PHOSPHOPROTEOMIC ANALYSIS OF FC GAMMA RECEPTOR IIB DEFICIENT MACROPHAGES IN ENHANCING THE SEVERITY OF ENDOTOXIN TOLERANCE

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จุฬาลงกรณ์มหาวิทยาลัย
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การวิเคราะห์ทางฟอสโฟโปรติโอมิกส์ของเซลล์แมคโครฟาจที่ขาดตัวรับชนิดเอฟซีแกมมาทูบี ในภาวะดื้อต่อการตอบสนองของเอนโดทอกซิน

น.ส.ธัญณิชา อ่อนดี

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Miss Thunnicha Ondee
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ธัญณิชา อ่อนดี : การวิเคราะห์ทางฟอสโฟโปรติโอมิกส์ของเซลล์แมคโครฟาจที่ขาดตัวรับชนิดเอฟซีแกมมาทูบี ในภาวะดื้อต่อการตอบสนองของเอนโดทอกซิน. (PHOSPHOPROTEOMIC ANALYSIS OF FC GAMMA RECEPTOR IIB DEFICIENT MACROPHAGES IN ENHANCING THE SEVERITY OF ENDOTOXIN TOLERANCE) อ.ที่ปรึกษาหลัก : ผศ. ดร. นพ.อัษฎาศ์ ลีฬหวนิชกุล

การติดเชื้ออย่างรุนแรงในกระแสเลือดเป็นหนึ่งในสาเหตุสำคัญของการเสียชีวิตในผู้ป่วยทั่วโลก ซึ่งเกี่ยวข้องกับความผิดปกติหรือความบกพร่องจากการตอบสนองของระบบภูมิคุ้มกัน สารเอนโดท็อกซินของแบคทีเรียหรือที่เรารู้จักกันในชื่อ lipopolysaccharide (LPS) จะมีคุณสมบัติเป็นตัวกระตุ้นในการทำให้เกิดการอักเสบและยังเกี่ยวข้องกับพยาธิสรีรวิทยาของการติดเชื้ออีกด้วย การที่ได้รับสาร LPS ซ้ำ ๆ สามารถทำให้เกิดผลการยับยั้งการทำงานของการตอบสนองของระบบภูมิคุ้มกันได้ ซึ่งเป็นที่รู้จักกันในชื่อของความทนทานต่อสารชนิดเอนโดท็อกซิน นอกจากนี้การตอบสนองของระบบภูมิคุ้มกันในระดับโมเลกุลต่อการติดเชื้อยังส่งผลต่อเป้าหมายทางภูมิคุ้มกันแบบเฉพาะ และอาจจะเกี่ยวข้องกับ Fc gamma receptor IIb (CD32b) ซึ่งเกี่ยวข้องกับโรคลูปัส บทบาทของ FcgRIIb ในโรคลูปัส อาจจะมีผลต่อการทนทานต่อสารเอนโดท็อกซินซึ่งทำให้เกิดการติดเชื้อง่ายขึ้นในผู้ป่วยกลุ่มนี้ การศึกษากลไกการทำงานพื้นฐานของความทนทานต่อเอนโดท็อกซินต่อการติดเชื้อในกระแสเลือดที่เด่นชัดในหนูทดลองที่ขาดตัวยับยั้งสัญญาณชนิด FcGRIIb (FcGRIIb - / -) อาจจะเกี่ยวข้องกับการติดเชื้อในโรคลูปัส

ผลจากการทดลองพบว่าความทนทานต่อสารชนิดเอนโดท็อกซิน ทำให้มีการผลิตไซโตไคน์ลดลงในหลอดทดลองในเซลแมคโครเฟสที่ถูกกระตุ้นด้วย LPS แบบต่อเนื่องสองครั้งในเซลล์ที่ขาด FcGRIIb รุนแรงกว่า Wild-type (WT) นอกจากนี้ การผลิตไซโตไคน์,การกินเชื้อ และการฆ่าเชื้อแบคทีเรีย ก็ลดลง สำหรับแบบจำลองการติดเชื้อในกระแสเลือดโดยวิธีทำ cecal ligation and puncture (CLP) หลังจากฉีด LPS ในหนูทดลองพบว่าหนูลูปัส ถูกชักนำให้เกิดการติดเชื้อที่รุนแรงมากกว่า Wild-type (WT) โดยวัดจากอัตราการตายของหนู, จำนวนแบคทีเรียในเลือด,ไซโตไคน์ในซีรั่ม, ครีเอตินีน และแอลละนินทรานอะมิเนส (การบาดเจ็บของไต และ ตับ) การวิเคราะห์ฟอสโฟโปรติโอมิค ในเซลที่เกิดความทนทานต่อเอนโดท็อกซิน พบว่าโปรตีนไคเนส C-β type II (PKCBII) มีค่าสูงกว่าในเซลแมคโครเฟสจากหนู FcGRIIb-/- การวิเคราะห์ western blot ของ macrophage ในหลอดทดลอง และ immunoblot ของม้ามของ FcGRIIb - / - ในหนูทดลองที่ได้รับ LPS แบบต่อเนื่องพบผลการทดลองในทำนองเดียวกัน ข้อมูลที่ได้จากการศึกษา FcGRIIb และความทนทานต่อสารเอนโดท็อกซินในงานวิจัยนี้อาจนำไปสู่กลยุทธ์ใหม่ในการป้องกันหรือรักษาโรคติดเชื้อแบคทีเรียในกระแสเลือดที่รุนแรงของผู้ป่วยได้ในอนาคต

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Sepsis is a severe response to systemic infection and one of the leading causes of death in patients world-wide. Bacterial endotoxin (lipopolysaccharide; LPS), is a potent inducer of inflammation that has been associated with the pathophysiology of sepsis. The repeated LPS exposure can induce a suppressive effect as refer to endotoxin tolerance. Fc gamma receptor IIb (CD32b), the only inhibitory receptor among FcGR family, associates with systemic lupus erythematosus (FcGRIIb defunctioning polymorphisms) which are commonly found in Asian populations. Because of an inhibitory nature of the signaling, endotoxin tolerance in lupus with FcGRIIb deficient (FcGRIIb-/-) is interesting and might be translated into the infection in lupus patients. Therefore, we aim to explore the endotoxin tolerance in FcGRIIb-/- mice and the underlying mechanisms. Indeed, several characteristics of endotoxin tolerance using two sequential LPS stimulations in FcGRIIb-/- bone marrow-derived macrophage (BMM) including cytokine production, phagocytosis and killing activity were lower than the wild-type (WT) cell. For in vivo, cecal ligation and puncture (CLP) induce sepsis after LPS preconditioning was more severe in FcGRIIb -/- mice, as measured by mortality rate, bacteria count in blood, serum cytokines, creatinine (kidney injury) and alanine transaminase (liver damage). Then the phosphoproteomic analysis, using a dimethyl labeling method, in endotoxin tolerance of FcGRIIb-/- macrophage revealed the prominently decreased protein kinase C-β type II (PKCBII) in comparison with WT cell. In addition, the validation analysis including western blot analysis of macrophage in vitro and the immunoblot of spleen from FcGRIIb-/- mice with sequential LPS administration demonstrated the lower PKCBII in LPS tolerance FcGRIIb-/- groups. The impact of FcGRIIb and endotoxin tolerance association with sepsis might lead to the new strategies for the prevention and/or treatment of bacterial infections in patients with lupus in the future

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Thunnicha Ondee

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LIST OF ABBREVIATIONS

Ab	Antibody
ACN	Acetonitrile
ALT	Alanine aminotransferase
ATP	Adenosine triphosphate
aPKC	Atypical isoforms
AUC-CR	Area under the curve of cytokine response
BCA	Bicinchoninic acid assay
bp	Base pair
BSA	Bovine serum albumin
BMM	Bone marrow derived macrophages
CASP	Colon ascenden stent peritonitis
cDNA	Complementary DNA
CLP	Cecal ligation and puncture
cPKC	Conventional isoforms
dNTPs	Deoxynucleotide triphosphates
DHB	2,5-dihydroxybenzoic acid
DTT	Dithiothreitol
E.Coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FA	Formic acid

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FcgR	Fc gamma receptor
GO	Gene ontology
GTP	Guanosine triphosphate
IA	Iodoacetamide
IFN-γ	Interferon-gamma
IL	Interleukin
IL-β	Interleukin-bata
IgG	Immunoglobulin G
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
LPS	Lipopolysaccharide
M	Molar
M. tuberculosis	*Mycobacterium tuberculosis*
MS/MS	Tandem mass spectrometry
MTS	Thiazolyl blue tetrazolium bromide
NIH	National Institutes of health
nPKC	Novel isoforms
NSS	Normal saline
PAMP	Pathogens associated molecular pattern
PBMC	Peripheral blood mononuclear cells

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PCR	Polymerase chain reaction
PKCβ	Protein kinase C-bata
PRKCβ	Protein receptor kinase C-bata
ppm	Parts per million
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cell
PTM	Post-translational modification
pThr	Phosphothreonine
pTyr	Phosphotyrosine
pSer	Phosphoserine
S. pneumoniae	*Streptococcus pneumoniae*
SD	Standard deviation
SDC	Sodium deoxycholate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SILAC	Stable isotope labeling by amino acids in cell culture
TEAB	Triethylammonium bicarbonate
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TGFβ	Transforming growth factor beta
TiO2	Titanium dioxide chromatography

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TMT	Tandem mass tags
TNF-α	Tumor necrosis factor alpha
WB	Western blotting assay

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CHAPTER I
INTRODUCTION

Fc gamma receptor (FcGR) is a receptor of Fc portion of immunoglobulin and FcGRIIb is the only inhibitory receptor among FcGR family (1, 2). The functional defect of FcGRIIb has been identified as one of the genetic causes of lupus and FcGRIIb-/- mice, with the loss of the inhibitory signaling, have been used as one of the representative lupus mouse model. Interestingly, FcGRIIb dysfunction polymorphisms are common in Asia, at least in part, explained by the genetic pressure from infection in the region (3). FcGRIIb polymorphisms are protective in malaria but, conversely, enhance susceptibility to lupus (4). In response to infection, the Fc portion of the immunoglobulin competitively binds with activating and inhibitory FcGRs to determine immune-response directions (5). Although the organism control of FcGRIIb -/- mice against most organisms are better than wild-type due to the hyper-responsive inflammatory responses, sepsis is more severe in these mice with the repeated antigen stimulation (6). Interestingly, the inhibition signaling defect in FcGRIIb-/- mice still permits effective eradication of *Plasmodium spp., Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (6-8). However, the immune response function possibly becomes exhausted after repeated infections. As such, the immune exhaustion after repeated or huge stimulation from pathogens as chronic infection and sepsis, respectively, is well-known (9, 10). While LPS tolerance is a helpful mechanism against LPS intoxication as the mice could be survived from the repeated LPS exposure due to the decreased responses against LPS which mainly reduced cytokine production. In sepsis, LPS tolerance is possibly a part of immune exhaustion phase of sepsis which patients are more susceptible to the opportunistic infection and monocytes from these patients fail to produce inflammatory cytokines (11-13). Moreover, not only the viable organisms, the repeated stimulations of bacterial molecule, such as LPS, might also induce the immune-response exhaustion. As such, the role of the immune-response defect after repeated LPS-stimulations, endotoxin tolerance, is a long-standing area of investigation (14-18). Indeed, elevated blood endotoxin is common in patients with

sepsis and the evidence of chronic endotoxin exposure is reported (19). Moreover, the huge responses against endotoxin followed with the prominent immune exhaustion in FcGRIIb-/- mice is reported (20). Likewise, patients with sepsis are susceptible to infection (21, 22) and the susceptibility toward infection is increased with immune exhaustion (23-25).

Hence, the immune exhaustion is, at least in part, one of the mechanisms leading to the increased infection susceptibility in sepsis. Indeed, the mortality rate of patients with sepsis from infection is very high even before the immune-suppression era (21). In addition, we have been reported that the insufficient inflammatory response in FcGRIIb-/- mice is, at least in part, due to the more prominent endotoxin (LPS) tolerance of FcGRIIb-/- macrophage, despite macrophage hyper-responsiveness after the single endotoxin stimulation (20). Perhaps, the more understanding in underlying mechanism of immune exhaustion in FcGRIIb-/- mice might be useful for the clinical management of infection in patients with sepsis. Unfortunately, the mechanism responsible for the prominent immune exhaustion in sepsis and FcGRIIB-/- mice is still unknown. Because FcGRIIb-/- of comparison with wild-type macrophage is hyper-response against the single LPS stimulation but prominently exhausted in the twice LPS stimulations, then it is possible that the alteration of cell energy is associated with these FcGRIIb-/- cell characteristics. In addition, the post-translational modification of phosphate groups in several proteins is one of the common mechanisms associated with cell energy.

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We hypothesized that FcGRIIb-/- mice might have hyper-elevated immune response to LPS but follow by vigorous endotoxin tolerance induce the higher bacterial susceptibility. Then we investigated this by mimicking multiple infections by sequential LPS injections. We examined whether endotoxin-tolerance in FcGRIIb-/- mice was more prominent than in wild-type. Additionally, we tested the potential clinical importance of endotoxin-tolerance with an *in vivo* sepsis model, cecal ligation and puncture (CLP), and with macrophage cytokines responses after LPS stimulations including phagocytosis and killing activity *in vitro*. Then, we performed phosphoproteomics analysis in macrophage of FcGRIIb-/- mice and wild-type after the single LPS stimulation

and twice LPS incubation (LPS tolerance) and demonstrated an interesting candidate protein from the analysis. We postulated that the phosphoproteomes of wild-type and FcGRIIb-/- will respond differently to LPS stimulation and performed phosphoproteomic analysis to test this hypothesis.

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CHAPTER II

OBJECTIVES

Research questions

1. Does endotoxin tolerance related with sepsis severity and Fc gamma receptor IIb deficient mice have a more prominent endotoxin tolerance?
2. Does macrophages have different signaling networks (explored by quantitative phosphoproteomics between Fc gamma receptor IIb deficient and wild type)?

Hypothesis

1. Endotoxin tolerance-related sepsis susceptibility is more severe in Fc gamma receptor IIb deficient mice than wild type.
2. Signaling network by quantitative phosphoproteomics in Fc gamma receptor IIb deficient is different from wild type.

Objective

1. To investigate if Fc gamma receptor IIb deficient mice play an essential role in increased endotoxin tolerance-related sepsis susceptibility.
2. To investigate the dynamic responses of Fc gamma receptor IIb deficient macrophages of endotoxin tolerance signaling network by quantitative phosphoproteomics.

