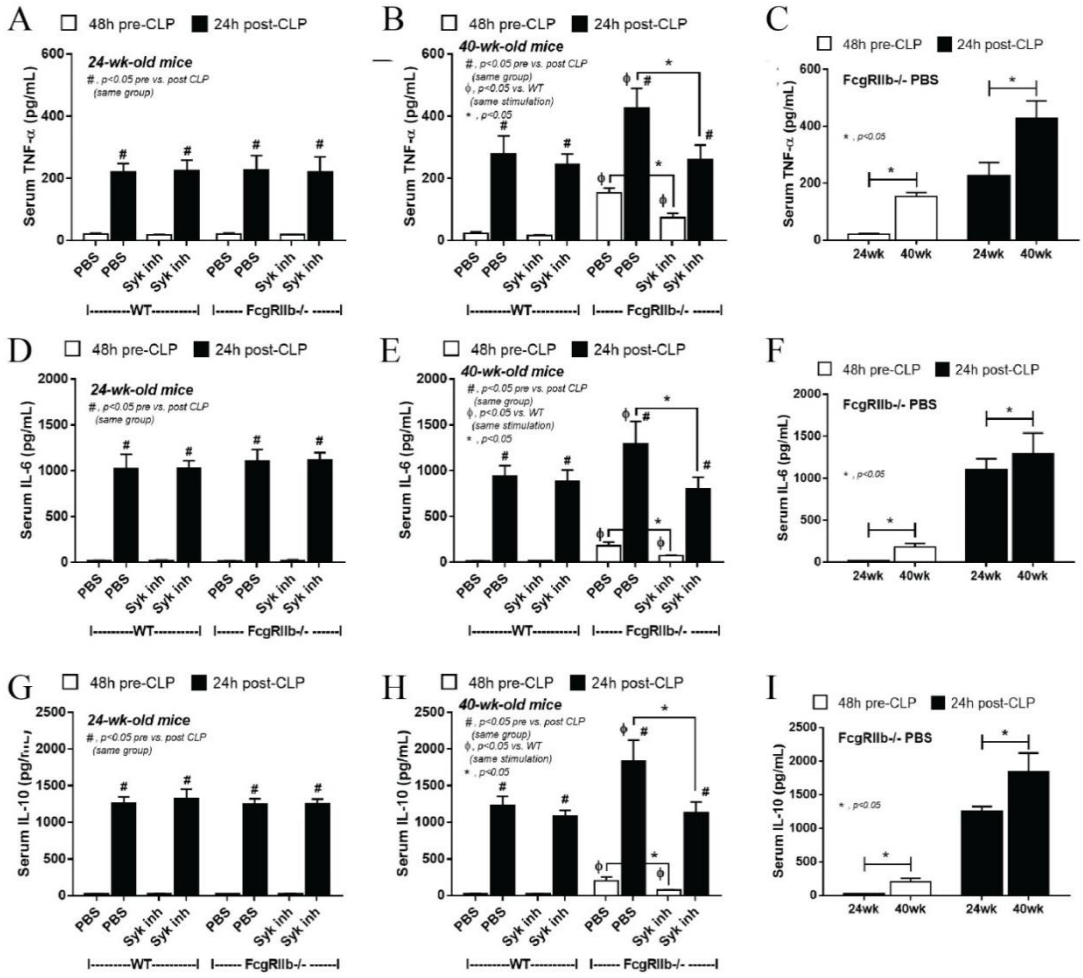


Figure 41 Systemic cytokines of mice at before and after cecal ligation and puncture (CLP) surgery in FcgRIIb$^{-/-}$ or wild-type (WT) group after 14 days of Syk inhibitor administration (Syk inh) or phosphate buffer solution (PBS) control in 24-wk-old and 40-wk-old mice as determined by serum TNF-**α** (A-C), serum IL-6 (D-F) and serum IL-10 (G-I) are demonstrated (n = 5-7/ group). Figure C, F, I and L are demonstrated for better visualization of the difference between sepsis of FcgRIIb$^{-/-}$ mice at 24 wks old versus 40 wks old.

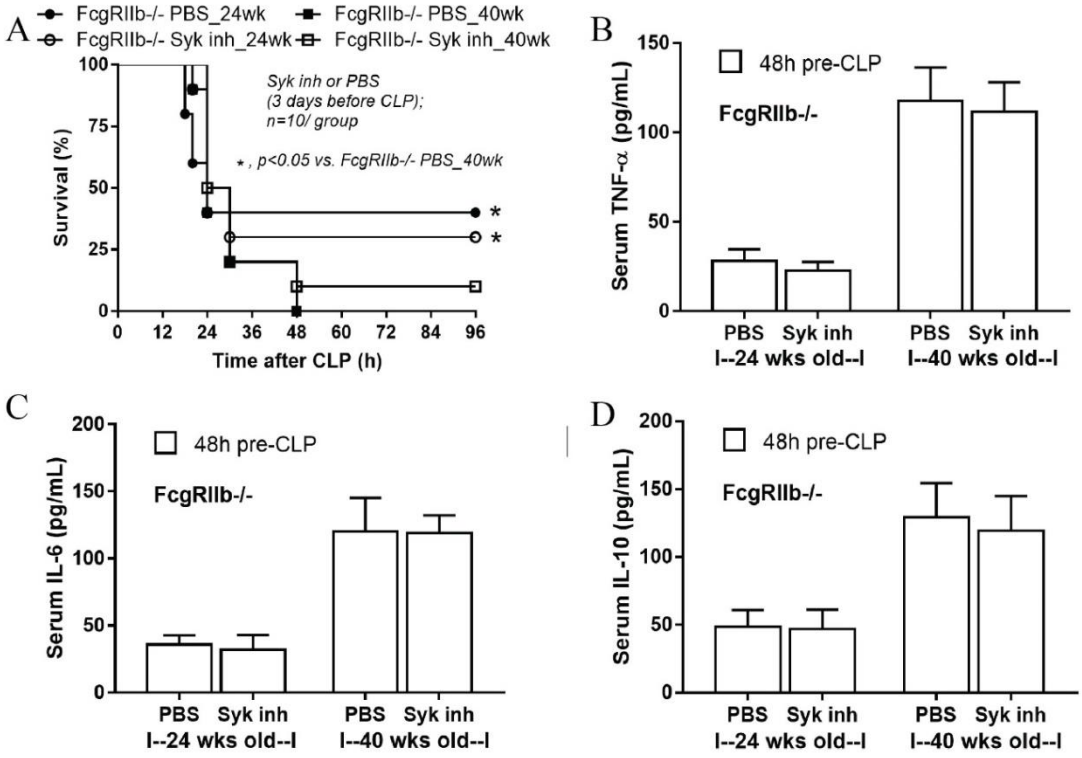


Figure 42 Survival analysis (A) of FcgRIIb$^{-/-}$ mice aged 24 and 40 weeks after 3 days of Syk inhibitor administration (Syk inh) or phosphate buffer solution (PBS) control (n = 10/group). Systemic inflammation determined by serum cytokines (B-D) before cecal ligation and puncture (CLP) surgery are shown (n=10/group).