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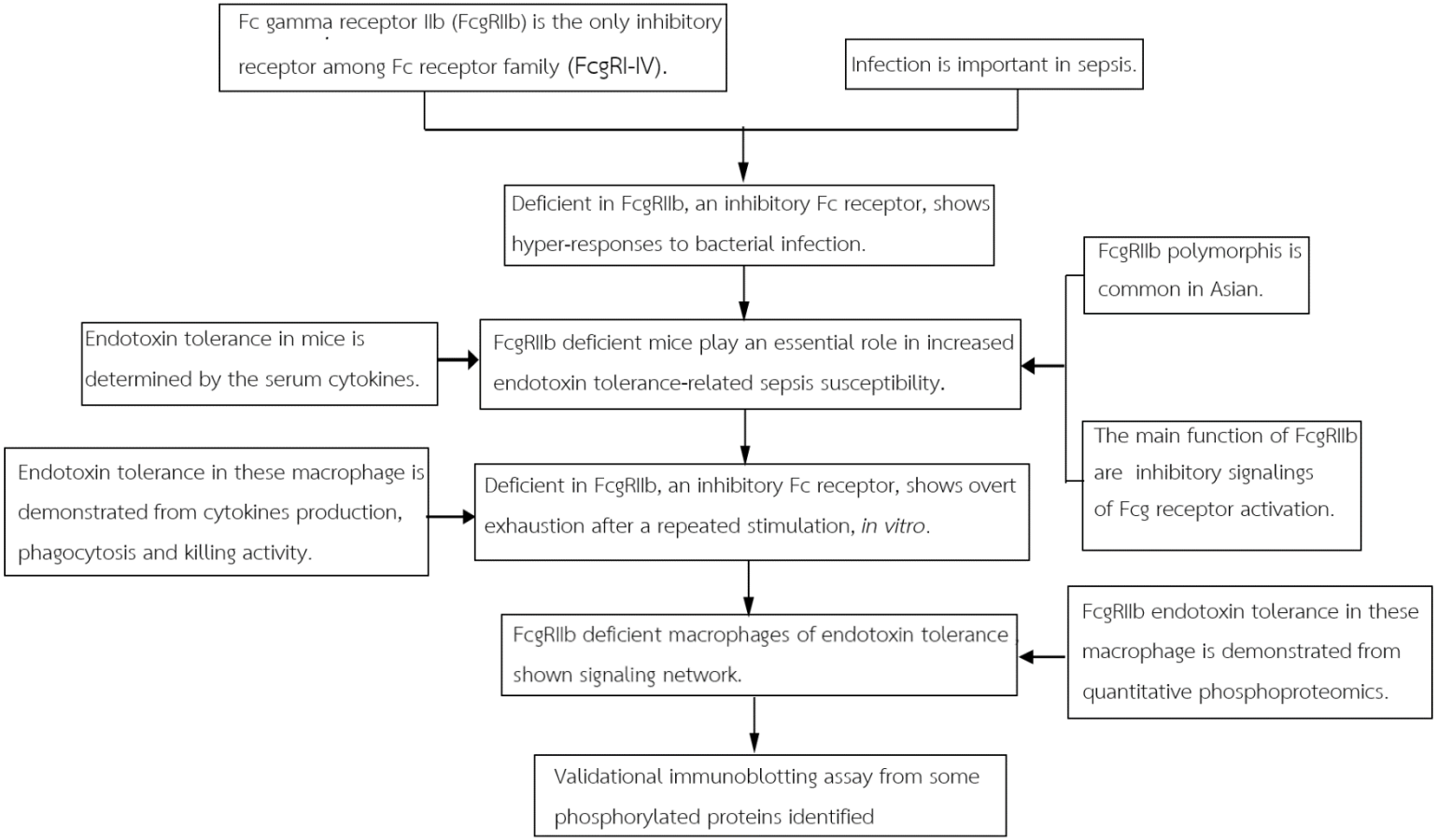
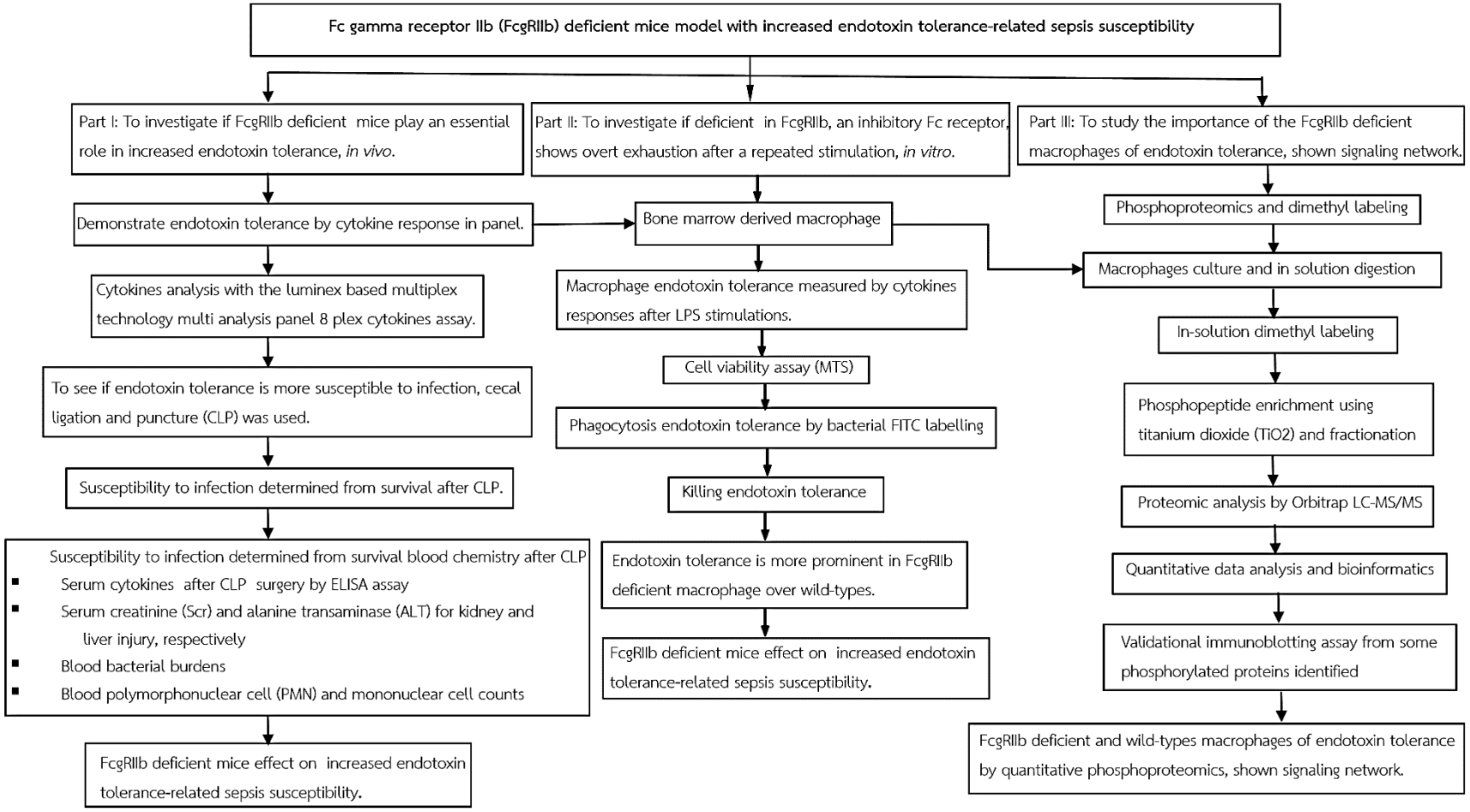
Conceptual framework



Methodology Workflow



CHAPTER III
LITERATURE REVIEW

Sepsis and bacterial infection

Sepsis continues to be the major infection related cause of death worldwide (26-28). This is caused by an immune response, triggered by infections, destroys tissues and organs of host (29-31). Despite modern medical advances including new antibiotics and vaccines, best practice treatments and well equipped intensive care units (32), sepsis mortality rates remain high (33, 34). The risk of death from sepsis is as high as 30%, from severe sepsis as high as 50%, and from septic shock as high as 80% (34). Most commonly, the majority causes of sepsis are due to bacterial infections. The sources of infection could be almost any organs or implanted device such as skin, lung (pneumonia, gastrointestinal tract (bacterial penetration or ruptured intestine from trauma, surgical site, and intravenous catheter. The organisms or/and their toxins spread directly or indirectly into bloodstream. Most commonly, bacterial causes of sepsis are gram negative bacteria such as *E. coli, P. aeruginosa, and Haemophilus* influenzae. Although nearly all bacteria have been known to cause sepsis, most sepsis is caused by gram negative bacteria possibly because of host responses to a major molecule of gram negative bacteria; lipopolysaccharide (also called the endotoxin) (35, 36).

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Endotoxin tolerance and bacterial sepsis

Bacterial endotoxin, or lipopolysaccharide (LPS), a glycolipid of the cell membranes in gram negative bacteria, is one of the most potent inducers of inflammation in immune responses (26, 37). It has been suggested that LPS triggers hyperinflammation in sepsis and the early life threatening cytokines storm cause of septic shock(38). Immune response to LPS, a systemic production of pro-inflammatory cytokines, aims to take out the invaded pathogens (39). Although these cytokines are necessary to the efficient control of the pathogen(40-42), the excessive inflammatory

response is potentially auto-destructive and may lead to hyper-inflammatory phases and immune dysfunction/immunosuppressive phase (43). These reactions cause tissue damage, septic shock, and eventually death (44, 45). One of the many attempts to characterize the immune response state in sepsis has suggested a role for endotoxin tolerance in the later stages of this process (46-48). Endotoxin tolerance, this phenomenon of is known from animal models after an initial low dose of LPS, animals are protected against the detrimental consequences of a subsequent high dose of LPS. This protection is associated with an attenuated cytokines response to LPS(49) because of a down-regulation of innate immune cells responsiveness(50). Moreover, endotoxin tolerance, also termed as cell reprogramming, can be defined as the severely reduced capacity of cells in response against LPS during the second exposure to this stimulus and represents an immune amnesia rather than an anti-inflammatory response, resulting in reduced inflammatory cytokines production in *in vivo* and similar *in vitro* (51, 52). Endotoxin tolerance is known to cause innate immune cells, like macrophages, to produce a decreased pro-inflammatory response to a pathogens associated molecular pattern (PAMP), like LPS, after the pre-stimulations. The innate immune cells involved in LPS tolerance have thought to be primarily monocytes and/or macrophages and possibly also in dendritic cells, neutrophils and T cells(53) . Toll-like receptor 4 (TLR4) is a member of the pattern recognition receptors (PRR) family which is responsible for recognizing gram-negative bacteria and their endotoxins, such as lipopolysaccharide (LPS). This recognition allows the initiation of the inflammatory response that is firmly regulated throughout the immune system to prevent uncontrolled inflammation (Figure 1), which can lead to shock, organ dysfunctions, tissue damage and pathologies(54). The immune system has developed a protection against repeated endotoxic activation therefore the preventing harmful reaction. This defined as the temporary hypo-responsiveness in which endotoxin tolerance is known as one of these mechanisms(55). The molecular mechanisms of endotoxin tolerance are vague. However, negative regulators such as SOCS1, IRAK-M and SHIP are believed to play an important role, along with the down regulation of toll like receptor 4 (TLR4) on cell surface and gene reprogramming (Figure 2). Endotoxin tolerance protects against a lethal challenge of LPS and prevents infection and ischemia reperfusion

damage. This is paralleled by a dramatic reduction of tumor necrosis factor (TNF) production and some other cytokines in response to LPS. Importantly, endotoxin tolerance is become apparent because of its clinical relevance in severe infections by gram negative bacteria (54, 56).

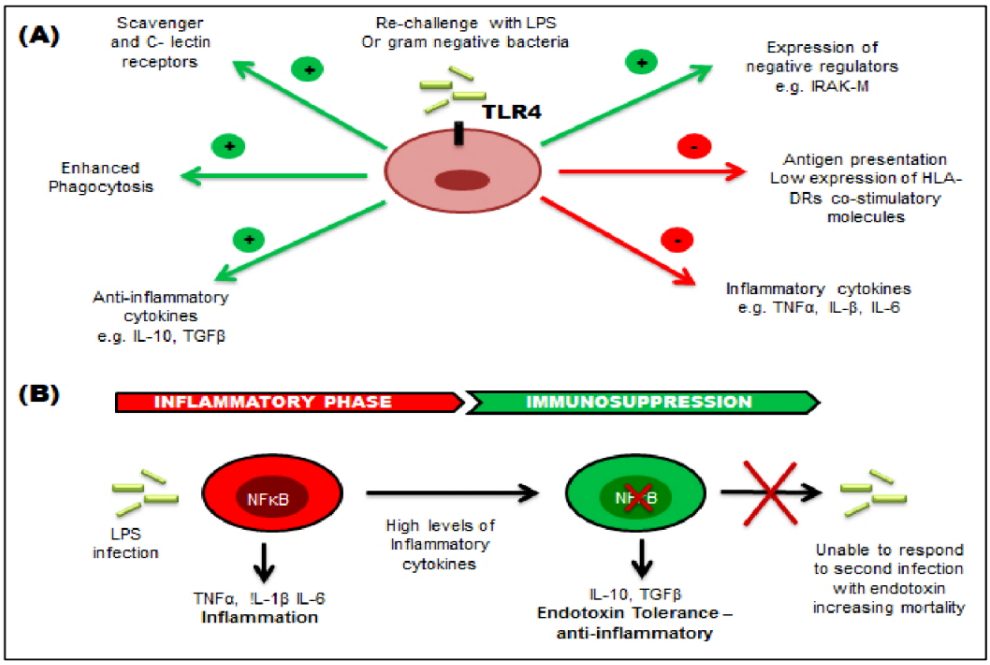

Figure 1. Endotoxin tolerance monocyte and/or macrophage.

The major down regulation of proinflammatory cytokines, TNF-α, IL-6, and IL-β, and the up-regulation of anti-inflammatory cytokines (IL-10 and TGFβ) after the endotoxin (LPS) re-stimulation is shown (A). Other changes when undergoing tolerance also represented. The 2 stages of immune responses are shown sepsis in monocytes and/or macrophages (B). Endotoxin tolerance is shown in the 2nd stage (immunosuppression) (53, 54, 56).

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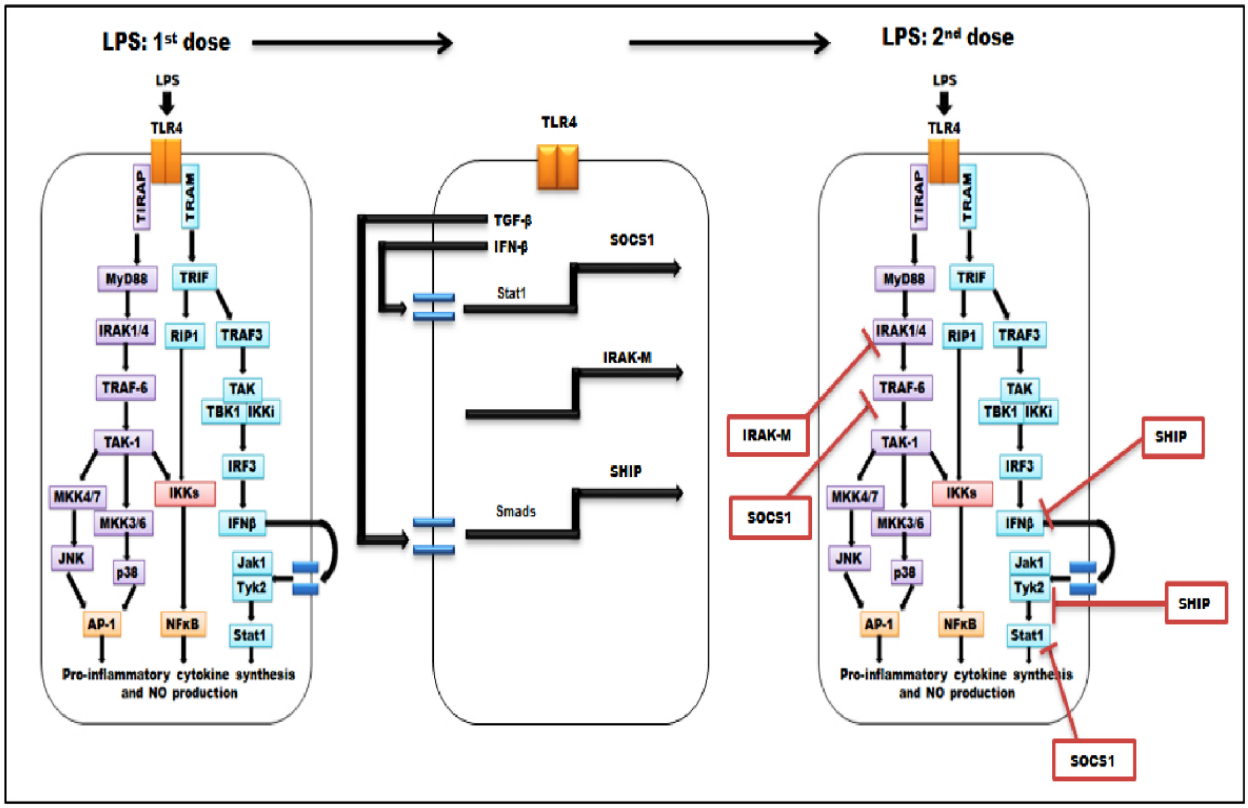

Figure 2. The signaling pathways of endotoxin tolerance.

The first diagram shows usual functional signaling, which occurs on first dose with LPS. The middle one shows the activation of inhibitory pathways. The left hand diagram shows an effect of the negative regulators on 2nd dose with LPS (53, 57).

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Fc gamma receptor IIb (CD32b)

Fc gamma receptor gene families consist of four classes, which are FcgRI (CD64), FcgRII (CD32), FcgRIII (CD16), and FcgRIV (58). Fc gamma receptor IIb (FcGRIIb), the only inhibitory receptor among FcGR family, expressed on cell surface of B lymphocytes, monocytes and/or macrophages, neutrophils, dendritic cells, basophils and natural killer cells (59-61). The Fc gamma receptors (Fcgr) response to the Fc portion of immunoglobulin G (IgG), consist of several subtypes but FcgrIIb is the only receptor that produce inhibitory signal (Figure 3) (58). The defect of FcgrIIb, inhibitory signal, leads to the hyper-responsiveness of adaptive immune response and

autoimmune disease. Accidentally, the prevalence of FcgrIIb polymorphisms is also common in Asia especially in Thai, Chinese and Japanese (62-65).

Fc receptor (mouse)	Human homolog	Type	Signaling motif	Affinity for IgG	Binding partner	Expression pattern
FcγRI	FcγRIA/B/C	Activating	ITAM	High 10^8–10^9 M^{-1}	IgG2a	Macrophages, dendritic cells, eosinophils, neutrophils
n/a	FcγRIIA/C	Activating	ITAM	n/a	IgG1, IgG2a	n/a
FcγRIIB	FcγRIIB	Inhibitory	ITIM	Low 10^6 M^{-1}	IgG1, IgG2a, IgG2b	B cells, macrophages, neutrophils, mast cells
FcγRIII	FcγRIIIA/B	Activating	ITAM	Low 10^6 M^{-1}	IgG1, IgG2a, IgG2b	Macrophages, mast cells, γδ Tcells, NK cells
FcγRIV	FcγRIIIA	Activating	ITAM	Medium 10^7 M^{-1}	IgG2a, IgG2b	Neutrophils, monocytes, macrophages, dendritic cells

Figure 3. Overview of Fc receptor gene families and comparative between murine and human genes (58).

The signal activation demands cross linking in the FcR chains with various linked Ig molecules such as Ig coated immune complexes or microbes (Figure 4). Signaling transduction begins by Src kinase mediated tyrosine phosphorylation of the ITAMs followed with SH2 domain mediated recruiting Syk family kinases of ITAMs, stimulation of PI-3 kinase, recruiting adapter molecules such as SLP-76 and BLNK stimulation including enzymes like phospholipase Cg as well as Tec family kinases. Therefore, IP3 and DAG are a generated and an intracellular free Ca2+ are mobilized. This signaling pathway of leukocytes activate in gene transcription on cytokines, microbicidal enzymes, inflammatory mediators, inducement in a cytoskeleton, cell migration all together for leading to a phagocytosis including degranulation(66).

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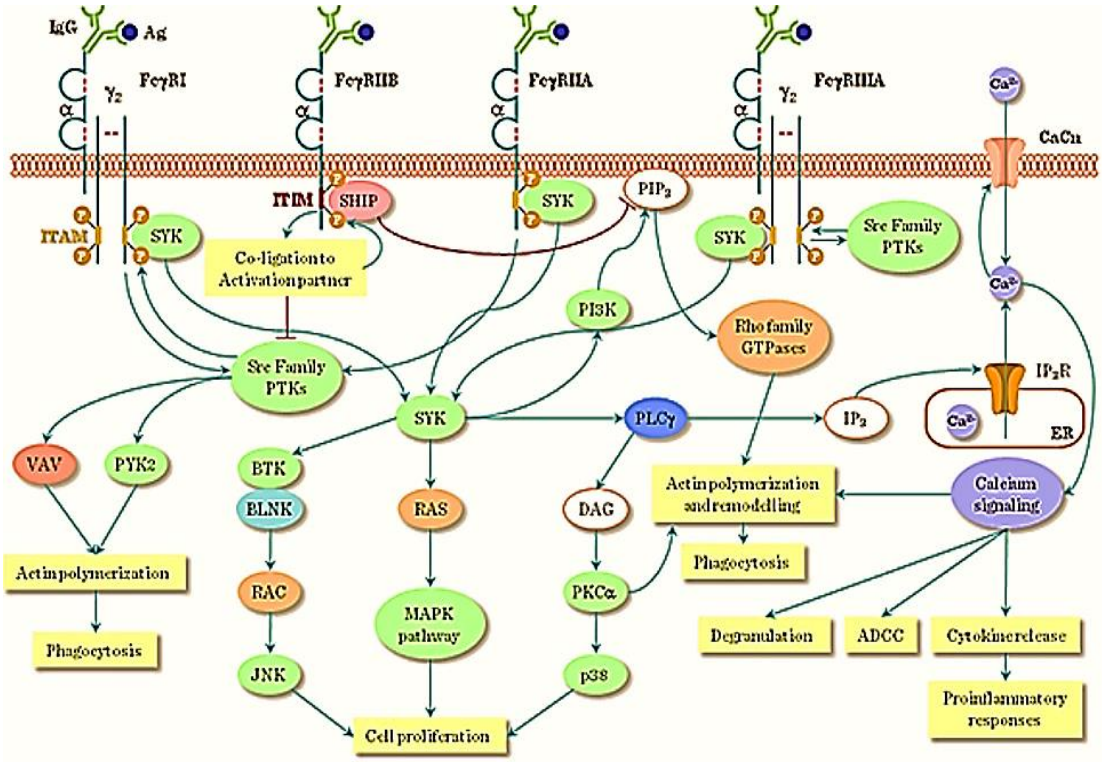

Figure 4. The overview in Fc gamma receptor signaling pathway (66).

The functional defect of FcGRIIb is one of the genetic causes of systemic lupus erythematosus (SLE) which is, at least in part, because of the loss of inhibitory function of B lymphocytes (2, 67). Interestingly, FcGRIIb loss of function polymorphisms are common in Asia, possibly, due to malaria based selection pressure in populations (68). FcGRIIb dysfunction polymorphisms are protective against malaria through the enhanced macrophage functions but, conversely, enhance susceptibility to patients possibly due to B lymphocyte hyper-functions. In response to infections, the Fc portion of the immunoglobulin competitively binds with activating and inhibitory FcGRs to determine immune response directions (5). Interestingly, the inhibitory signaling defect in FcGRIIb-/- mice still permits effective eradication of *Streptococcus pneumoniae*, *Plasmodium spp*. and *Mycobacterium tuberculosis* (7, 8).

Claworthy *et al.* hypothesized that people with the Fcgrllb polymorphisms are less susceptible to malaria which is supported by *Plasmodium spp*. infection in FcGRIIb

-/- mice is less severe than wild type (69). It is interesting that the zone of the world regions with FcgrIIb polymorphisms pre-dominance is the tropical zone with high prevalence of malaria. Hence, the susceptibility of FcgrIIb alteration to infection depends on specific organisms. FcgrIIb polymorphisms showed less susceptible to malarial infection (70). Likewise, in *Streptococcal pneumonia* model, FcgrIIb -/- mice shows ability to clear organisms very effectively through the circulating immune complex receptor but the cytokines responses in immunized mice are more severe than wild type control (71). However, sepsis from *Streptococci* or other gram positive bacteria are not as common as gram negative sepsis (72). Nevertheless, immune responses of the function become exhausted after the repeated and/or chronic infections (73, 74) or with the enormous micro-organism burdens (10, 43, 75). Not only viable organisms, repeated stimulations of bacterial molecule, such as LPS, but also induce the immune response exhaustions, as refer to endotoxin tolerance (15-18, 76).

Interestingly, endotoxin tolerance has been demonstrated to be associated with immune exhaustion (immunosuppressive stage). The characteristics of gene expression signature in peripheral blood mononuclear cells (PBMC) of patients at immunosuppressive-phase of sepsis are similar to the expression in PBMC of human volunteers with repeated endotoxin challenges (26). In addition, isolated PBMC from patients at late-phase sepsis and endotoxin tolerance of macrophages in vitro demonstrated similar cytokine production profiles (48, 52). In parallel, the repeated or persistent activation of LPS might induce endotoxin tolerance and immune exhaustion in patients, resulting in the high susceptibility to bacterial infections. Indeed, elevated blood endotoxin is common in patients and the evidence of chronic endotoxin exposure in such patients is reported (77).

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Cecal ligation and puncture (CLP) animal model

The current standard models of sepsis including lipopolysaccharide injection model (LPS model), cecal ligation and puncture model (CLP model), colon ascendens stent peritonitis (CASP) and bacterial injection model (Table 1) with several advantages and disadvantages.

Animal models of sepsis

Animal model	Advantage	Disadvantage
LPS injection	Simple, sterile; some similarities with human sepsis pathophysiology	Early and transient increases in inflammatory mediators more intense than in human sepsis
CLP or CASP	Early silent period; moderate and delayed peak of mediators; multiple bacterial flora	Age and strain variability; early hemodynamic period in some models
Clinically relevant CLP	Replication of clinical risk factors	Difficulty in analyzing pathophysiological pathways
Infusion or instillation of exogenous bacteria	Early hyperdynamic state	No change in intrarenal microcirculation; need large animals; labor-intensive

Table 1. The advantage and disadvantage of different sespsis models (78).

For example, The LPS model is easy and rapid model but less physiologic compare with human condition; the LPS toxin act as directly PAMP, there are proteinuria from direct LPS effect to podocyte, the inflammatory cytokines surge rapidly in very level compare with real sepsis patients, etc. The CLP model need the surgery with resemble to the rupture gastrointestinal organ in human. In short, the

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cecum is exposed and punctured through and through with a needle and squeeze some feces into peritoneum. Then the local peritonitis follows with sepsis occurs. The severity of the model can be adjusted by the size of needle and the length of ligated cecum (Figure 5).

CLP is the currently most popular sepsis model with the more physiologic response in comparison with human sepsis; cytokines are gradually increase with the peak level less than LPS model, blood bacteria can be detected with the multi-organisms, etc. In parallel, colon ascendens stent peritonitis (CASP) depend on the same principle, generation of the localized peritonitis from intestinal leakage but is less popular compare with CLP due to the need for the stent and the less standard protocol. Another sepsis model is the exogenous bacterial infusion model. The animals show vital sign alteration but not necessary to have sepsis. Moreover, the bacterial infusion model show limited reproducibility in small animal and mostly used with large animal such as sheep or piglets. In this study, we used CLP as the main method for sepsis model. The worsen mortality rate after CLP in LPS stimulated FcGRIIb-/- over wild-type (Lower) (78).

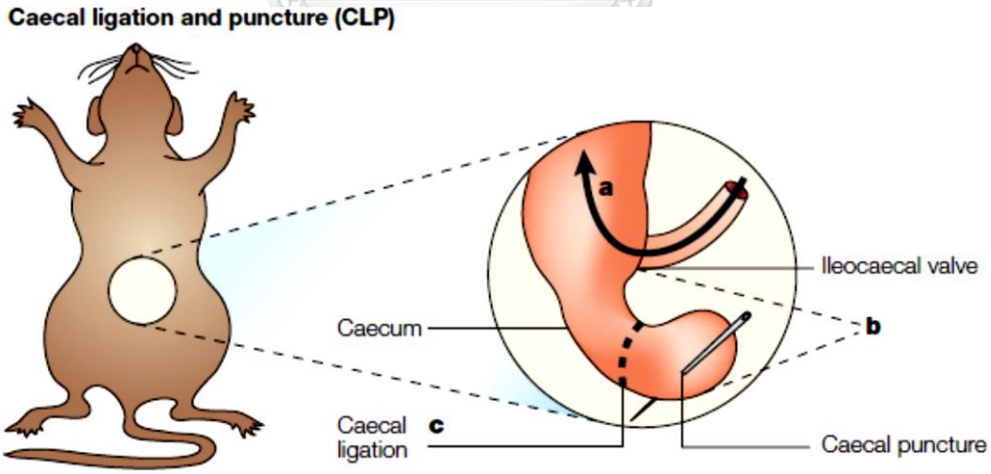

Figure 5. The Cecal ligation and puncture model (79).

FcGRIIb deficient mouse and the susceptibility to the infection

Several studies had been done on this topic with limited number of literatures. As show in table 3, most of the studies demonstrates that FcGRIIb deficiency reduce the severity of infection. However, there is no study on gram negative and fungal infection in the list. Then, this research will performed experiment on polymicrobial sepsis with CLP model after the 2nd LPS injection for endotoxin tolerance (Table 2).

Pathogen	Result	reference
Plasmodium spp.	Reduced susceptibility	*Clatworthy MR, et al. (2007) PNAS 104:7169– 74* (68)
S. pneumoniae	Increase severity after stimulating by Streptococcal vaccine	*Clatworthy, et al. (2004) J. Exp. Med. 199, 717-23* (7)
M. tuberculosis	Reduced susceptibility	*Maglione, et al. (2008). J. Immunol. 180, 3329–38* (8)

Table 2. Conclusion of the previous studies on FcGRIIb -/- mice and infection (7, 8, 68, 80).

FcGRIIb-/- mice and macrophages susceptible to endotoxin tolerance and bacterial sepsis

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We previously report that pro-inflammatory cytokines (such as TNF-α, IL-6) of FcGRIIb -/- mice and macrophages are hyper-responsive to once LPS stimulation but highly exhausted after the 2nd LPS stimulation (20). Interestingly, the prominent IL-6 of serum mice from LPS stimulation in FcGRIIb-/- over wild-type after once LPS stimulation and more exhaust after 2nd LPS stimulation, *in vivo*. Macrophage endotoxin tolerance evaluated in cytokine responses IL-6 in supernatant media from macrophages of FcGRIIb-/- or wild-type (FcGRIIb +/+) after activation, and the difference between a single high dose (N/LPS100) versus twice high dose of LPS (LPS100/100) were demonstrated. The prominent IL-6 of supernatant macrophages from LPS stimulation in FcGRIIb-/- over wild-type after once LPS stimulation and more

exhaust after 2nd LPS stimulation, *in vitro* (20). More interestingly, twice LPS stimulation in FcGRIIb-/- mice shows more susceptible to bacterial sepsis induced by cecal ligation and puncture (CLP) procedure (20, 78).

Phosphoproteomics and cell signaling study

We previously demonstrated the important role of FcGRIIb-/- mice and macrophages which is susceptible to endotoxin tolerance and bacterial sepsis (81) . The high throughput proteomics is the new tools to study the correlation among the different protein receptors/adaptors in the easier, less time consuming method. Instead of one molecule to one or few molecules correlation of the signaling, nearly all of the protein alteration detected and use the bioinformatic method to link the possible correlation. This allows us to see the correlation among several signaling and possible to select the interesting correlation for the further studies. The understanding in such correlation should lead to the prediction of the key pathway responsible for sepsis severity in patients and the intervention as the proper therapeutic strategies. The large scale identification of the phosphorylated proteins with the advance bio-informatic methods make it possible to predict the correlation of the signaling protein in the diagram (82, 83).

Protein phosphorylation and phosphoproteomics

Protein phosphorylation is one of the most important post translational modifications (PTMs) accountable to regulating many biological processes along with signal transduction, metabolism, protein synthesis, transcriptional and translational regulation, proliferation, differentiation, and apoptosis (84, 85). This reversible process is controlled tightly by 2 types of enzymes which are kinases and phosphatases in response to inducement from both inside and outside of the cells. The kinase catalyzes a high energy organic compound such as ATP (adenosine triphosphate) and GTP (guanosine triphosphate) to donate phosphate group to side chain of certain amino acid residues (tyrosine, serine or threonine in mammalian). There are 518 kinases in human kinome. Moreover, it has been estimated that phosphotyrosine (pTyr) is relative low abundance (1.8%) compared to phosphoserine (pSer, 86.4%) and phosphothreonine (pThr, 11.8%). Because of polarization and hydrophilic properties

of phosphate group, the phosphorylated proteins change their conformation leading to activation, deactivation or change their interaction with the other proteins. On the other hand, protein phosphatase remove phosphate group from phosphoprotein by hydrolyzing phosphoester bond. This process is also known as dephosphorylation. Most of mechanisms in signal transduction involve in this PTM; therefore, study of phosphoproteins may gain knowledge more than those studies on protein expression (85, 86).

Many methods are used to detect phosphoproteins. The phosphorylated protein can be detected by technique western blotting (WB) using antibodies against general pSer, pThr, or pTyr residues. Although phosphoproteins can be detected by WB using phosphosite specific antibodies or can be visualized by 1D-gels and 2D-gels using phosphospecific staining or autoradioactive labeling, it is difficult to identify phosphorylation sites and novel phosphoproteins. The large scale analysis by mass spectrometer is applied in study phosphorylations. Moreover, the instruments of mass spectrometer have been developed in improve their resolution and accuracy resulting in detecting greater than 20,000 phosphorylation sites in one experiment. However, phosphoproteins are generally low abundance and an ambitious for phosphoproteomics (87-90).

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Phosphopeptide enrichment from Titanium dioxide chromatography (TiO2)

In fact, phosphopeptides present in a few proportion along with lower ionization efficiency of phosphopeptides. Therefore, these need to be enriched before using mass spectrometer analysis. Interestingly, titanium dioxide chromatography (TiO2) is the one technique which has very high affinity, selectivity, chemical stability and rigidity properties to phosphopeptides. In recent years, this has become one of the most strategical popular methods of the phosphopeptide enrichment from complicated biological samples. This strategical method, the peptides in acidified buffer are loaded into packed TiO_2 column and eluted by an alkaline buffer. Nevertheless, loading solution with low pH, to promote binding phosphopeptides into a partisphere column, and also maintain nonphosphorylated acidic peptides. To improve upon specificity of TiO_2, used very acidic buffer by adding 2,5-

dihydroxybenzoic acid (DHB), phthalic acid or glycolic acid into loading buffer for reduce non-phosphorylated containing acidic amino acid residues binding to resin. Moreover, the phosphopeptides are more efficiently eluted from a resin after using high pH elution buffer in pH at11.3 (87, 90).

Quantitative proteomics using stable isotope dimethyl labeling

Mass spectrometer is the potent tool for detective protein identification. Nevertheless, it has not provided a quantitative information from the peptide intensities. Due to the efficiency of ionization, generated signals from mass spectrometer are variable from peptide to peptide. The quantitation strategies are used stable isotope labeling provide qualitative from protein identification and quantitative information. Currently, these techniques are presented in the phosphoproteomics field in order to determine the changes of phosphorylation levels of different cellular states. The stable isotope labeling allows samples more than two different states which be analyzed in a single run. Chemical and physical properties of one isotope labeled peptides are identical the other isotope labeled peptides. Moreover, the differences in mass are showed by isotope labeling that can be distinguished by mass spectrometer. However, the relative intensities of different isotopes generated by mass spectrometer reflect the relative quantity of the peptides in different states each as samples in experiment (87, 88, 91) .