The Prominent effect of Syk inhibitor on FcgRIIb-/- macrophages: RNA sequencing analysis.

As we know that the sepsis severity of in FcgRIIb$^{-/-}$ mice. The Syk inhibitor can decrease pro-inflammatory cytokine involved in the pathogenesis of SLE and pathogenesis of sepsis in SLE. We further examined the prominent anti-inflammatory effect of Syk inhibitor on the FcgRIIb$^{-/-}$ macrophages. Therefore, RNA sequencing was performed to investigate differential gene expression and potential pathway in FcgRIIb$^{-/-}$ macrophages after activation by BG+LPS with or without Syk inhibitor compare with negative control (no-treatment) (Figure 43). According to RNA sequencing result, we found 15402, 15078, 14727 genes expressed in control, BG+LPS and BG+LPS+Syk

inhibitor respectively and the expression pattern between each condition was similar (Figure 43A). However, Treatment with Syk inhibitor suppressed genes in BG+LPS activated FcgRIIb$^{-/-}$ macrophages (Figure 43B).

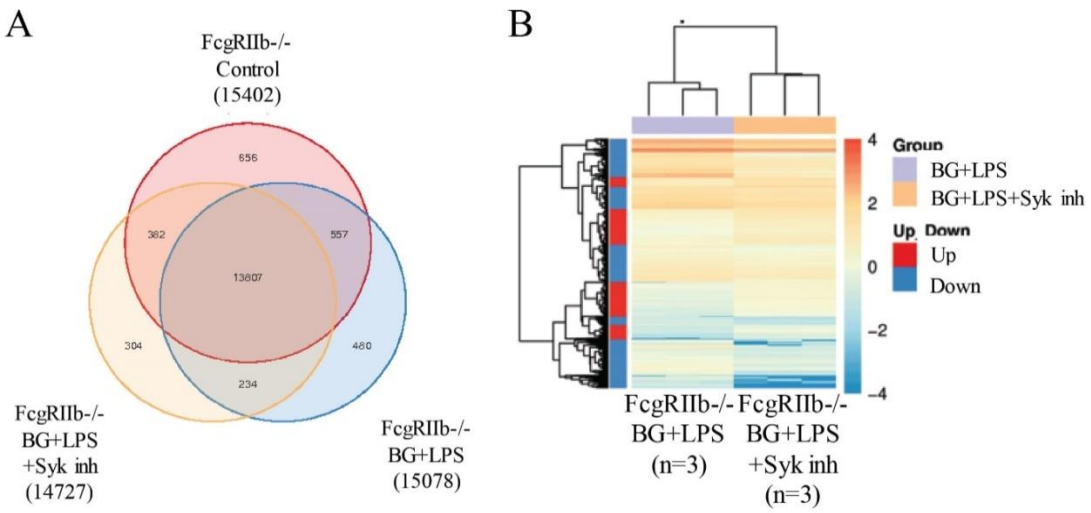


Figure 43 Comparison between gene expression profiles from three groups of FcgRIIb$^{-/-}$ macrophages. A) Venn Diagram showing number of genes expression in FcgRIIb$^{-/-}$ macrophages activated with BG+ LPS with or without Syk inhibitor compared with no treatment control. B) Heat map indicated the differrential gene expression of BG+LPS activated FcgRIIb$^{-/-}$ macrophages with and without Syk inhibitor treatment.

The pathway analysis showed that Syk inhibitor suppressed most of the expressed gene in BG+LPS activated FcgRIIb$^{-/-}$ macrophages. In addition, the comparison of deferentially expressed genes (DEGs) in FcgRIIb$^{-/-}$ macrophages with negative control FcgRIIb$^{-/-}$ macrophages after stimulation with BG+LPS demonstrated the upregulation of pro-inflammatory genes (*TNF-α*, *NF-κB*, and *MAPK*) and downregulation of genes in metabolic pathways (Figure 44). In groups treated with Syk inhibitor, DEGs in most pathways involved the signaling pathways of TNF-α, Toll-like receptor, and NF-κB were downregulated (Figure 44).