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Interestingly, stable isotope dimethyl labeling is a very simple, reliable, fast and inexpensive quantitative proteomics method (92). In this strategy, the principle of this technique is reductive amination. The formaldehyde interacts with **ε** amino group of side chain of lysine residue or the N-terminus of a peptide in primary amine to form Schiff base. Moreover, this intermediates are subsequently reduced by sodium cyanoborohydride to form a secondary amine and convert to dimethyl amines which shown on the figure 6.

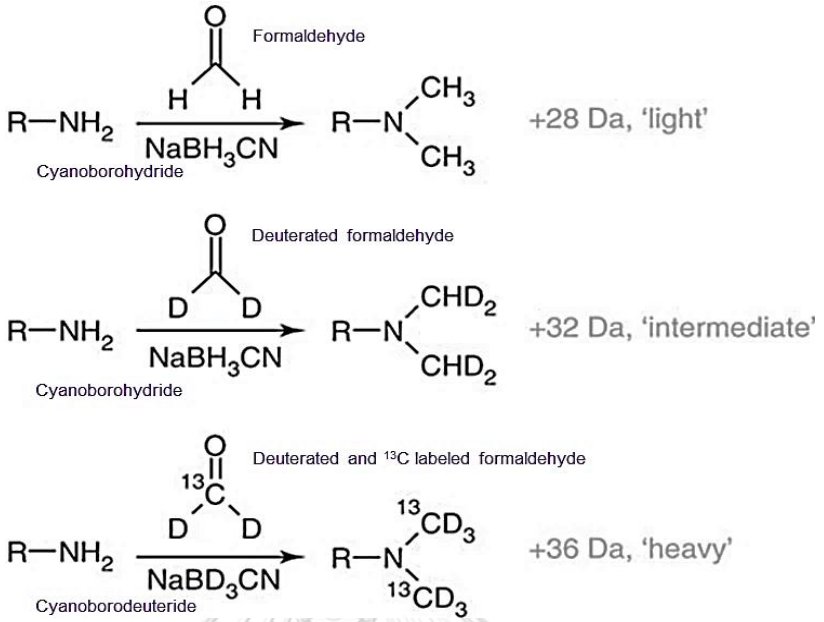

Figure 6. This the pictures show reaction of dimethyl labeling.
They are reduced by sodium cyanoborohydride to form a secondary amine and convert to dimethyl amines (93).

Moreover, stable isotope dimethyl labeling, multiplex at least 3 labeling, can be generated by different combinations of isotopomers of formaldehyde and cyanoborohydride such as $CH_2O/NaBH_3CN$ (light), $CD_2O/NaBH_3CN$ (medium) and $^{13}CD_2O/NaBD_3CN$ (heavy). Firstly, the combinations can generate with mass increase of 28 Da while the medium and heavy labeling generate a mass increase at 32 Da and 36 Da, respectively. However, the labeled peptide differ from mass with only at 4 Da between light and medium labeling and between medium and heavy labeling whereas light and heavy labeling have mass difference at 8 Da.(90-94) .

Interestingly, the previous study show that Protein kinase C regulates macrophage tumor necrosis factor secretion and direct activation restores TNF-**α** production endotoxin tolerance by phorbol myristate acetate (PMA) (95). Recently, our

study show that the endotoxin tolerance of protein kinase C -β type II was selected as an interesting protein because it have main cytokine control function and still lack of the exploration in the current literatures as demonstrated by macrophage phosphoproteomic analysis(20, 96).

Protein Kinase C structure and function

One of all the kinase families is protein kinase C (PKC) of protein serine or threonine kinase centrally involved in intracellular signal transduction. PKC isoforms have divided in three major subfamilies depend on their stimulation requirements. Three PKC isoforms are 1.) conventional isoforms (cPKC): PKC-α,-βI, -βII, and -γ, require diacylglycerol, calcium and phosphatidylserine; 2.) novel isoforms (nPKC): PKC-δ, ,-η, –θ and-ε require diacylglycerol and phosphatidylserine but are calcium independent; and 3.) final isoforms, atypical isoforms (aPKC): PKCζ and λ/ι, require just only phosphatidylserine (97, 98). which shown on the figure 6. The PKC isoforms have different, and some conflicting, effect on this disease states. Moreover, different PKC isoforms are associated with such important role functions as differentiation, cell growth, cell survival, secretion, apoptosis and motility. Consequently, this enzymes can have been implicated of numerous disease states such as plethora of diseases, cancer in different organs (99-101) , including cardiovascular disease (98, 102-105). PKC of roles on cancer are complicated with often opposing, a tissue specific, effects on a different isoforms in apoptosis and cell cycle. Moreover, absence and /or dysregulation of PKC may be lead to different pathologies, heart failure (106, 107), allergy (108, 109), diabetes (110, 111), Parkinson, Alzheimer (112, 113), inflammatory diseases (114, 115), including some of autoimmune diseases (116-119). Likewise, PKC of roles on heart diseases are complexity because elements of the disease such as cardiac function, myocyte hypertrophy, fibrosis and inflammation which are affected of different ways from a different isoforms (120).

The previous study show that PKC inhibitors can reduce LPS activated cytokines secretion with macrophages and linking PKC stimulation in TLR signaling (Figure 7). The

great number of studies have shown PKC inhibition or their reduction with long term treatment by phorbol esters, reduce LPS activated cytokine secretions (95, 121-123). Consequently, acute stimulation on PKC by phorbol esters increase cytokine secretion (95, 122, 124, 125). Current research has shown multiple steps of TLR pathways which directly involved with PKC-α, -ε, -ζ and -δ. Beginning of evidence in the PKC involvement for TLR signaling become from observations which modify PKC activity within cells from the innate immune systems affected on cytokine secretions. Toll like receptors (TLRs) are the pattern recognition family receptors which bind pathogens and activate a secretion of cytokines. Their PKC involved with receptor complex such TLR or proximal components. These isoforms are involved in a downstream stimulation in MAPK, RhoA, TAK1, including NF-**K**B. Therefore, PKC stimulation are closely associate with the innate immune responses and TLR signaling. Accordingly, LPS and other TLR ligands were shown that stimulated most of the PKC isoforms expression of neutrophils, dendritic cells, monocytes and macrophages (121, 124, 126-128).

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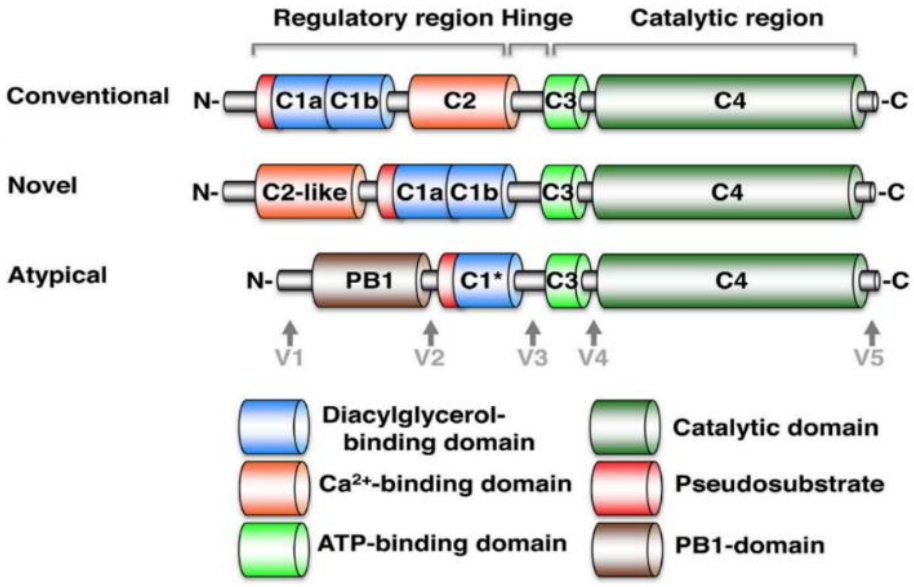

PKC Subfamily	PKC Isoforms	Activators
Conventional	PKCα, βI, βII, γ	Diacylglycerol, lipid, Ca^{2+}
Novel	PKCδ, ε, η, θ	Diacylglycerol, lipid
Atypical	PKCγζ, ι/λ,	PIP_3, Par6-CDC42

Figure 7. The explanatory diagram of the structure in mammalian protein kinase C (PKC) family. The mammalian of PKC isoforms are divided in 3 functionally and structurally difference subfamilies according to their regulatory domains, including 1.) a conventional (classical) isoforms (cPKC), 2.) novel isoforms (nPKC), and 3.) atypical isoforms (aPKC). Carboxy terminus of all isoforms involves the catalytic and ATP binding domain which are linked via the hinge region (V3) into a membrane targeting amino terminal regulatory region. The pseudosubstrate motif within an amino terminus are similar to an optimal PKC sequenceing substrates but lacks the serine/threonine phosphoacceptor residues. For resting state, they interacts by the substrate binding crevice in a catalytic domain, thereby blocking they access to potential substrates and inhibiting their catalytic activity. Inhibition of a catalytic domain have relieved upon recruitment of an enzymes into the plasma membrane, where their 2 distinct regulatory elements interact by in membrane constituents. These include diacylglycerol (DAG) binding tandem C1 domains in the cPKC and nPKC, a Ca2+-dependent phospholipidbinding C2 domain in the cPKC and nPKC, and a partitioning defective 6 (Par6)-CDC42 binding Phox/Bem 1 (PB1) domain in the aPKC. The single C1 (C1*) domain in the aPKC doesn't bind DAG, and the C2- ike (C2*) domain in nPKC doesn't bind Ca2+ or phospholipids. For the aPKC PB1 is a protein-protein binding domain which mediates hetero-dimerization or homo-oligomerization. In mouse PKC**λ** and human PKC**ι** are orthologs had 98% amino acid sequence identity and referred to as PKC**ι/λ**(107).

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Protein kinase C-β type II function and activity

A study performed in human tumor colon by Dowling et al. (129) provided a mechanism of explanation for the PKCβII role in tumor suppression, and activation on PKCβII demonstrated the downregulation of signals induced with IGF-1, a critical regulator of cell survival and a major player on colorectal carcinogenesis. PKC expression of cancer and normal tissue samples from tumor colon in patients revealed to the significant and selective decrease on PKCβII in tumor colon cells. Tissue microarray analysis substantiated the findings and provided a decrease of protein PKCβII expression in the both of stroma and epithelia of the tissue disease. In addition, the direction of correlation was demonstrated between an expression level of PKCβII in the normal tissue in colon tumor patients and the patients' 10 years for survival rates. The tumor suppressive activity of PKCβII was proved by the HCT116 colorectal carcinoma cell line. Then, overexpression PKCβII gene led to downregulation in colony forming ability and inhibition of invasion and migration in the cancer cells in *in vitro* assays. These study show that target of PKC of cancer patients by a variety of PKC inhibitory drugs may be counteraction and which in the future therapies should be consider focusing of restoring rather than inhibitor by PKC activity. However, studies in the future are required clearly whether a suppressor activity of PKC is the general phenomenon of all types in cancers and the ability to suppress cancer growth are a common trait which is shared by all or just only a selected PKC isoforms group(107).

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CHAPTER IV
MATERIALS AND METHODS

Animal and animal models

The FcgRIIb -/- mice on C57BL/6 background were provided by Dr. Silvia Bolland for our research unit due to the connection with the National institute of health (NIH), Maryland, USA. Other mice, wild type mice were purchased from the National Laboratory Animal Center, Nakhon Pathom Province, Thailand. Female, 8-week-old C57BL/6 mice were used in the experiments and following the procedures approved by the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand in accordance with the National Institutes of Health (NIH) criteria (SST 002/2559, May 2016)(96).

Salting out procedure for extracting DNA

Lyse the mouse tail in 500 ul of lysis solution, added 20 ul of Proteinase K (20 mg/ml). Incubate at least 4-6 hours at 55 °C (Optional; Add 250 ul of saturated Nacl, shake well for 10 secs and let it stand for 15 min on ice). Centrifuge 10 to 15 min at maximum speeded, room temperature. Transfer the clear DNA solution (supernatant) into a clean tube and precipitate it with 750 ul of ethanol. Pellet gently by spinning at room temperature in microfuge setting for 1 min. After then, washed the DNA precipitate with 75% ethanol and air dry for a few minutes. Dissolve it in 100 ul TE. DNA could put into solution rapidly by soaking tubes at 55 C for 15 min. Then, genotyping was confirmed from this DNA extraction in next step.

Confirmed molecular genotyping

The animals were genotyped by PCR using primers design to distinguish the targeted allele from the wild-type by the sizes of the reaction products. FcgRIIb -/- mice genotyping was confirmed as previous described by PCR (130). Accordingly, The DNA was isolated from fetal tail tips. Each 50 μL PCR reaction contained 100 ng sample template DNA, 200 μM dNTPs, 1× PCR buffer with 1.5 mM MgCl2, and 1 unit of Taq

DNA polymerase. The third primers in this study were used concentrations in each as 10 uM following primers of FcREC1: 5'AAAGGCTG TGGTCAAACTCGAGCC-3', OL4143: 5' CTCGTGCTTTACGGTATCGCC-3' and OL4080: 5'TTGACT GTGG CC TTAAACGT GTAG-3'. Thermocycler conditions were one cycle of preliminary denaturation at 98°C for 3 min, followed by 35 cycles of denaturation at 98°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min. The final amplification one cycle included an addition of at 72°C for 5 min and stores in 4 °C for forever until use. The PCR products were resolved on agarose gels and stained with ethidium bromide. In accordance with above, wild type allele and FcgRIIb -/- targeted allele were generated at 173 bp and 232 bp respectively (130). The animals were confirmed molecular genotyping before start experiments in all times.

Endotoxin-tolerance model and serum cytokine analysis

To explore if there was the difference in immune exhaustion between FcGRIIb-/- mice and wild-types, endotoxin-tolerance model was performed in both groups. Endotoxin (LPS) of *Escherichia coli* 026:B6 (Sigma-Aldrich, St Louis, Mo) was administered intraperitoneally in two separate doses at 0.8 mg/ kg (day 0) and 4 mg/kg at 5 days later (day 5). To measure the time-course of serum cytokine concentrations, 50ml of blood was collected through tail vein nicking. To see if there was hyperimmune response after the first dose of LPS and become exhausted after the second dose, serum cytokines were measured by a Luminex-based multiplex technology multi-analysis panel 8-plex cytokine assay (four pro-inflammatory cytokines; TNF-a, IL-6, IL-1β, IFN-γ, and four anti-inflammatory cytokines; IL-2, IL-4, IL-5, IL-10) (Bioplex, Bio-RAD, Hercules, Calif). Additionally, area under the curve of cytokine response (AUC-CR) after each time of LPS stimulation was calculated to determine the degree of endotoxin tolerance. Then the percentage of the ratio of AUC-CR after second LPS stimulation/ AUC-CR after first LPS administration were used to stratify the degree of endotoxin tolerance into low and high degree with the ratio ≥50% and <50%, respectively. The lower of the AUC-CR ratio (lower cytokines in the

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second response compared with the first response) represented the higher degree of the endotoxin tolerance(20).

Cecal ligation and puncture with LPS-preconditioning model and serum analysis

Two separate doses of LPS were used as LPS-preconditioning for the induction of endotoxin tolerance as mentioned above. Then CLP was done at 12 h after the second dose of LPS to see the impact of endotoxin tolerance to bacterial infection. In parallel, the same volume of normal saline (NSS) in two separate doses was administered in the same schedule as the control group (CLP without LPS preconditioning). CLP procedures were slightly modified from the previous publication (77, 131). Briefly, cecum was ligated at 10mm from cecal tip with silk 2-0, punctured twice with a 21-gauge needle through an abdominal incision under isoflurane anesthesia. NSS at 2mL/kg was administered subcutaneously for the fluid replacement at post-operation. Then serum parameters were explored only in CLP with LPS preconditioning but not CLP without LPS administration due to the scope of the interest. To measure time-course parameters of CLP with LPS-preconditioning model, 50mL of blood was collected through tail vein nicking at the indicated time-point. Blood was also collected at the time of sacrifice through cardiac puncture under isoflurane anesthesia. Then, the following serum parameters were explored at 18 h of CLP with LPS preconditioning in FcGRIIb-/- and wild-type mice. Blood bacterial burdens were determined by bacterial colony enumeration in blood agar (Oxoid, Hampshire, UK) after incubation at 378C for 24 h. Blood polymorphonuclear cell (PMN) and mononuclear cell counts were performed by a previously published method (17). Serum cytokines (TNF-α, IL-6, and IL-10) after CLP surgery were measured by ELISA assay (ReproTech, NJ). Serum creatinine (Scr) (QuantiChrom Creatinine Assay, DICT-500, BioAssay) and alanine transaminase (ALT) (EnzyChrom ALT assay, EALT-100, BioAssay) were measured for kidney and liver injury, respectively.