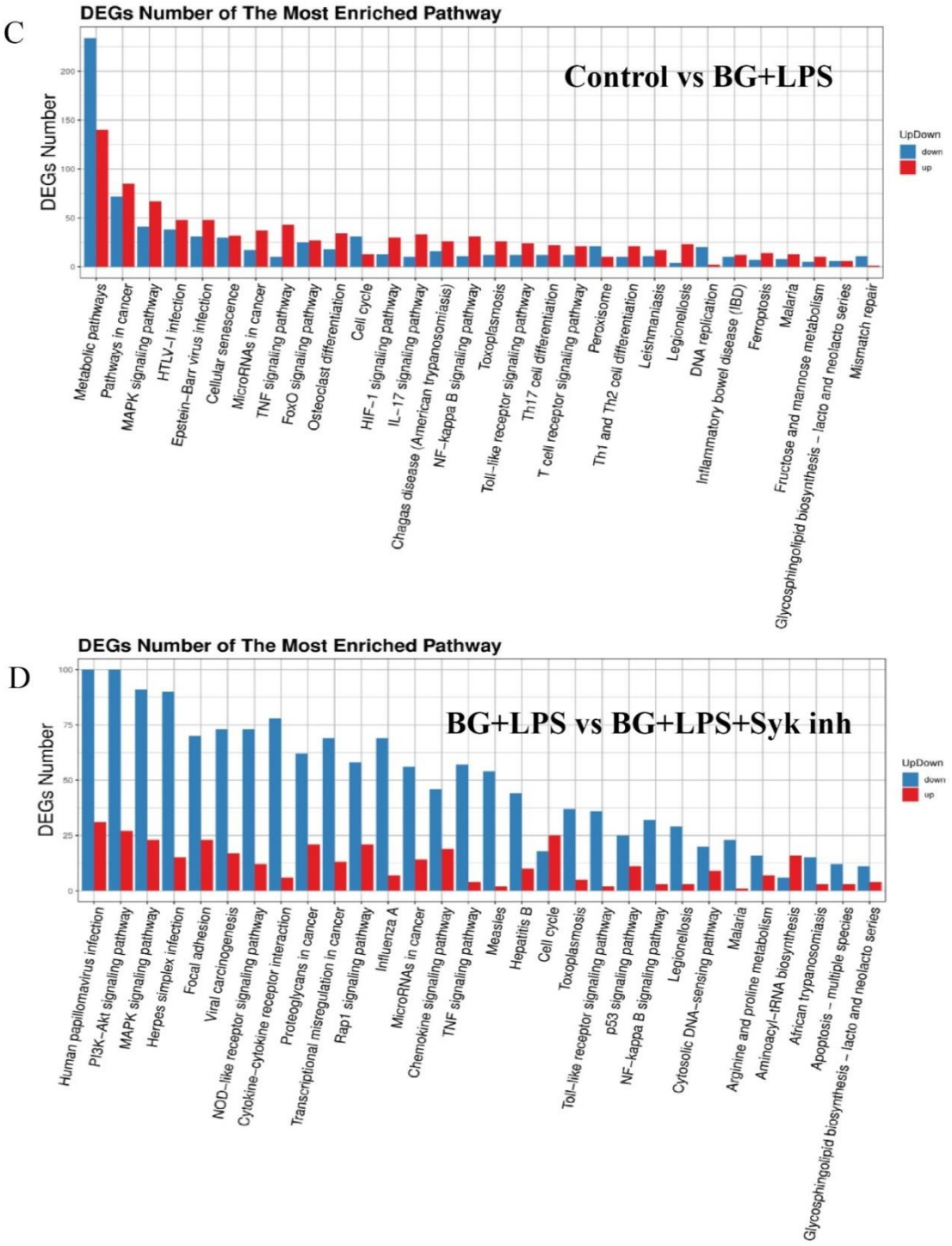


Figure 44 The representative of the differential expresses genes and the most enrichment pathway A) the enrichment pathway of BG+ LPS activated FcgRIIb$^{-/-}$ macrophages compare to control B) the enrichment pathway of BG+ LPS activated FcgRIIb$^{-/-}$ macrophages compared to BG+LPS+Syk inhibitor.

Interestingly, the direction of gene expression (up- or downregulation) in BG+LPS activated FcgRIIb$^{-/-}$ macrophages, compared with the negative control, was similar to genes associated with high mortality rate in patients with sepsis (Table 3). According to the RNA sequencing result, we found 31 genes expressed in BG+LPS activated macrophage (table 4). Most upregulated genes in patients with severe sepsis, except *PLK1* and *CD-163*, were also upregulated in WGP+LPS activated FcgRIIb$^{-/-}$ macrophages (Figure 44 control vs WGP+LPS). Furthermore, most downregulated genes in patients with severe sepsis, except for *AIM2*, *GSTM1* and *VNN3*, were downregulated in WGP+LPS activated FcgRIIb$^{-/-}$ macrophages (Figure 44 Control vs BG+LPS). Of note, the Syk inhibitor reversed the direction of the expressed genes in WGP+LPS activated FcgRIIb$^{-/-}$ macrophagesThis data suggested that there is a similar pattern between gene expressed in patients with sepsis and those expressed in BG+LPS activated FcgRIIb$^{-/-}$ macrophage (Figure 44 BG+LPS vs WGP+LPS+Syk inhibitor) indicating that Syk inhibitor might be a potential candidate for sepsis treatment in lupus.

Table 3 List of mortality gene in sepsis via gene expression analysis.

Model name	Direction of change in patients with mortality	Genomic features
Duke	Up (5 genes)	*TRIB1, CSK2, MKI67, POLD3, PLK1*
	Down (13 genes)	*TGFBI, LY86, CST3, CBFA2T3, RCBTB2, TST, CX3CR1, CD5, MTMR11, CLEC10A, EMR3, DHRS7B, CEACAM8*
Sage LR	Up (9 genes)	*CFD, DDIT4, DEFA4, IFI27, IL1R2, IL8, MAFF, OCLN, RGS1*
	Down (9 genes)	*AIM2, APH1A, CCR2, EIF5A, GSTM1, HIST1H3H, NT5E, RAB40B, VNN3*
Sage RF	Up (13 genes)	*B4GALT4, BPI, CD24, CEP55, CTSG, DDIT4, G0S2, MPO, MT1G, NDUFV2, PAM, PSMA6, SEPP1*
	Down (4 genes)	*ABCB4, CTSS, IKZF2, NT5E*
Stanford	Up (8 genes)	*DEFA4, CD163, PER1, RGS1, HIF1A, SEPP1, C11orf74, CIT*
	Down (4 genes)	*LY86, TST, OR52R1, KCNJ2*

From: Sweeney TE *et. al* Nature communications .2018 Feb 15;9)1:(694).

Table 4 List of mortality prediction gene in sepsis via gene expression analysis found in BG+LPS activated macrophage.

No.	Gene name	Direction of change in patients with mortality
1	*MKI67*	Up-reglulate
2	*PLK1*	Up-reglulate
3	*CFD*	Up-reglulate
4	*DDIT4*	Up-reglulate
5	*IFI27*	Up-reglulate
6	*IL1R2*	Up-reglulate
7	*MAFF*	Up-reglulate
8	*OCLN*	Up-reglulate
9	*RGS1*	Up-reglulate
10	*B4GALT4*	Up-reglulate
11	*BPI*	Up-reglulate
12	*NDUFV2*	Up-reglulate
13	*PSMA6*	Up-reglulate
14	*CD163*	Up-reglulate
15	*PER1*	Up-reglulate
16	*RGS1*	Up-reglulate
17	*HIF1A*	Up-reglulate
18	*LY86*	Down-reglulate
19	*RCBTB2*	Down-reglulate
20	*CX3CR1*	Down-reglulate
21	*MTMR11*	Down-reglulate
22	*DHRS7B*	Down-reglulate
23	*AIM2*	Down-reglulate
24	*CCR2*	Down-reglulate
25	*GSTM1*	Down-reglulate

26	*HIST1H3H*	Down-reglulate
27	*NT5E*	Down-reglulate
28	*VNN3*	Down-reglulate
29	*ABCB4*	Down-reglulate
30	*IKZF2*	Down-reglulate
31	*KCNJ2*	Down-reglulate