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Bone Marrow Derived Macrophages

Macrophages were derived from bone marrows (BM) follow the established procedure (20, 132). In short, BM cells from FcGRIIb-/- and wild type mice obtained from femurs are centrifuged at 1,000 rpm in 4 °C for 10 min. Then, total cell were incubated in high glucose DMEM supplement with 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin, HEPES with sodium pyruvate and 20% L929-conditioned media in a humidified 5% CO_2 incubator at 37 °C for 7 days. The fresh medium were added up to the cell cultures at day 4 from 7 days. The cells were harvested at the end of the culture period using very cold PBS and then confirmed macrophages morphology with 1.5 x10 ^{3} cells/ slide by cytospin centrifuge at 600 rpm in 37 °C for 5 min (Shondon cytospin@4 cytocentrifuge, Thermo electron corporation,USA)and stained with Wright's stain (Sigma Aldrich, St. Louis, USA). After that, macrophage phenotypes were confirmed macrophage markers with anti-F4/80 and anti-CD11c antibodies (BioLegend, CA, USA) by flow cytometry. The aforementioned flow cytometry, cells were washed by cold PBS once. Then cells were re-suspended in 200 ul staining buffer (FACS staining buffer; 1x PBS and 1% (v/v) Fetal Calf Serum or FBS), and fluorochrome-conjugated antibody was added to the cell suspension (1ul CD11b PE (FL2) or 1 ul F4/80 Biotin and add 10 ul Strepavidin ECD (FL3)/sample each as 30 min) in the dark. Then, cells were washed by 3x in staining buffer and directly analyses on flow cytometer.

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Macrophage endotoxin tolerance to culture supernatant cytokine analysis and cell viability assay

The macrophage endotoxin tolerance protocol followed a previously published method (133). Briefly, endotoxin (LPS) *Escherichia coli* 026:B6 (Sigma) at 10 or 100 or 1,000 ng/mL in 100ml/well were used to activate macrophage at a density of 1x10^5 cells/well in 96 well polystyrene tissue culture plate. To observe the difference between single or double sequential LPS stimulations, 2 groups of experiments were performed. For the single LPS stimulation (N/LPS), there were no endotoxin at the 1st 24h of the incubation, the plate is washed with phosphate buffer

solution (PBS), refilled fresh media and treated with LPS at 100 ng/ml (N/LPS100). For the double LPS stimulation (LPS/LPS), LPS at 100 ng/ml were treated for the 1st 24h and treated with the 2nd dose of LPS at 100ng/ml. The culture supernatant was collected at 1, 2, 4, 6 and 24h after the 2nd LPS incubation in all groups and stored at -80 ℃ until cytokine (proinflammatory cytokines; TNF-α, IL-6, and 4 anti-inflammatory cytokines; IL-10) determination by ELISA assays (eBioscience) according to the kit manufacturers' instructions. After the incubation, cell viability was measured by MTS assay based on the formation of blue formazan metabolized from colorless MTS in the CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay Kit (One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) according to the manufacturer's instruction (134). In short, 20 μl of MTS was added to the culture plates for 2h at 37°C in a humidified, in 5% CO2 atmosphere incubator and then read the amount of soluble formazan produced by cellular reduction of MTS, proceed immediately. Record the absorbance with microplate photometers with a wavelength at 450 nm using a 96-well plate reader. In addition, AUC-CR measured from culture supernatant after LPS stimulation in a single dose (N/LPS) and in two separated doses (LPS/LPS) was analyzed for the degree of macrophage endotoxin tolerance. The degree, determined by the percentage of the ratio of AUC-CR in supernatant after two doses of LPS (LPS/LPS)/AUC-CR of culture supernatant after a single LPS (N/LPS), was stratified into no tolerance, low and high degree with the ratio of ≥100%, ≥50%, and<50%, respectively. The lower ofthe AUCCR ratio (lower cytokine after two doses of LPS compared with the level after a single LPS activation) represented the higher degree of endotoxin tolerance. The ratio of AUC-CR was calculated from LPS stimulation in low dose [AUC-CR (LPS10/10)/AUC-CR (N/LPS10)] and high dose [AUC-CR (LPS100/100)/AUCCR (N/LPS100)].

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Phagocytosis endotoxin tolerance with bacterial FITC -dextran labelling

A phagocytic activity assay was performed following a previous study (135). Briefly, 200 µg/ml of zymosan conjugated with 40 kDa fluorescein isothiocyanate dextran (FITC-dextran) (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells, which were then incubated for 1 h at 37 °C in 5% CO2. Following this, the extracellular fluorescence and non-ingested FITC-dextran were quenched and removed by adding trypan Blue and vigorous washing with phosphate buffer solution (PBS), respectively. The fluorescent positive cells representing FITC-phagocytized cells were analyzed using a fluorescent intensity reader at 492 nm excitation and 518 nm emission wavelengths (Verioskan Flash microplate reader, Thermo-Scientific, Rockford, IL, USA) and an Olympus IX81 inverted fluorescence microscope (Tokyo, Japan). Macrophage microbicidal activity was also measured, as per previously described protocol [10], with the incubation of a 1×10^7 colony forming unit (CFU) of *E. coli* (American Type Culture Collection, ATCC, Manassas, VA, USA) in BMM at 1×10^5 cell/ well (96-well plate) using 25 µL of normal mouse serum as an opsonin. Then, the non-phagocytized bacteria were removed by washing and incubated further for 1 h with 100 µL of gentamicin 100 µg/mL to eradicate viable extracellular bacteria. Subsequently, the plate was washed and induced cell lysis by 200 µL of sterile water/well, before being plated on trypticase soy agar in the serial dilution. Bacterial count was evaluated after 24 h of 37 °C incubation.

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Killing endotoxin tolerance

The protocol followed a previously published method (136). In brief, $1x10^5$ cells of BM derived macrophages were plated in 96-well plate. The LPS-stimulation regimens were performed as described above in step 8.3. Then $1x\ 10^7$CFU of *E. Coli* were added to each well in 25 µl of normal mouse serum (added as an opsonin) and incubated for 15 min followed by washing to remove non-phagocytized microorganisms. Of note, normal mouse serum were pooled from several mice and kept at -80°C and were used for all experiments. Subsequently, gentamicin 100 µg/ml at 100 µl were added for 1h to eradicate the viable extracellular bacteria. After then

the plate was washed, gently scraped and cell lysis will be induced with 200 µl of distilled water. Serial dilutions of the lysate were streaked on tryptic soy agar, incubated at 37 °C for 16 h to determine bacterial colony count. The number of bacteria from the cell lysate were inversely associated with the intracellular killing activity.

Phosphoproteomics and dimethyl labeling

Macrophage culture and in solution digestion

BM derived macrophages ($1x10^7$ cells) were plated in 12-well plate. The endotoxin (LPS) *E. Coli* 026:B6 (Sigma) at 100 ng/ml stimulation regimens are performed as described above in step 8.3. Then, all cells in 5% deoxycholic acid sodium salt (SDC, Merck) (protease inhibitor and phosphatase inhibitor) were sonicated. Then 300 micrograms of total proteins from each as sample were mixed with dithiothreitol (DTT, GE Healtcare) at a final concentration of 10 mM for 30 minutes at 37°C. Subsequently, iodoacetamide (IA, Sigma) was added to alkylate the samples for 30 minutes at room temperature in the dark. After further quenching the reaction with 40 mM DTT, the samples were diluted with 25 mM triethylammonium bicarbonate (TEAB, Sigma) to reduce the concentration of SDC from 5% to 0.5% prior to trypsin digestion which used ratio 1:50 of trypsin: protein for 16 h at 37°C. Trifluoroacetic acid (TFA, Merck) were used to stop the reaction and remove SDC from the digested peptides. The concentration of peptides were determined by peptide quantification kit (Thermo SCIENTIFIC) as manufacturer's instruction. After that, the peptides were adjusted into an equal amount and air dried.

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In-solution dimethyl labeling

The dried peptides were redissolved in 100 µl of 100 mM TEAB. The sample was mixed with 15 µl of 4% (v/v) formaldehyde isotype of CH_2O 37% (light dimethyl labeling) for wild type cell labeling and formaldehyde isotype of CD_2O 20% (intermediate dimethyl labeling) for knockout cell labeling, respectively. Then, 15 µl of 0.6 M of sodium cyanoborohydride ($NaBH_3CN$) was added. These mixtures were

allowed to react with gentle agitation for an hour. Thirty microliter of 1% (v/v) ammonia solution was used to quench the reaction and these samples were acidified with formic acid (FA, Sigma). Each sample was tested its labeling efficiency by mass spectrometer before combination.

Phosphopeptide enrichment and fractionation

The dimethylated peptides of different labeling was combined in 1:1 ratio and fractionated by high pH reversed phase peptide fractionation kit (Thermo SCIENTIFIC) according to instruction manual to reduce the complexity of the samples. Phosphopeptides were had low abundance and enriched from the samples using titanium dioxide (TiO_2). Firstly, acetonitrile equilibrated titanium dioxide microspheres are applied into C8 ziptip. After that, the column was equilibrated with loading buffer (1M glycolic acid, 80% acetonitrile and 5% TFA) and the fractionated peptides were slowly applied into the column followed by washing solution [loading buffer with washing buffer (80% acetonitrile and 5% TFA)]. The phosphopeptides were eluted by elution buffer (0.5% Ammonium solution) and dried in vacuo. Finally, the elutes from each fraction were dried in a SpeedVac centrifuge before LC-MS/MS analysis.

Proteomic analysis by Orbitrap LC-MS/MS

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The phosphopeptides were resuspended with 20 µl of 0.1% FA. Ten microliter of each sample was analyzed on an EASY-nLC1000 1100 system coupled to Q Exactive Orbitrap Plus mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nano-electrospray ion source. The ten most intense ions are sequentially isolated and fragmented in the higher energy collision induced dissociation cell. The MS methods included a full MS scan at a resolution of 70,000, followed by 10 data-dependent MS2 scans at a resolution of 17,500. The normalized collision energy of HCD fragmentation was set at 30%. An MS scan range of 400 to 1600 m/z was selected and precursor ions with unassigned charge states, a charge state of +1, or a charge state of greater than +8, were excluded.

Quantitative data analysis and bioinformatics

A dynamic exclusion of 30 s was used. Using Maxquant Software (version 1.5.1.2), the MS raw data files were searched against a composite database containing the forward and reversed peptide sequences of the Mouse Uniprot Database. The search parameters were set up for the following fixed modifications: carbamidomethylation of cysteine (+57.02146 Da), as well as light and medium dimethylation of N-termini and lysine (+28.031300 and +32.056407 Da). For variable modification, the oxidation of methionine (+15.99491 Da) and phosphorylation of serine, threonine, and tyrosine were set. A maximum of four modifications and two missed cleavages per peptide were allowed. Parent and fragment mono-isotopic mass errors were set at 10 and 0.2 ppm, respectively. A target–decoy approach was used to limit the false discovery rate of the identified peptides to less than 1%. The wild-type mice were used as denominators to generate ratios of (FcGRIIb−/−)/(wild-type) mice. Significantly differentially regulated-proteins were determined by unpaired t-tests with a p-value < 0.05. In addition, Gene ontology analysis (GO) and functional enrichment of the significance of proteins in the molecular function, biological process, and cellular component categories were performed with(http://david. abcc.ncifcrf.gov/) (137) and the Protein Analysis through Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org) (138). The Kyoto encyclopedia of genes and genomes (KEGG) PATHWAY (http://www.genome. jp/kegg) (139) was used to perform signaling pathway enrichment analysis with $p < 0.05$ as a cut-off criterion.

Western Blot Analysis

BMM or RAW264.7 cells were pelleted and washed with 1× PBS. Once cells were pelleted, the supernatants were removed. The cells were re-suspended and lysed in RIPA lysis buffer, 1× protease inhibitors, and 1× phosphatase inhibitors (Thermo-Scientific Rockford, IL, USA), and spleens were homogenized for PRKCB detection. The cell mixture or spleen preparations were sonicated on ice and then centrifuged at 1500× g at 4 °C and the protein quantification was performed by the BCA assay (Pierce BCA Protein Assay, Thermo Scientific). The protein (20 μg) was separated in 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to a

nitrocellulose membrane, detected by Phospho-PRKCB (Thr641) primary antibodies (Thermo-Scientific Rockford, IL, USA), probed with a rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology), and detected by an enhanced chemiluminescence rapid step chemiluminescence detection system (Thermo-Scientific Rockford, IL, USA). The rabbit monoclonal antibodies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (Cell Signaling) were used as the housekeeping genes for the preparations of cells and spleens, respectively.

Statistical analysis

The data were shown as the mean ± SE and differences between groups are examined for statistical significance using the unpaired Student t-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with 2 and 3 groups, respectively. The repeated measures analysis of variance with Bonferroni post hoc analysis for the analysis of data with two groups and the time-course experiments, respectively. Survival analyses were evaluated using the log-rank test by observation and recorded every 6 to 24 h and then all mice were sacrificed at 96 h after CLP. *P* values < 0.05 are considered statistically significant. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis. In addition, the AUC of cytokine response to LPS stimulation was calculated with Prism4.0, Graphpad Software and compared between the mouse strains with the unpaired Student t test (SPSS).

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CHAPTER V
RESULTS

Endotoxin tolerance observed in both FcGRIIb-/- and wild-type mice

At 8 weeks, FcGRIIb-/- mice had normal serum creatinine with neither proteinuria nor anti-dsDNA, which was consistent with other studies (7, 67). Cytokine responses were explored with the Luminex-based multiplex system after single or sequential LPS challenge in both wild-type and FcGRIIb-/- mice. After the first dose of LPS, most pro-inflammatory and anti-inflammatory cytokines were significantly higher in FcGRIIb-/- mice, especially after the first hour postadministration (Figure 8). These results are consistent with the prominent cytokine responses in FcGRIIb-/- mice reported previously (7). Interestingly, after the second LPS administration with a five times higher dose of LPS, cytokines of FcGRIIb-/- mice were significantly less than the wild-types only with TNF-a and IL-6, and that difference diminished after the first time-point (Figure 8). Nevertheless, the degree of cytokine decrease in the knockout animals was significantly more than observed in the wildtypes (Figure 8, inset). The alteration of cytokine levels (cytokines levels after first LPS dose minus the levels after second dose, at the matched-time-points) was more prominent in FcGRIIb-/- mice in all cytokines except IL-5 (Figure 8, inset). Subsequently, the ratio of the AUC-CR after each LPS stimulation was used to determine the degree of endotoxin tolerance (see the Methods).

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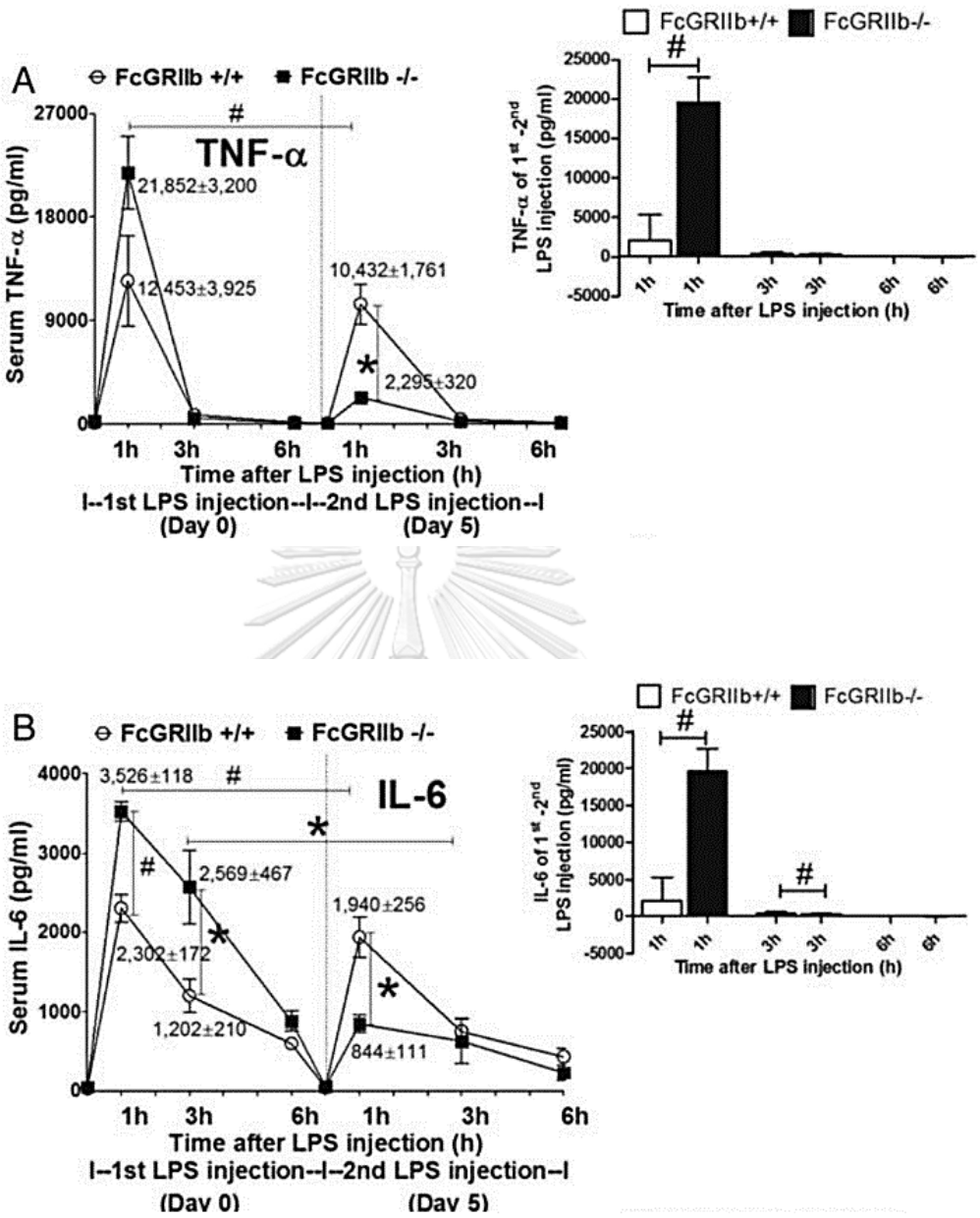
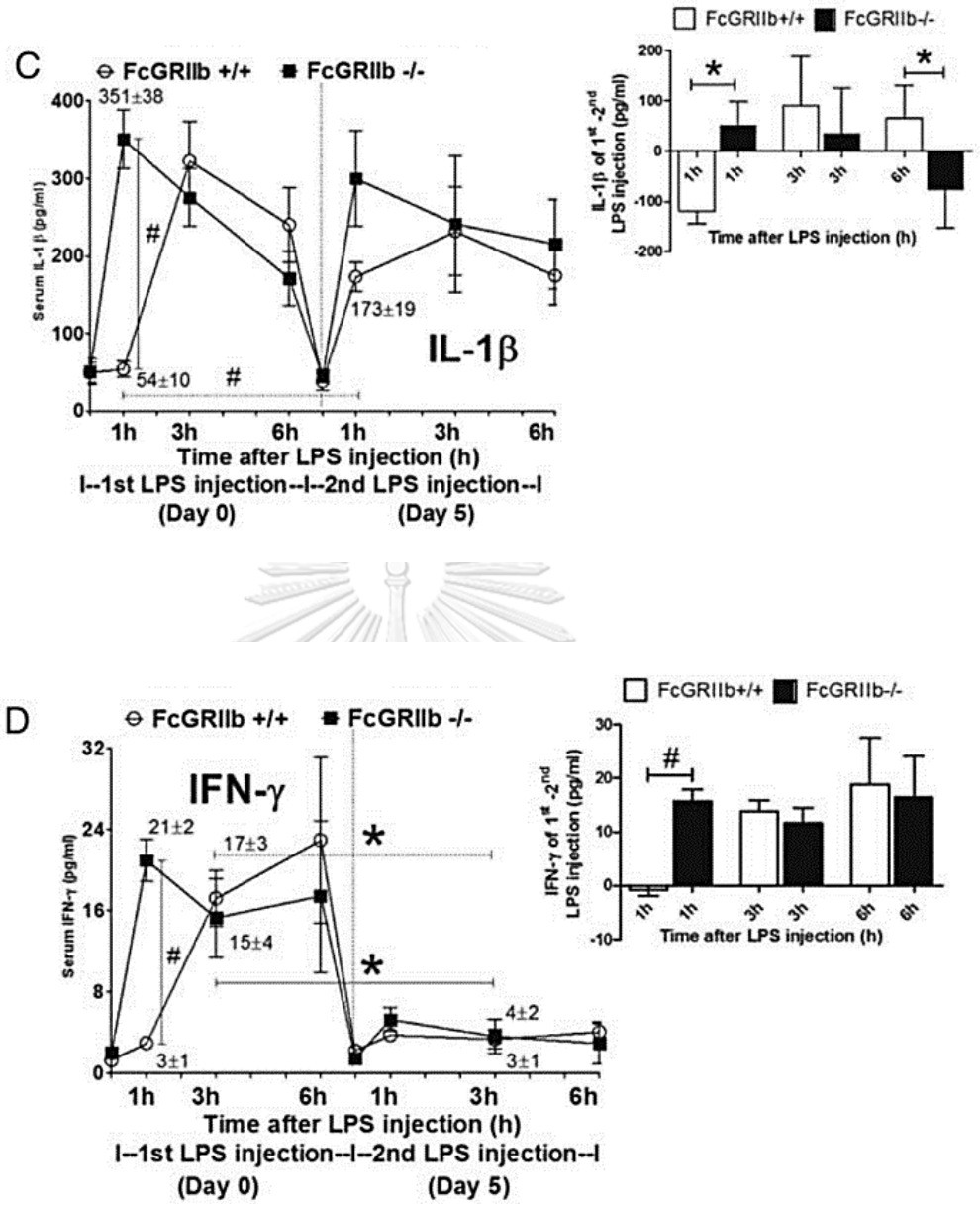


Figure 8. Cont.



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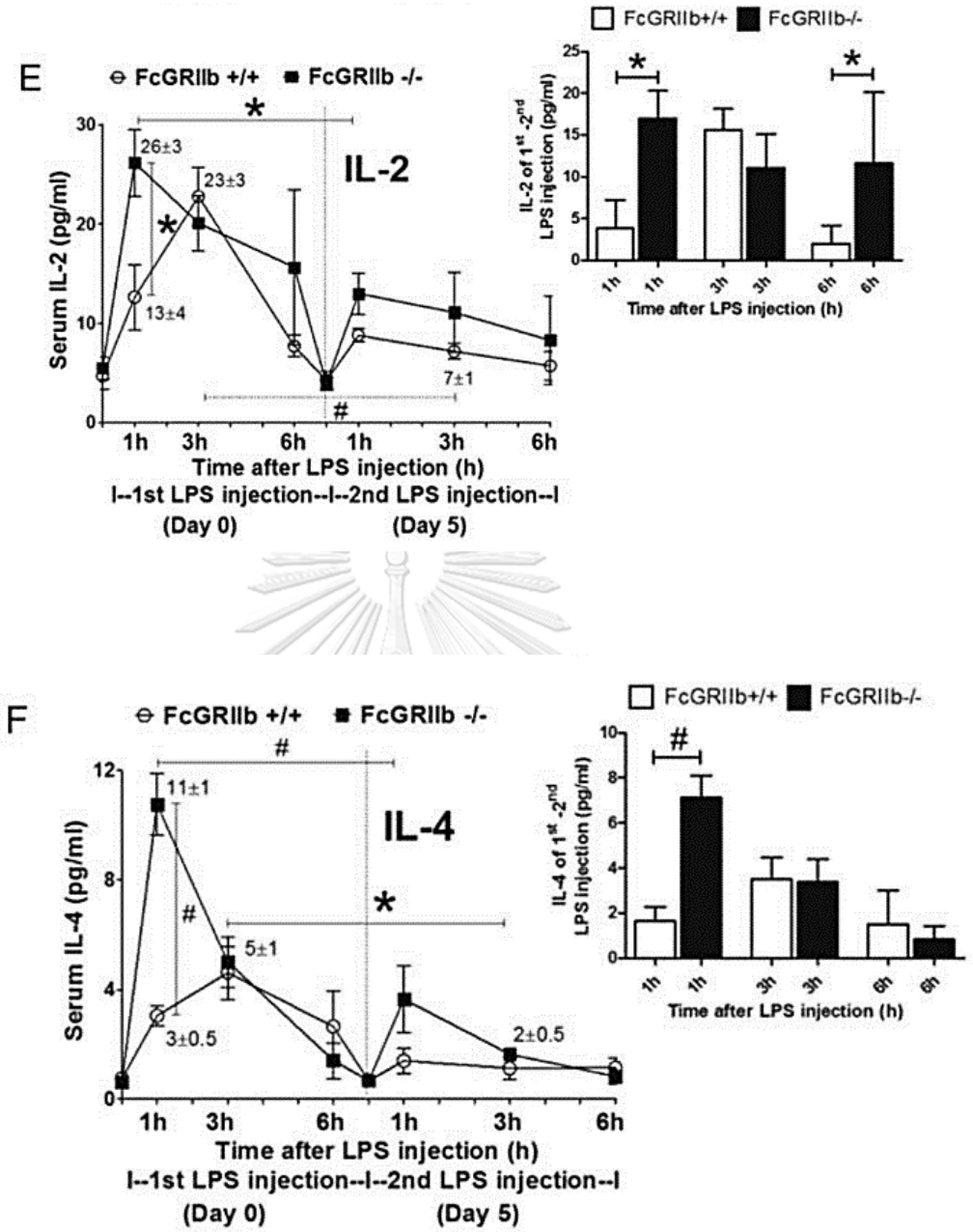

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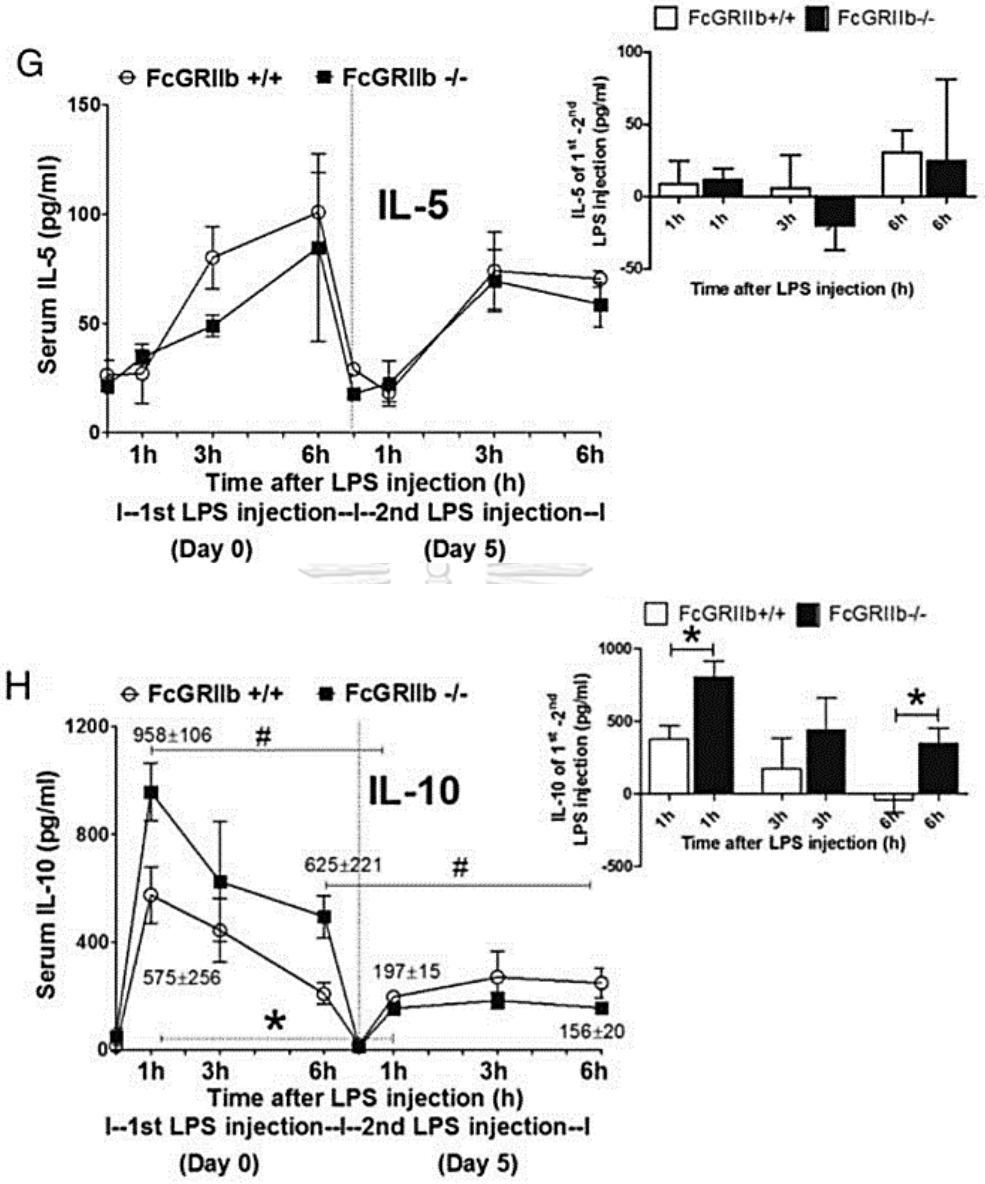

Figure 8. Serum cytokines in wild-type (FcGRIIb+/+) or FcGRIIb-/- mice.

Result of serum cytokines in the both wild-type or FcGRIIb-/- mice after at 1, 3, and 6h after first LPS injection (0.8 g/kg) (day 0) and second LPS injection (4 g/kg) (day 5) as measured by TNF-α (A), IL-6 (B), IL-1β (C), IFN-γ (D), IL-2 (E), IL-4 (F), IL-5 (G), and IL-10 (H). To emphasize the difference of serum cytokines after first and second doses of LPS, the delta change of serum cytokine response at the matched-timepoints after both LPS injection was presented as inset graphs (n¼5–7 per group). Repeated measures ANOVA with Bonferroni post hoc analysis was performed; *, P<0.05; #, P<0.01. ANOVA indicates analysis of variance; IFN-γ, interferon-gamma; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

Indeed, high degree of endotoxin tolerance in FcGRIIb-/- mice was demonstrated with most of the cytokines including TNF-α, IL-6, and IL-10, the important cytokines of sepsis-physiology (Figure 9). In contrast, wild-type mice showed high degree of the exhaustion with only a few cytokines (Figure 9). Fc gamma receptor IIb-deficient mice are more susceptible to cecal ligation and puncture sepsis with LPS preconditioning. We then modeled a clinical context for endotoxin tolerance using the induction of polymicrobial bacterial infection by CLP. LPS preconditioning prior to CLP resulted in a higher mortality rate in both wild-type and FcGRIIb-/- mice compared with CLP with non-LPS NSS control (Figure 10, A and B). The survival rate in CLP without LPS was not different between wild-type and FcGRIIb-/- mice, at 30% and 22%, respectively (Figure 10C). The higher sepsis severity was shown in FcGRIIb-/- mice over the wild-types in CLP with LPS preconditioning as determined by the mortality rate (Figure 10D).

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Serum cytokines	AUC-CR in serum after second dose of LPS/AUC-CR after the first dose (%)		Degree of endotoxin tolerance*,†		*P* value‡
	FcGRIIb+/+	FcGRIIb−/−	FcGRIIb+/+	FcGRIIb−/−	
TNF-α	100±22	12±2	Low	High	0.007
IL-6	68±6	22±4	Low	High	0.002
IL-1β	85±19	80±10	Low	Low	0.810
IFN-γ	29±10	23±7	High	High	0.652
IL-2	44±5	36±16	High	High	0.313
IL-4	38±13	33±5	High	High	0.705
IL-5	61±7	86±17	Low	Low	0.229
IL-10	51±11	24±4	Low	High	0.045

Figure 9. The degree of endotoxin tolerance in *in vivo*.