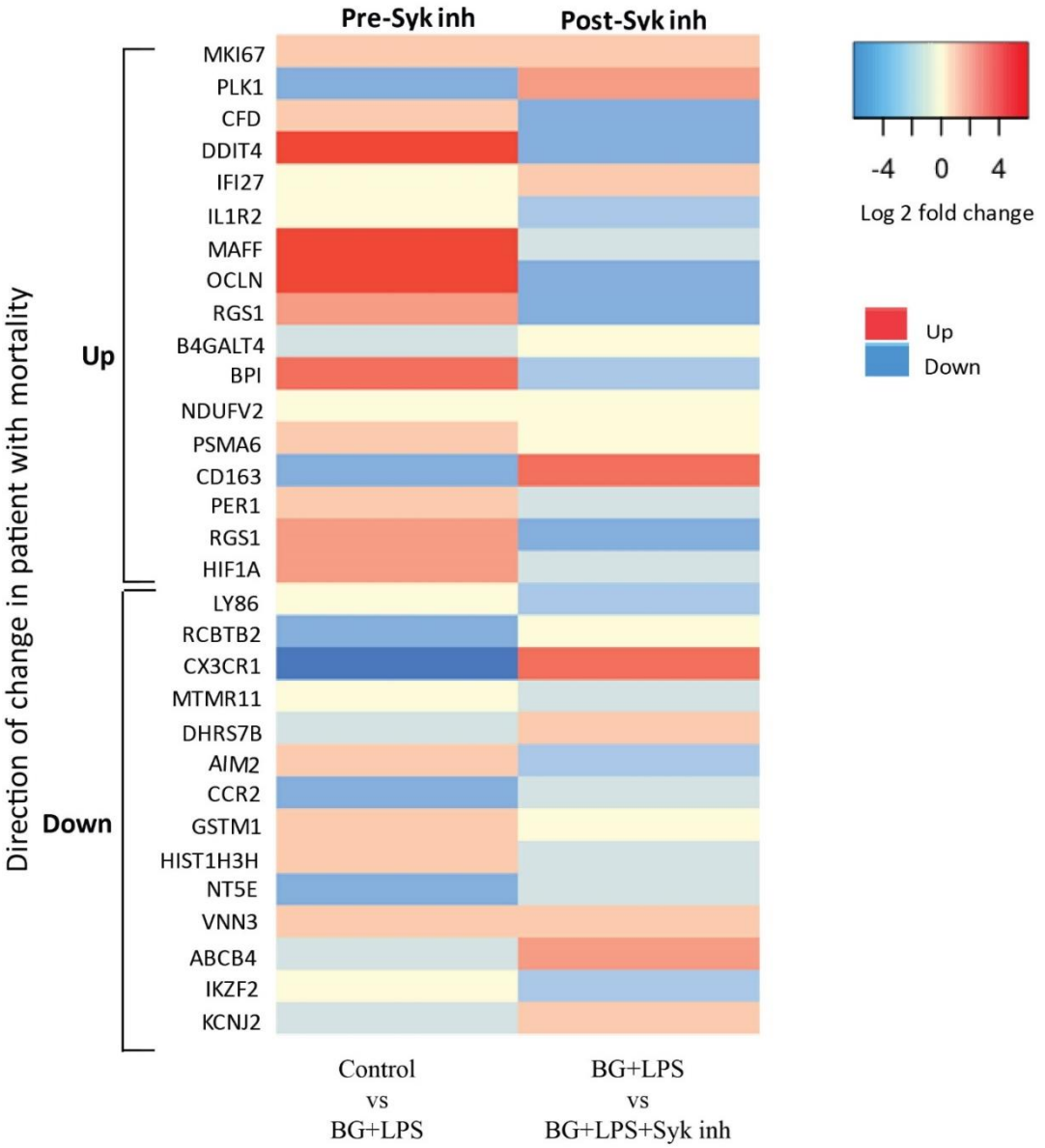


Figure 45 A heat map comparison of the up- and down- regulated genes in macrophages associated with genes expressed in patients with high mortality rate sepsis. Left column - fold-change in gene expression after activation of FcgRIIb$^{-/-}$ macrophages BG, a representative of (1⟶3)-β-D-glucan, and lipopolysaccharide (LPS) (BG+LPS) versus Control. Right column - fold-change in gene expression of FcgRIIb$^{-/-}$ macrophages activated by BG+LPS with Syk inhibition (BG+LPS+ SYK inh) versus no Syk inhibition (BG+LPS).

CHAPTER VI

DISCUSSION

Spontaneous gut leakage and increases LPS and BG in the serum of symptomatic FcgRIIb$^{-/-}$ mice showed an impact of gut leakage on sepsis severity. Prominent synergy responses of FcgRIIb$^{-/-}$ mice and FcgRIIb$^{-/-}$ macrophages to the LPS and BG were demonstrated. Our data support the importance of gut leakage in the SLE background.

Gut leakage in lupus nephritis (LN)

LN is a condition that present of the Curculating immune complex (CIC) including the kidney and GI surface area causing inflammation [143]. The obvious GI manifestations are rarely found in the lupus [144], but the immune complex deposition in the gut and the spontaneous endotoxemia (the indirect marker of gut leakage) can be found in SLE patients [3]. Moreover, the serum BG > 60 pg/mL (a negative cut-off for invasive fungal infection) without fungal infection was also found in SLE patients. Presentation of BG and LPS the foreign molecules in the blood indicated that GI permeability defect. The direct measurement of GI peemeability defect in the patients was diffifult, we demonstrated it in FcgRIIb$^{-/-}$ mice by using FITC-dextran gavage feeding into the mice. The leaky gut was observed in 40-wk-old mice, but not in the younger group. Moreover, the presentation of FITC-dextran in the serum we also observed together with BG and LPS in the serum. In symptomatic (40-week-old) gut leakage was found and mice showed high level of proteinuria and kidney injury.

The impact of gut permeability defect and translocation of LPS and BG from the gut on sepsis in FcgRIIb$^{-/-}$ mice.