All results determined by the percentage of the ratio of the area under the curve of cytokine response (AUC-CR) in serum after the second LPS stimulation/ the AUC-CR after the first LPS administration (data from Figure. 1). *Determined by ratio of AUC-CR (see the Methods); Low, low degree (the ratio ≥50%) and High, high degree (the ratio <50%). †All mice showed endotoxin tolerance due to the relatively low percentage of the ratio despite the five times higher dose of the second LPS (see the Methods). ‡The ratio of individual mice between groups tested by the unpaired Student t test. AUC-CR indicates area under the curve of cytokine response; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

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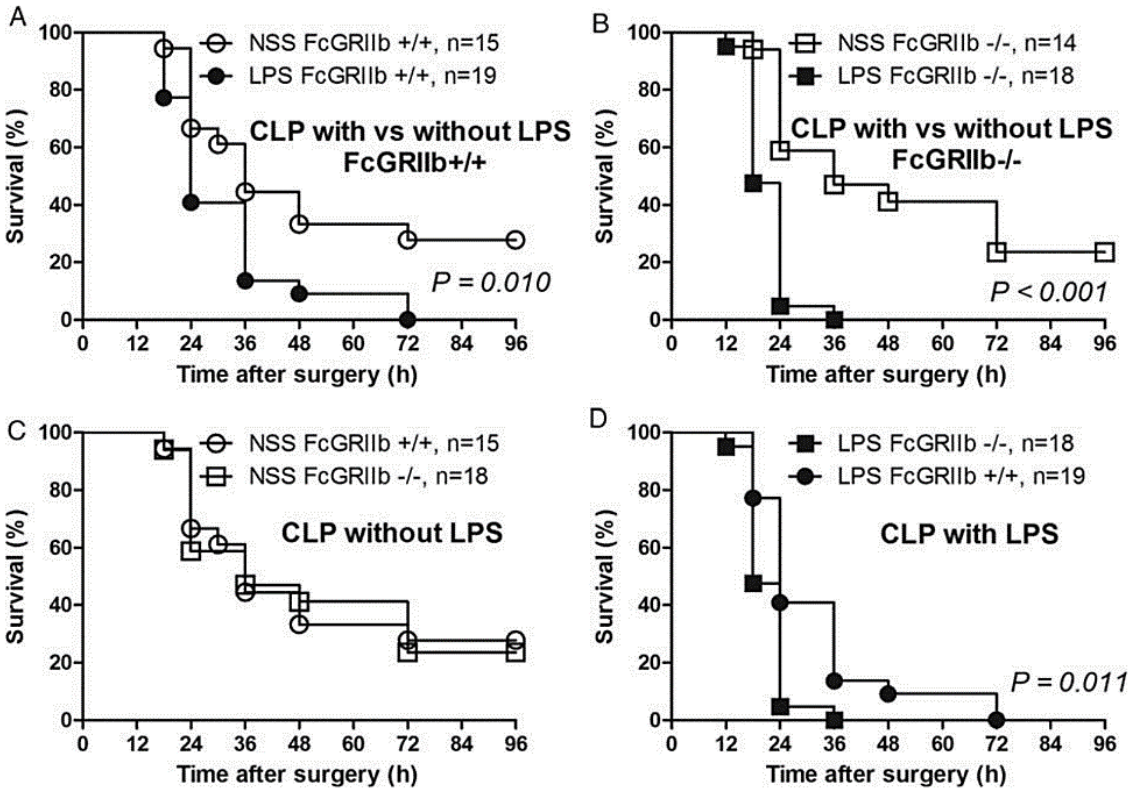

Figure 10. Survival analysis of cecal ligation and puncture (CLP) with or without LPS.

Survival analysis of CLP with or without LPS preconditioning in wild-type (FcGRIIb+/+) (A) and FcGRIIb-/- mice (B) and survival analysis between wild-type and FcGRIIb -/- mice after CLP without (C) and with LPS preconditioning (D) was shown. Survival analyzed by the log-rank test. LPS indicates lipopolysaccharide; NSS, normal saline.

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Other serum parameters in figure 11 show that blood bacterial burdens in FcGRIIb-/- mice were higher than wild-type at all selected time-points after CLP (Figure 11). Blood mononuclear cells, but not PMN cells, were lower in FcGRIIb-/- mice at 18 h after CLP but not at the earlier time-points (Figure 12 and 13). In addition, serum cytokines of FcGRIIb-/- mice were lower at the early time-points of sepsis (3 and/or 6 h) but higher at 18 h of CLP (Figure 14, A-C). Moreover, organ injury as measured by Scr and ALT were also higher in FcGRIIb-/- mice (Figure 15, A and B). Of note,the direct influence to CLP of serum cytokines induced by LPS was less likely because the cytokines declined to the base-line levels before CLP operation (12 h of the second LPS dose). The baseline of serum cytokines was shown as the 0 h time point of CLP in Figure 14,A–C.

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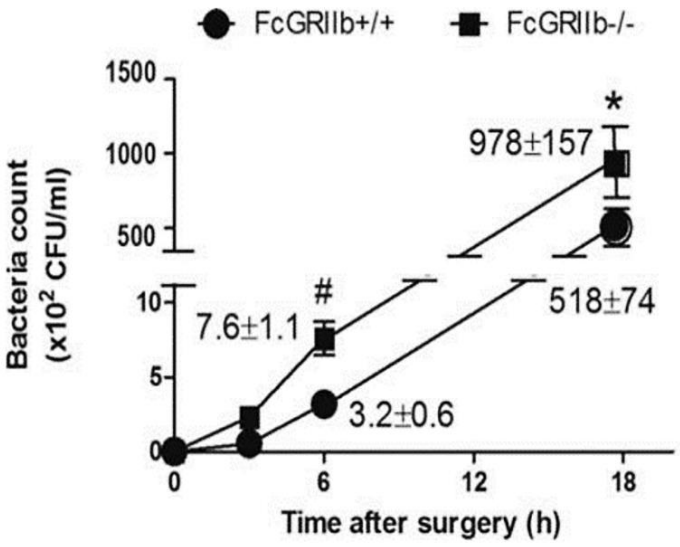

Figure 11. Serum parameters for the determination of sepsis severity of blood bacterial count. Sepsis severity of cecal ligation and puncture (CLP) after LPS preconditioning by time course of the blood bacterial count, in wild-type (FcGRIIb+/+) or FcGRIIb-/- mice was shown. Repeated measures ANOVA with Bonferroni post hoc analysis was performed; *, P<0.05; #, P<0.01.

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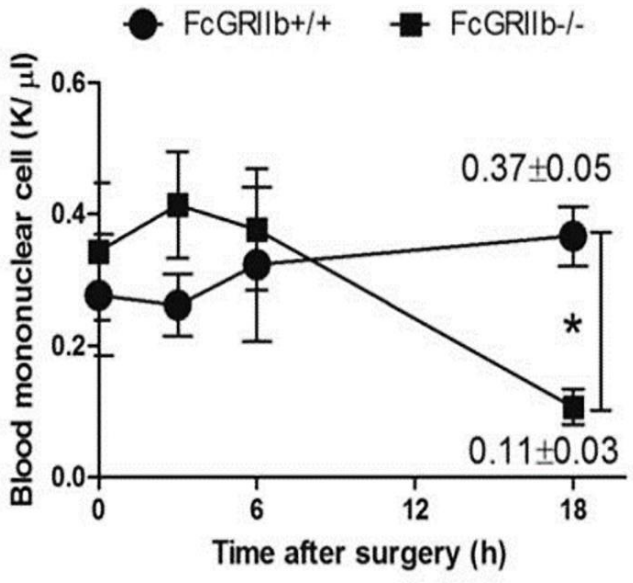

Figure 12. Serum parameters for the determination of sepsis severity in blood mononuclear cells. Sepsis severity cecal ligation and puncture (CLP) after LPS preconditioning by time course of blood mononuclear cells, in wild-type (FcGRIIb+/+)or FcGRIIb-/- mice was shown. Repeated measures ANOVA with Bonferroni post hoc analysis was performed; *, P<0.05.

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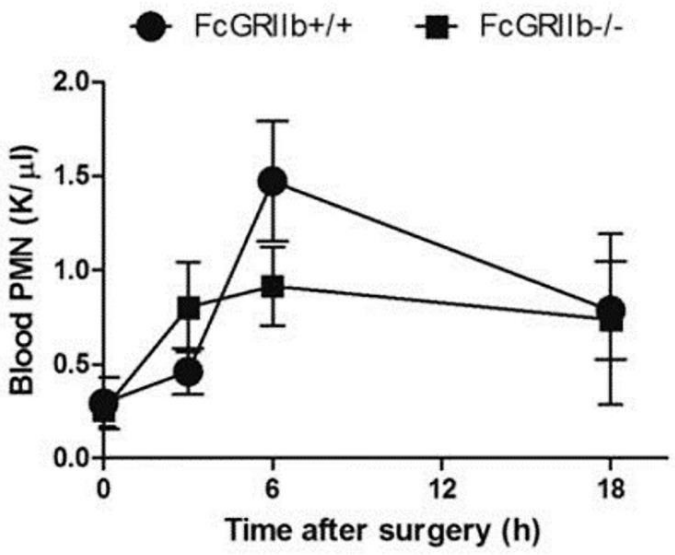

Figure 13. Serum parameters for the determination of sepsis severity of neutrophils (PMN). Sepsis severity of cecal ligation and puncture (CLP) after LPS preconditioning by time course of neutrophils (PMN), in wild-type (FcGRIIb+/+) or FcGRIIb-/- mice was shown. Repeated measures ANOVA with Bonferroni post hoc analysis was performed.

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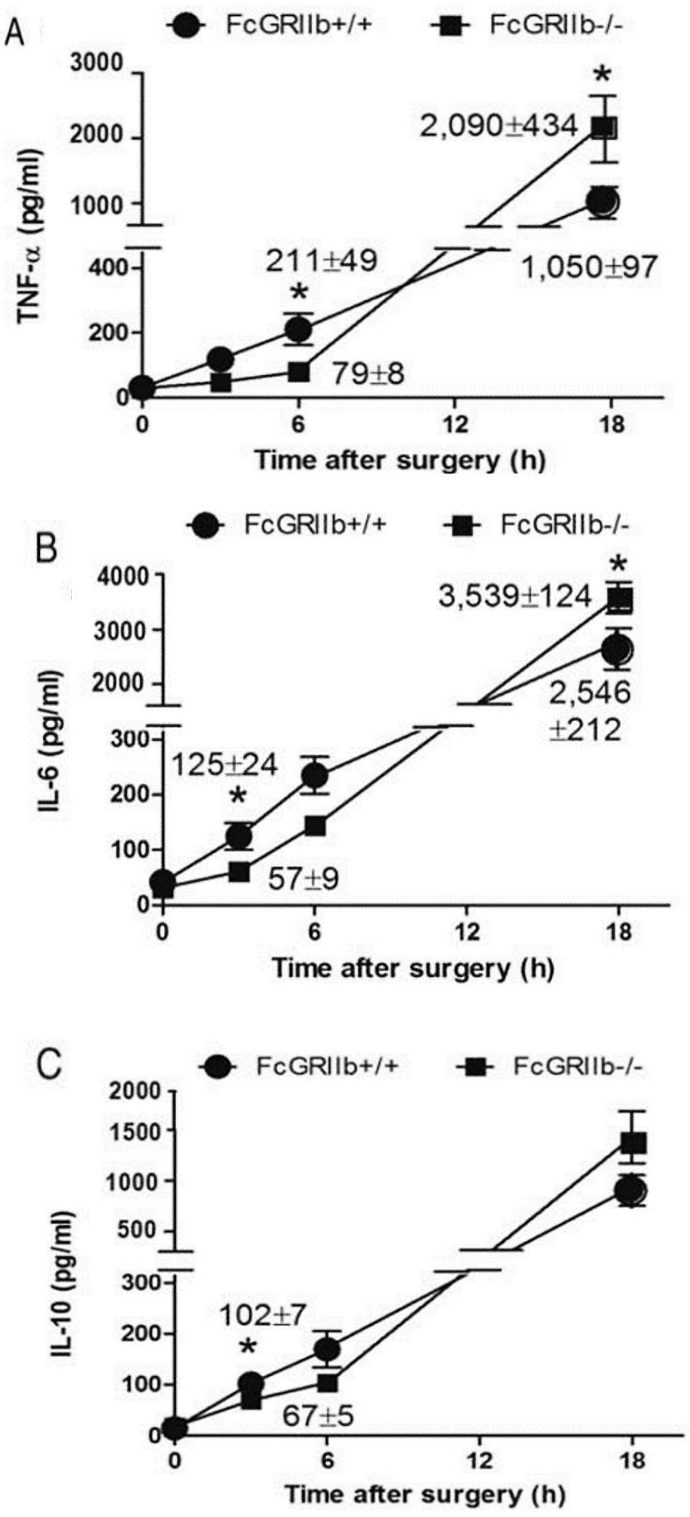

Figure 14. Serum parameters for the determination of sepsis severity of IL-10 serum cytokines. Sepsis severity of cecal ligation and puncture (CLP) after LPS preconditioning by time course of serum cytokines (TNF-**α**, IL6, andIL-10 (A–C)), in wild-type (FcGRIIb+/+)or FcGRIIb-/- mice was shown. Repeated measures ANOVA with Bonferroni post hoc analysis was performed for (A) to (C); *, P<0.05.

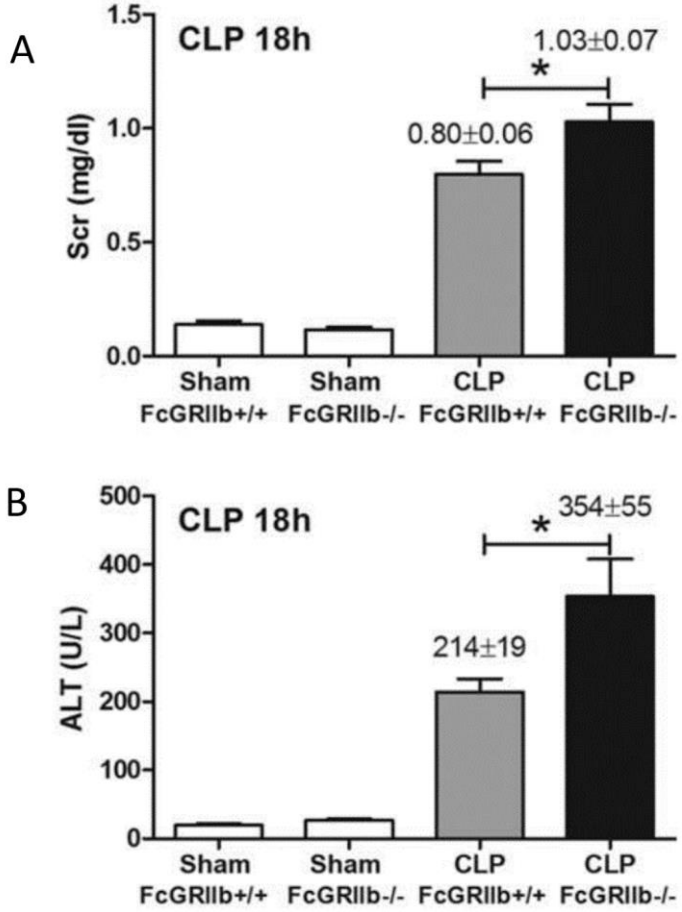

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Figure 15.. Serum parameters for the determination of sepsis severity of organ injury biomarkers. Sepsis severity of cecal ligation and puncture (CLP) after LPS preconditioning by time course of organ injury biomarkers at 18h; serum creatinine (Scr) (A) and alanine transaminase (ALT) (B), in wild-type (FcGRIIb+/+)or FcGRIIb-/- mice was shown. Repeated measures the unpaired Student t test was used for (A) and (B); *, $P<0.05$.

Bone marrow-derived macrophages of FcGRIIb-/- mice showed high cytokine responses in the single LPS incubation but lower responses in the sequential LPS stimulations

Because endotoxin tolerance is demonstrable in macrophages (16) and FcGRIIb receptors are present in macrophages (7), we tested endotoxin tolerance in vitro. Selected cytokine concentrations (TNF-a, IL-6, and IL-10) in culture media were compared between FcGRIIb-/- and wild-type cells. Cytokines in FcGRIIb-/- macrophage supernatants were higher than wild-type after the single low-dose LPS stimulation (N/LPS10) and were lower in the second low-dose stimulation (LPS10/10) in several time-points (Figure 16-18). Although the difference in cytokine levels between FcGRIIb-/- macrophages and the wild-types was not obvious (Figure 16-18).

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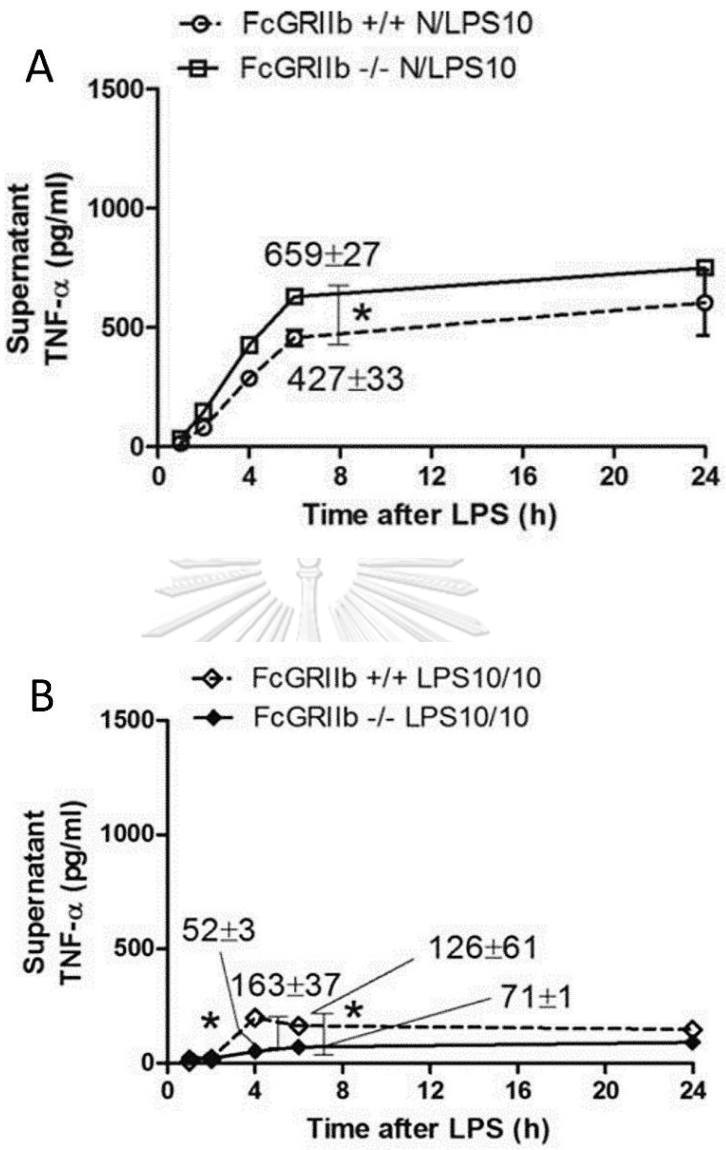

Figure 16. Cytokine responses of TNF-α in supernatant media from macrophages.
FcGRIIb-/-or wild-type (FcGRIIb+/+) after activation with a single low dose LPS (N/LPS10): TNF-α (A). Cytokines after stimulation with a second low-dose LPS (LPS10/10): TNF-a (B). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni posthocanalysis was performed), *, P<0.05. ANOVA indicates analysis of variance; TNF, tumor necrosis factor.