Gut leakage induced by DSS administration caused spontaneous endotoxemia and elevated serum BG in wild-type mice and in 8-week-old FcgRIIb$^{-/-}$ mice (asymptomatic mice with nonsapontaneous gut leakage). Interestingly, DSS administration, sepsis severity after CLP induction was compatible in both strains. After DSS administration, sepsis severity is more severe than those without DSS administration in both age group of FcgRIIb$^{-/-}$ mice, but sepsis severity was enhanced only in 40-week-old of wild-type mice. DSS showed a greater effect on the sepsis severity in FcgRIIb$^{-/-}$ more than wild type mice. As we know that the gram-negative bacteria and fungi consider as a normal microbiota in the gut [5, 145]. Translocation of LPS and BG from the gut to the blood, these two molecules acted as PARMPs for activation of host immune system. Moreover, the synergistic effect of LPS and BG was prominent in FcgRIIb$^{-/-}$ mice more than wild-type. This phenomenal may occur because of the hyperresponsive of FcgRIIb$^{-/-}$ mice including defective regulatory signaling [21]. This finding was supported by injection of BG, LPS, and BG+LPS without CLP induction, FcgRIIb$^{-/-}$ mice demonstrated a higher level of serum cytokine more than wild-type mice. Although, BG alone induced a small level proinflammatory cytokine in the serum, BG showed synergistic effect with LPS. In order to determine if there was a synergy in the sepsis, BG, LPS, or BG+LPS was injected to asymptomatic mice follow by CLP induction. The LPS administration with or without BG enhanced sepsis severity in FcgRIIb$^{-/-}$ mice more than wild type mice.

In parallel, macrophages stimulated with BG, LPS or BG+LPS showed the synergistic effect by induced higher level of proinflammatory cytokine. This finding supports the synergistic effect of these two molecules in several previous studies [19, 20]. Although, BG alone could not induce any cytokine production in both strains of mice this data indicated that a limited independent proinflamatory cytokine potential [146, 147].

The prominent Syk signaling in FcgRIIb-/- mice and macrophages.

The increased expression of FcgRs after LPS activation demonstrated that the connection between the innate and adaptive immune system as previously described [138, 139]. It seems that LPS stimulation is a natural pre-conditioning to prime macrophage for the up-coming adaptive immune responses by preparing the FcgRs receptor expression for the Fc portion of immunoglobulin. Actually, in the natural condition with recent organism challenge, before the adaptive immune activation, the pathogens are opsonized with natural antibodies and simultaneously engage both innate and FcgRs to initiate the signaling cross-talk between innate and FcgRs receptors [148-150].

Interestingly, Spleen tyrosine kinase (Syk) and the NF-κB (a well-known transcription for innate immune responses) are the possible shared- downstream signaling of FcgR, TLR4 and Dectin-1[130] which could be activated by specific stimulus including anti-dsDNA, LPS, and BG in seum of the FcgRIIb$^{-/-}$ mice. Thus the prominent inflammatory responses of FcgRIIb$^{-/-}$ mice compare to wild-type was due to the signaling activation of circulating immune complex (CIC) from auto-antigen, LPS in the serum, and BG in the serum from the gut leakage. Moreover, the over response in FcgRIIb$^{-/-}$ may due to the absene of inhibitory signaling. Indeed, Syk is i) a direct downstream signaling of Dectin-1 receptor[151] ii) alternative non MyD88 signaling pathway by induction of TIR-domain-containing-adaptor-inducing interferon-β (TRIF) protein through the activation of CD-14, TLR4 and Syk activated by LPS has been demonstrated [152] and iii) a recruitement molecule by activating FcgRs through scr family kinase resulting in phosphorylation of tyrosine-based activation motifs (ITAMs) [153]. In contrast, the activation of inhibitory receptor FcgRIIb inhibits the cross-talk signaling between innate receptors and FcgRs via immunoreceptor tyrosine-base inhibitory motifs (ITIMs) [154]. Furthermore, Wild type macrophages stimulated with BG+LPS increased expression of FcgRs in both the activating receptors (*FcgRI, FcgRIII, and FcgRIIV*) and the inhibitory receptor (*FcgRIIb*), when compared with baseline control. Upregulation of activation or inhibitory receptor also means having a balance of the immune activation. In contrast, FcgRIIb$^{-/-}$ macrophages with lacking inhibitory

receptor resulting in upregulation of activating *FcgRs* without the inhibitory signaling leading to hyper-responsiveness of FcgRIIb$^{-/-}$ macrophages. Further studies about the mechanism in this topic are interesting.

The high abundace of Syk expression in the spleen of FcgRIIb$^{-/-}$ of full brown FcgRIIb$^{-/-}$ lupus mice and BG+LPS activated macrophage and the synergy of the cytokine production when compared with LPS stimulation. Moreover, the inhibitor against the signaling protein including Syk, Dectin-1, and NF-**κ**B, but not Raf-1 suggesting the downstream signaling cross-talk between TLR4 and Dectin 1 was mainly through Syk dependent signaling pathway, but not via Raf1. This data was similar to the previous publication [103].

The therapeutic effect of Syk inhibitor in the FcgRIIb$^{-/-}$ mice, the impact of pathogenesis and treatment outcome.

As we know that Syk may play a crucial role in response BG+LPS activation. Moreover, the permeability defects in lupus mice resulting in presentation of these two molecules in the blood of the symptomatic mice. Syk inhibitor may provide the therapeutic benefit for FcgRIIb$^{-/-}$ mice. Although Syk inhibitor attenuates lupus progression has been demonstrated [155, 156]. It has never been tested in FcgRIIb$^{-/-}$ mice. After 4-week-administration of Syk inhibitor attenuated proteinuria, pro-inflammatory cytokines, anti-dsDNA and glomerular immune complex deposition in FcgRIIb$^{-/-}$, but not improve renal function as demonstrated by serum creatinine and gut leakage in FcgRIIb$^{-/-}$ mice. The attenuated the inflammatory responses possibly due to the translocation of the pathogenic molecules from the gut to the blood. Thus, the Syk inhibitor might be interesting candidate for treatment of active lupus especially in patients with FcgRIIb dysfunction-polymorphism. Syk inhibitor might be an interesting new candidate for treatment.

High Syk signaling in FcgRIIb$^{-/-}$ mice and the anti-inflammatory effects of Syk inhibitor in lupus mice.

LPS and BG also found in the sepsis, the impact of these two molecules also affect the severity of sepsis especially in symptomatic (40-wk-old FcgRIIb$^{-/-}$ mice) compare to wild-type. The prominent Syk expression was found in the spleen of FcgRIIb$^{-/-}$ mice. According to the signaling crosstalk, Syk act as a key protein during crosstalk between TLR4, Dectin1, and FcGRs. Moreover, the level of Syk dramatically increased after CLP surgery suggesting the potential role of Syk in the sepsis. Although it was previously showed that Syk inhibitor attenuated sepsis in LPS model (a model induced by exogenous LPS injection without bacteremia)[157], it has never been tested in CLP sepsis model (a model with both bacteremia and endotoxemia) [158]. Interestingly, the 14 days Syk administration but not 3-day oral gavage of a Syk inhibitor decreased lupus-systemic inflammation at baseline (pre-CLP surgery) and attenuated sepsis severity in 40-wk-old FcgRIIb$^{-/-}$ mice. In contrat, the Syk inhibitor had a reduced anti-inflammatory effect in 24-wk-old of both strains after CLP surgery. This might be explained by the lack of gut leakage and/or systemic inflammation at baseline in 24-wk-old FcgRIIb$^{-/-}$ mice and WT mice (both 24- and 40-week-old age groups). Although, increased level of anti-dsDNA antibodies in 24-wk-old FcgRIIb$^{-/-}$ mice, 14 –day administration of Syk inhibitor did not reduce baseline anti-asDNA antibody titers (pre-CLP surgery) or sepsis severity, implying a lesser impact of Syk induction through FcgR, by the activation from immune complex of anti-dsDNA antibodies, upon sepsis severity.

The anti-inflammatory effects of Syk inhibitors might also be related to blockade of FcgR, Dectin1, and TLR4 receptor, as well as interfering with other proinflammatory signaling pathways. RNA sequencing of WGP+LPS activated macrophages treated with Syk inhibitor demonstrated reduced gene expressions of proinflammatory pathway, including Akt, MAPK, NOD, and cytosolic DNA. Interestingly, the gene expression in BG+LPS activated macrophages was similar to a large number of genes associated moltarity prediction in sepsis patients [159] suggesting the potential influence of immune activation by BG+LPS, the representative of pathogenic molecules, in sepsis patients. Therefore, increased serum BG levels were observed in

patients with sepsis even without fungal infection, via sepsis-induced gut leakage resulting in translocation of BG [17]. Some of the genes co-expressed by BG+LPS upregulated DNA damge inducible transcript 4 (*DDIT4*) and occluding (OCLN), gene responsible for hypoxic responses and epithelial morphological stability, respectively [160, 159], and downregulated CX3C chemokine receptor 1 (CXC3CR1), a gene responsible for leukocyte migration and monocyte survival [161]. Nevertheless, the Syk inhibitor downregulated DDIT4 and OCLN and upregulated CXC3CR1 in BG+LPS activated macrophages, whereas sepsis was attenuated in 40-wk-old FcgRIIb$^{-/-}$ mice, suggesting the benefit of Syk inhibitor for sepsis treatment.

According to the potential clinical translation, Syk inhibitors are approved for treatment of several autoimmune diseases including lupus and rheumatoid arthritis [112, 162, 113]. Although the therapeutic effect of Syk inhibitors against polymicrobial sepsis was low in short-term treatment, our data indicated that susceptibility against bacterial infection might be lower with a longer Syk administration, possibly during the treatment of lupus disease activity. Of note, the current drug available for treatment of lupus, such as steroids and mycophenolate mofetil, are based upon immunosuppression, which can cause several opportunistic infections [163]. According to the effect of Syk inhibitors on the lupus progression [112, 162, 113] together with our data on the reduction of sepsis severity, Syk inhibitors might be an interesting candidate for the treatment of active lupus patients with FcgRIIb dysfunction polymorphisms.

CHAPTER VII

CONCLUSION

Gut leakage increased sepsis severity in FcgRIIb$^{-/-}$ mice.

The results show spontaneous gut leakage was found in most of patients with active lupus nephritis by increasing the level of LPS and BG in the blood. Moreover, it is found that spontaneous gut leakage was also observed in 40-weeks-old of FcgRIIb$^{-/-}$ mice as demonstrated by Dextran-FITC, and elevated the level of LPS and BG in the blood. The translocation of these 2 molecules affect sepsis severity especially in FcgRIIb$^{-/-}$ mice and our data support the hypothesis that

- Gut leakage may present without obvious GI manifestations.

- Gut translocation of LPS and BG are associated with increased inflammatory state.

- LPS and BG, in synergy, enhanced sepsis severity in FcgRIIb$^{-/-}$ mice more than wild type.

SYK inhibitor attenuates inflammation in FcgRIIb$^{-/-}$ mice

The results show that translocation of the foreign molecules (LPS and BG) from the gut activated innate immune cells especially macrophages. BG+LPS triggers the innate immune response via TLR4 and Dectin1 receptor and the synergy of BG+LPS signaling occurs via a Syk/NF-ΚB-mediated signaling pathway, but not via Raf1. Furthermore, the prominent activation of Syk was found in 40-week-old FcgRIIb$^{-/-}$ mice suggesting Syk might be a potential target for lupus treatment. To test this hypothesis, the oral administration of Syk inhibitor was performed and the results suggested that Syk inhibitor attenuated lupus progression including:

- The 4-week-administration of Syk inhibitor attenuated proteinuria inflammatory cytokines, anti-dsDNA in 40-week-old FcgRIIb$^{-/-}$ mice
- The glomerular immune complex was improved after Syk inhibitor treatment, but not renal histology score.

SYK inhibitor attenuates sepsis severity in 40-week-old of FcgRIIb$^{-/-}$ mice

The Syk protein level is high in the spleen of FcgRIIb$^{-/-}$ mice after CLP surgery. Moreover, in CLP surgery the level of Syk protein also increased. Thus, Syk inhibitor in sepsis might have an impact on the sepsis condition. To test this hypothesis, we induced sepsis using CLP surgery model in FcgRIIb$^{-/-}$ mice (24-week-old and 40-week-old). Our data suggested that Syk inhibitor attenuates sepsis severity including:

- 14-day administration, but not 3-day Syk inhibitor administration reduced lupus-induce systemic inflammation at baseline (pre-CLP surgery) and attenuated sepsis severity in 40-week-old FcgRIIb$^{-/-}$ mice.
- RNA sequencing analysis from BG+LPS activated macrophages demonstrated a set of genes that are associated with the mortality gene prediction in sepsis patients (Table 3). Moreover, macrophages treatment with Syk inhibitor demonstrated reduced gene expression involved proinflammatory pathway, including Akt, MAPK, NOD and cytosolic-DNA suggesting the potential influence of immune activation by pathogenic molecule (LPS and BG) in sepsis patients.

Although short-term treatment of Syk inhibitor, the results showed that the improvement was low in CLP model, our results suggested that a longer duration for treatment with Syk inhibitor, possibly during the treatment of lupus disease.

Taken together, this study can be concluded that identification of gut leakage by measuring the level of LPS and BG in patients with lupus might be beneficial. Theses findings provide proof of concept inhibition of Syk signaling is a promising therapeutic

target for SLE patients. In addition, Syk inhibitor appears to be an alternative drug for the treatment of lupus with a counter on sepsis. Further studies are interesting.

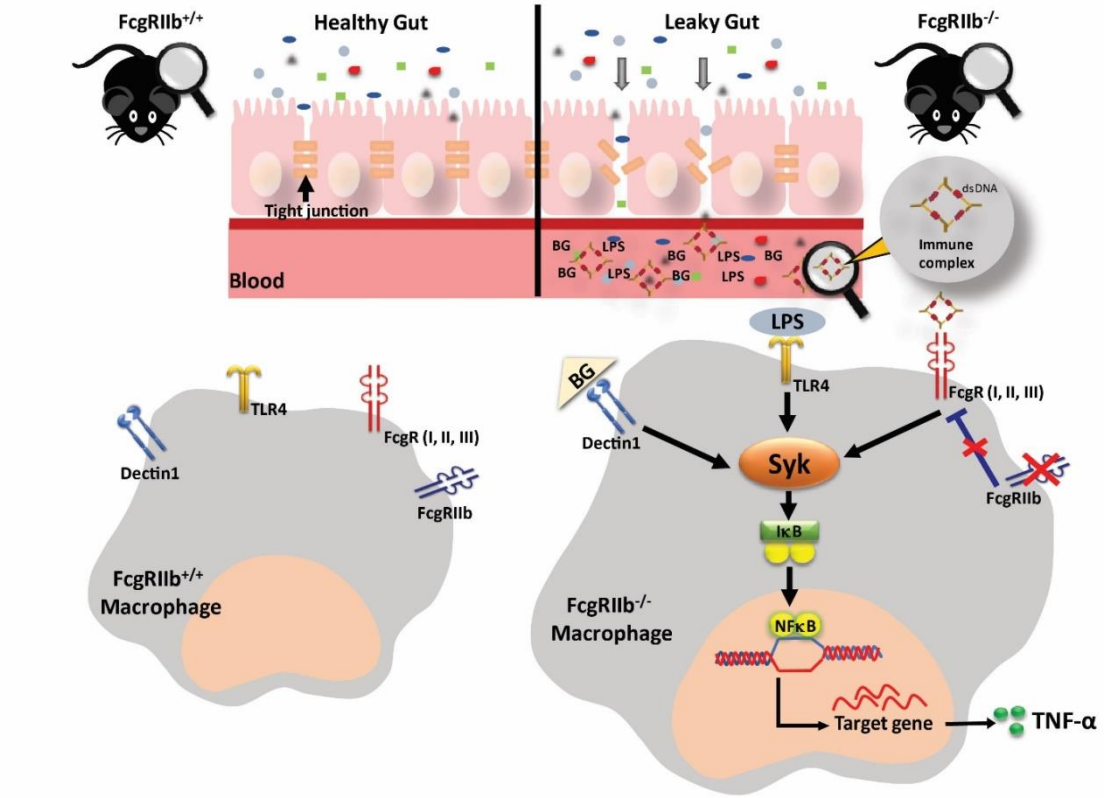


Figure 46 The proposed mechanism of the inflammatory hyper-responsiveness of FcgRIIb-/- lupus macrophages was demonstrated. Circulating immune complex (CIC), mostly from dsDNA and auto-antibodies, causes several injuries in FcgRIIb-/- lupus mice including tight junction of gut-epithelium that leads to gut leakage. Then translocation of lipopolysaccharide (LPS) and (1→3)-β-D-glucan (BG) from cell wall of Gram negative bacteria and fungi, respectively, from gut contents into blood is induced in FcgRIIb-/- mice. Simultaneous presence of LPS, BG and CIC in FcgRIIb-/- mice provides the possible collaboration among TLR-4 (LPS receptor), Dectin-1 (BG receptor) and FcgRs (CIC receptors) through Spleen tyrosine kinase (Syk), a common down-stream signaling. However, FcgRIIb presentation in wild-type (FcgRIIb+/+) macrophages attenuates inflammation through Syk down-regulation while lack of inhibitory-FcgRIIb signaling in FcgRIIb-/- macrophages induces hyper-inflammatory responses. In addition, there was no Syk activation in WT macrophages due to the absence of both gut leakage and autoimmune-induced CIC in wild-type mice.

APPENDIX

1.Genotyping

DNA extaction

1.1 Reagent

DNA extraction kit (qiagen, Hilden, Germany) the DNA extraction procedure were performed according to the manufacturer's instruction.

1.2 Primer:

FcREC1 5'-AAGGCTGTGGTCAAACTCGAGCC-3'

OL4143: 5'-CTCGTGCTTTACGGTATCGCC -3'

OL4080: 5'-TTGACTGTGGCCTTAAACGTGTAG -3'

1.3 PCR mixture

2x Master Mix (ul) 7.5 µl.

Nuclease free water 4.5 µl.

10 µM FcREC1 0.38 µl.

10 µM OL4143 0.38 µl.

10 µM OL4080 0.75 µl.

1.4 PCR condition

Step	Tempurature (C °)	Time	Cycle(s)
1	98	3 min	1
2	98	30 sec	35
3	60	30 sec	

4	72	60 sec	
5	72	5 min	1
6	4	Infinity	1

1.5 Gel electrophoresis

3 µl of PCR products were run on 2 % agarose gel.

WT (FcGrIIb $^{+/+}$) shold give 173 bp of PCR product only.

Heterologous (FcGrIIb $^{+/-}$) should give 173 bp and 232 bp PRC products.

Knockout (FcGrIIb $^{-/-}$) should give only 232 bp of PCR product (Figure 47)

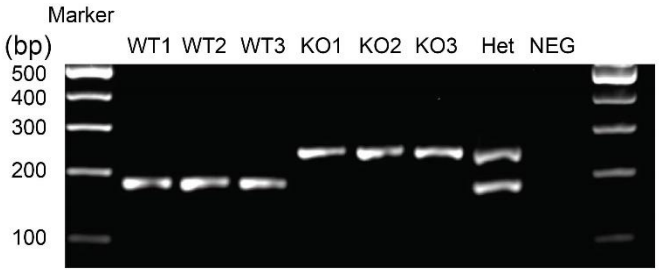


Figure 47 The respresentative of mouse genotype wild type (FcGrIIb $^{+/+}$), Heterologous (FcGrIIb $^{+/-}$), Knockout (FcGrIIb $^{-/-}$) respectively.

In this study, we confirmed the expression of FcgRIIb on BMDms (F4/80+ and CD11c+). Accourding to the result, we confirm that the FcgRIIb$^{-/-}$ macrophages showed no FcgRIIb protein expression in BMDMs cells (Figure 48)

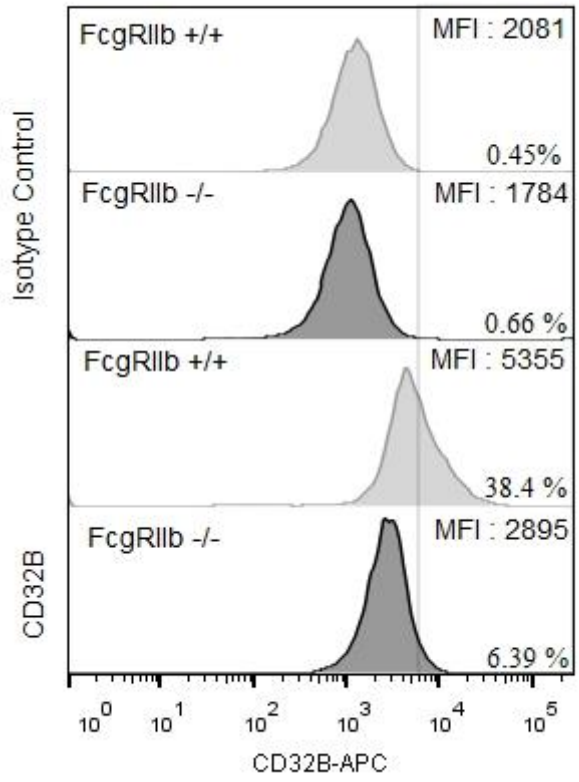


Figure 48 The representative of protein expression level in wild type and FcgRIIb$^{-/-}$ macrophages.

2. Material for gene expression

2.1 Mastermix for cDNA synthesis (1 reaction)

10X RT buffer	2 µL
25X dNTP Mix (100 mM)	.8 µL
10X Randomb Primers	2.0 µL
Mutiscribe^{TM} Reverse Transcriptase	1.0 µL
Nuclease-free H_2O	3.2 µL
Total RNA (final concentration 0.3 µg)	10 µL

Total	20 µL

2.2 PCR cycle for cDNA synthesis

Step	Tempurature (C°)	Time	Cycle(s)
1	25	10 min	1
2	37	120 sec	
3	85	5 sec	
4	4	infinity	

2.3 Mastermix for real time PCR (1 reaction)

PowerUpTM SYBR® Green Master Mix	10 µL
Forward primer 10 µM	0.5 µL
Reverse primer 10 µM	0.5 µL
Nuclease-free water	7 µL
cDNA	2 µL
Total volumn	20 µL

2.3 Real time PCR cycle

Step	Tempurature (C°)	Time	Cycle(s)
1	98	10 min	1
2	98	120 sec	40
3	60	5 sec	
4	Melting-cruve	Default	1

3.Cell culture

3.1Complete DMEM (100 mL) (Store at 4 °C)

DMEM (high glucose)	87	mL
FBS	10	mL
10000 U/mL Penicilin/Streptomycin	1	mL
100X L-glutamine	1	mL
100X Hepes buffer	1	mL

3.3 BMDMs Medium'

Complete DMEM	100	mL
Recombinant m-csf (final concentration 25 ng/mL)	25	µL

4.Western blot reagents

4.1 1.5 M Tris-HCl pH 8.8

Tris base	181.7	g
Milli-Q water	700	mL

Adjusted pH to 8.8 using conc. HCl and the milliQ water was added to 1000 mL

4.2 1 M Tris-HCl pH 6.8

Tris base	121.1	g
Milli-Q water	700	mL

Adjusted pH to 6.8 using conc. HCl and the milliQ water was added to 1000 mL

4.3 10% SDS

SDS	10	g

Add Milli-Q water to 100 mL

4.4 10% Ammonium persulphate (APS)

Ammonium persulfate	0.05	g
Milli Q	1	mL

4.5 10X Tris/Glycine/SDS

ultrapure Tris	30.3	g
glycine	144.1	g

Add milli-Q, to 1 L filter and store at RT

4.6 1X Tris/Glycine:

10X Tris/Glycine	100	mL
Milli-Q water	900	mL

4.7 10 % SDS-PAGE (2 gel)

Milli Q 4,175 µl

40% Acrylamide gel	3,125	µL
1.5 M Tris-HCl pH 8.8	2.5	mL
10% SDS	100	µL
10% APS	100	µL
TEMED	5	µL

4.8 4% stacking gel (2 gel)

Milli-Q	3,650	µL
40% Acrylamide gel	625	µL
1 M Tris-HCl pH 6.8	625	µL

10% SDS	50	µL
10% APS	50	µL
TEMED	5	µL

4.9 10X Transfer buffer (1000 mL)

Tris base	30.3	g.
Glycine	47.1	g.

For 1X transfer buffer (fresh prepare)

10X transfer buffer	100	mL
Methanol	200	mL
Milli-Q	700	mL

4.10 Antibodies dilution reagent

1 % BSA in TBS T

4.11 Loading dye for protein

Add 60 mg of DTT in 1 mL of loading dye

4.12 10X Running buffer (1000 mL)

Tris-base	30	g.
Glycine	144	g.
SDS	10	g.

5.Software and programs

GraphPad Prim 6.0

Adobe Illustrator CC

EndNote X5

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VITA

NAME Jiraphorn Issara-Amphorn

DATE OF BIRTH 31 August 1989

PLACE OF BIRTH chonburi Thailand

INSTITUTIONS ATTENDED

2017-present Doctor of philosophy (Medical Microbiology)
Chulalongkorn University, Bangkok, Thailand

2011-2014 Master of Science (Biochemistry) Cum. G.P.A 3.67
Kasetsart University, Bangkok, Thailand

2007-2011 Bachelor of Science (Biochemistry) Cum. G.P.A 2.82
Kasetsart University, Bangkok, Thailand

HOME ADDRESS 4 M.3 Nongkayad Panasnikhom Chonburi Thailand 20140

PUBLICATION

1. Waiyaput W, Payungporn S, Issara-Amphorn J, Panjaworayan NT. Inhibitory effects of crude extracts from some edible Thai plants against replication of hepatitis B virus and human liver cancer cells. BMC complementary and alternative medicine. 2012 Dec 6;12:246.
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AWARD RECEIVED

September 2014 The best thesis award, Graduate school Kasetsart University

Apirl 2017 The travelling award, FIMSA Advance Course

March 2018 3 rd student oral presentation, The 34th Annual Meeting AAIAT 2018