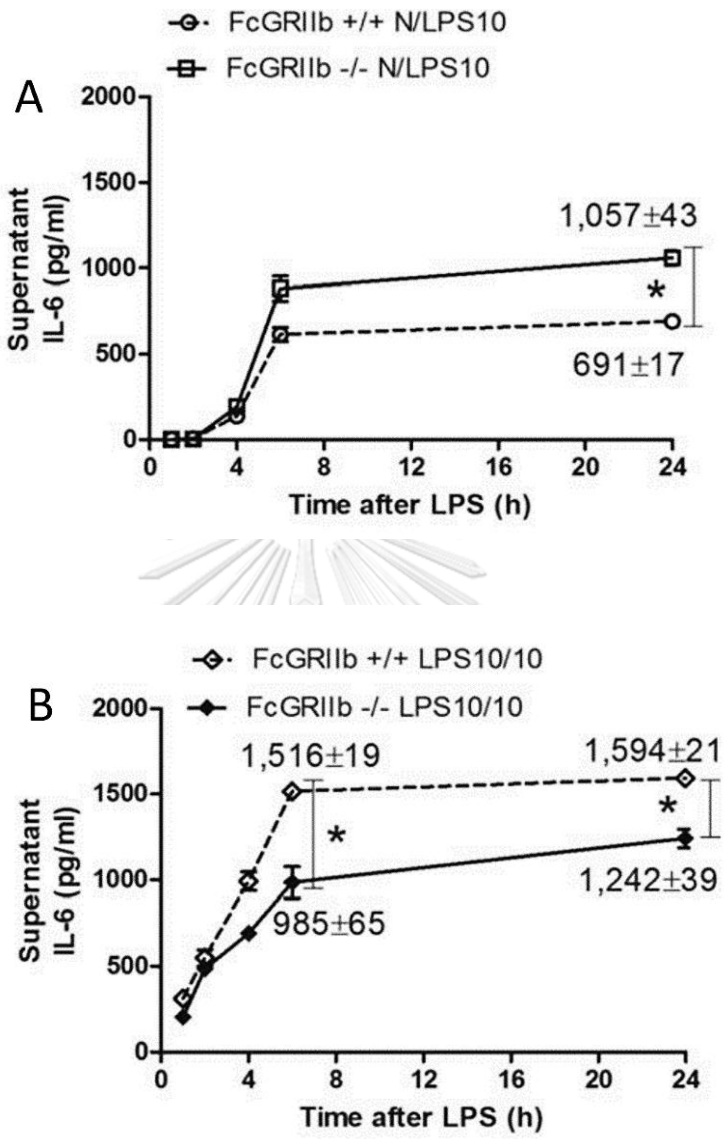

Figure 17. Cytokine responses of IL-6 in supernatant media from macrophages.

FcGRIIb-/- or wild-type (FcGRIIb+/+) after activation with a single low dose LPS (N/LPS10): IL-6 (A). Cytokines after stimulation with a second low-dose LPS (LPS10/10): IL-6 (B). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni posthocanalysis was performed), *, P<0.05. ANOVA indicates analysis of variance; IL, interleukin.

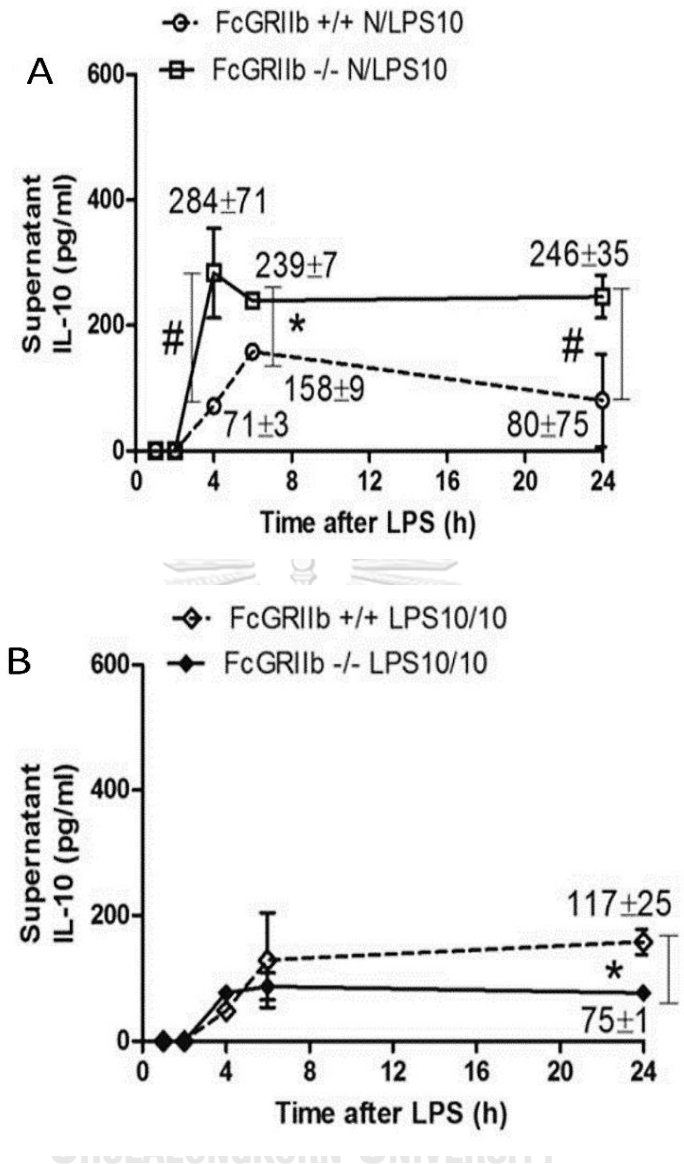

Figure 18. Cytokine responses of IL-10 in supernatant media from macrophages.

FcGRIIb-/-or wild-type (FcGRIIb+/+) after activation with a single low dose LPS (N/LPS10): IL-10 (A). Cytokines after stimulation with a second low-dose LPS (LPS10/10): IL-10 (B). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni posthocanalysis was performed), *, P<0.05; #, P<0.01. ANOVA indicates analysis of variance; IL, interleukin.

Endotoxin tolerance could be demonstrated by TNF-**α** and IL-10 but not IL-6 (Figure 19). To evaluate dose–response effects, a higher dose of LPS was used. Then, FcGRIIb-/- macrophages showed higher responses in the single high-dose LPS (N/LPS100) (Figure 20-22, A). Interestingly, cytokine levels in supernatant of macrophages primed with high-dose LPS were dependent on the levels of second LPS doses. In the high dose of LPS followed by the low dose (LPS100/10), all cytokines were detected at the very low levels with the non-significant difference between groups (Figure 20-22, B). But, with the higher second dose of LPS (LPS100/100), FcGRIIb-/- cells produced lower cytokines than wild-type with larger differences between groups (Figure 20-22, C).

	AUC-CR after two separated LPS (LPS/LPS)/AUC-CR after a single LPS (N/LPS) (%)		Degree of endotoxin tolerance*		
Cytokines from culture supernatant	FcGRIIb+/+	FcGRIIb−/−	FcGRIIb+/+	FcGRIIb−/−	*P* value†
Low dose of LPS; AUC-CR (LPS 10/10)/AUC-CR (N/LPS10)					
TNF-α	31.9 ± 4.0	11.9 ± 0.1	High	High	<0.001
IL-6	258.1 ± 5.1‡	125.5 ± 14.7‡	No	No	<0.001
IL-10	118.5 ± 12.5‡	33.1 ± 1.1	No	High	<0.001
High dose of LPS; AUC-CR (LPS 100/100)/AUC-CR (N/LPS100)					
TNF-α	139.7 ± 17.2‡	33 ± 5	No	High	<0.001
IL-6	38 ± 13	78.0 ± 2.5	High	High	<0.001
IL-10	100.6 ± 17.0	34.4 ± 2.1	No	High	<0.001

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Figure 19. The degree of endotoxin tolerance in *in vitro*.

All results determined by the percentage of the ratio of the area under the curve of cytokine response (AUC-CR) in culture supernatant after two separate LPS stimulation (LPS/LPS)/the AUC-CR after a single LPS administration (N/LPS) (data from Figure 4 and 5). Determined by the ratio of AUC-CR (see the Methods); No (no tolerance; the ratio ≥ 100%); Low, low degree (the ratio <50%) and High, high degree (the ratio <50%). †Tested by the unpaired Student t test. ‡The second cytokine response was higher than the first response. AUC-CR indicates area under the curve of cytokine response.

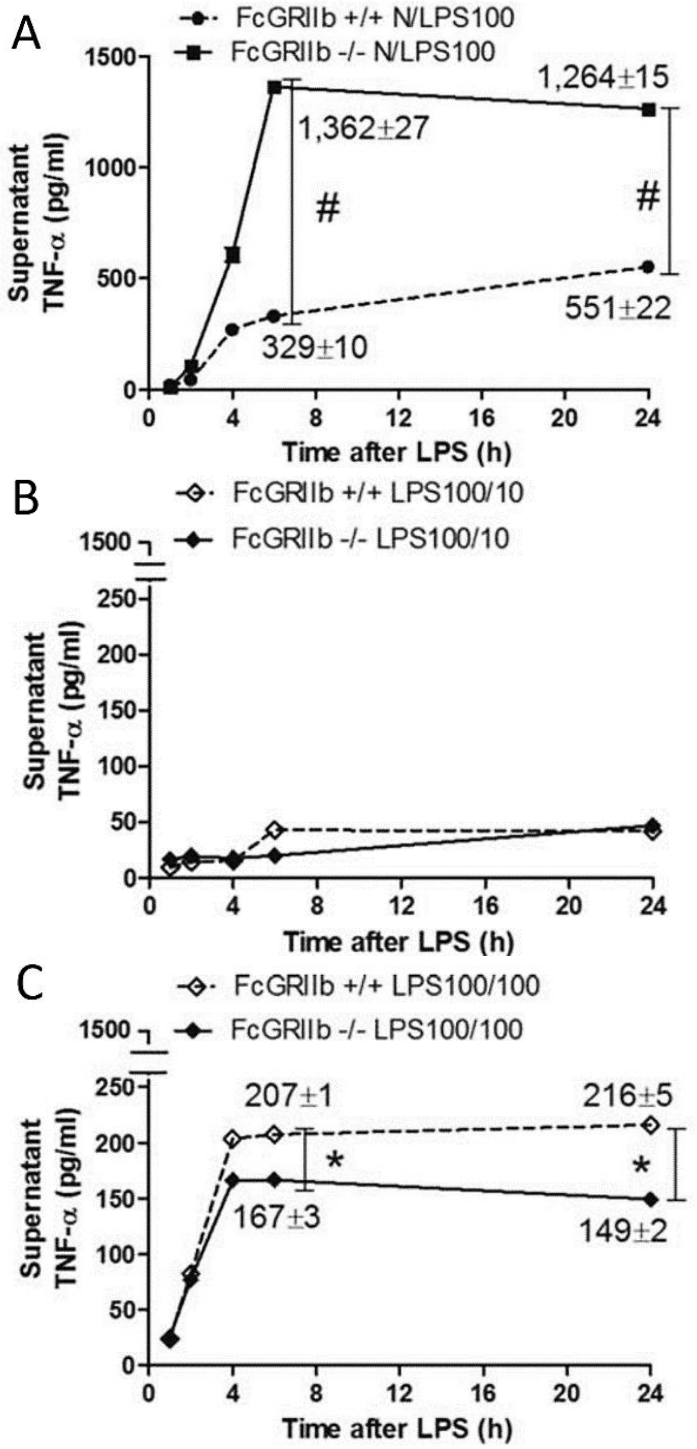


Figure 20. Cytokine responses of TNF-α in supernatant media from macrophages**.**

FcGRIIb-/- or wild-type (FcGRIIb+/+) after activation with a single high-dose LPS (N/LPS100), TNF-α (A) and sequential LPS doses, low and high-dose LPS (LPS100/10 (B) and LPS100/100 (C), respectively). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, P<0.05; #, P<0.01.

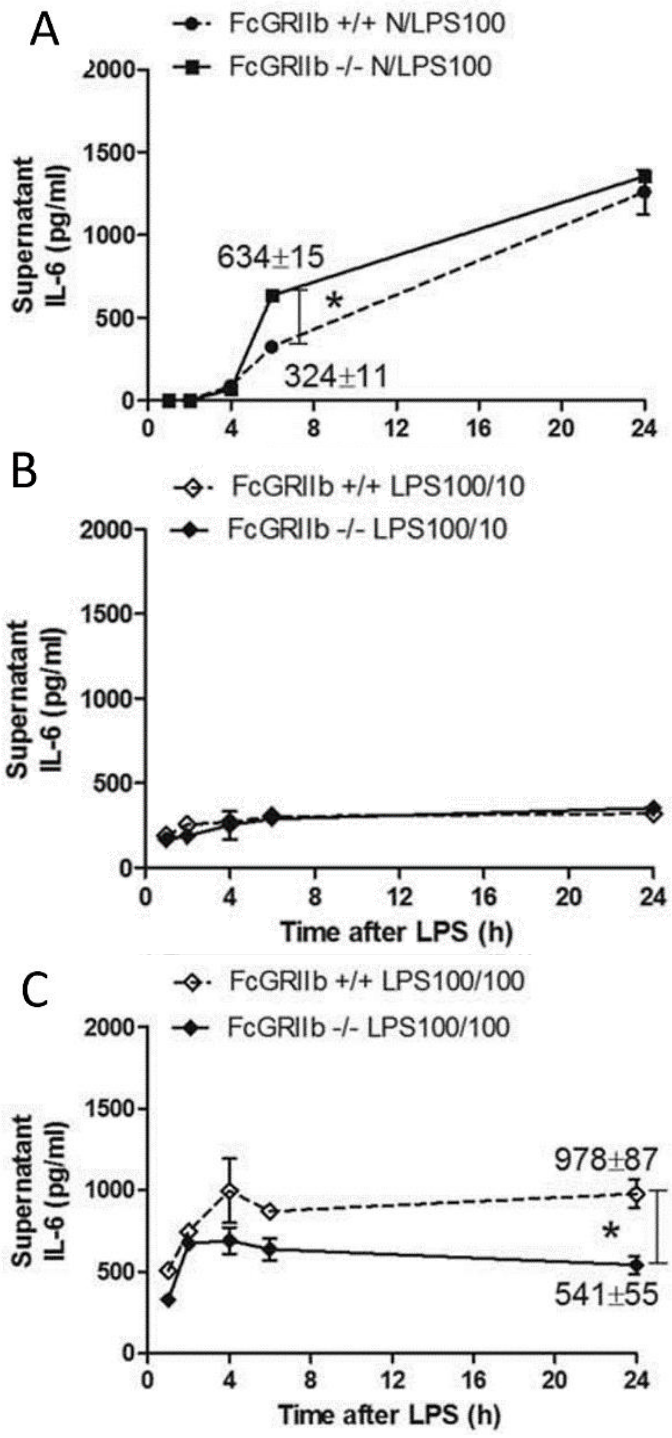


Figure 21. Cytokine responses of IL-6 in supernatant media from macrophages. FcGRIIb-/- or wild-type (FcGRIIb+/+) after activation with a single high-dose LPS (N/LPS100), IL-6 (A) and sequential LPS doses, low and high-dose LPS (LPS100/10 (B) and LPS100/100 (C), respectively). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, $P<0.05$; #, $P<0.01$.

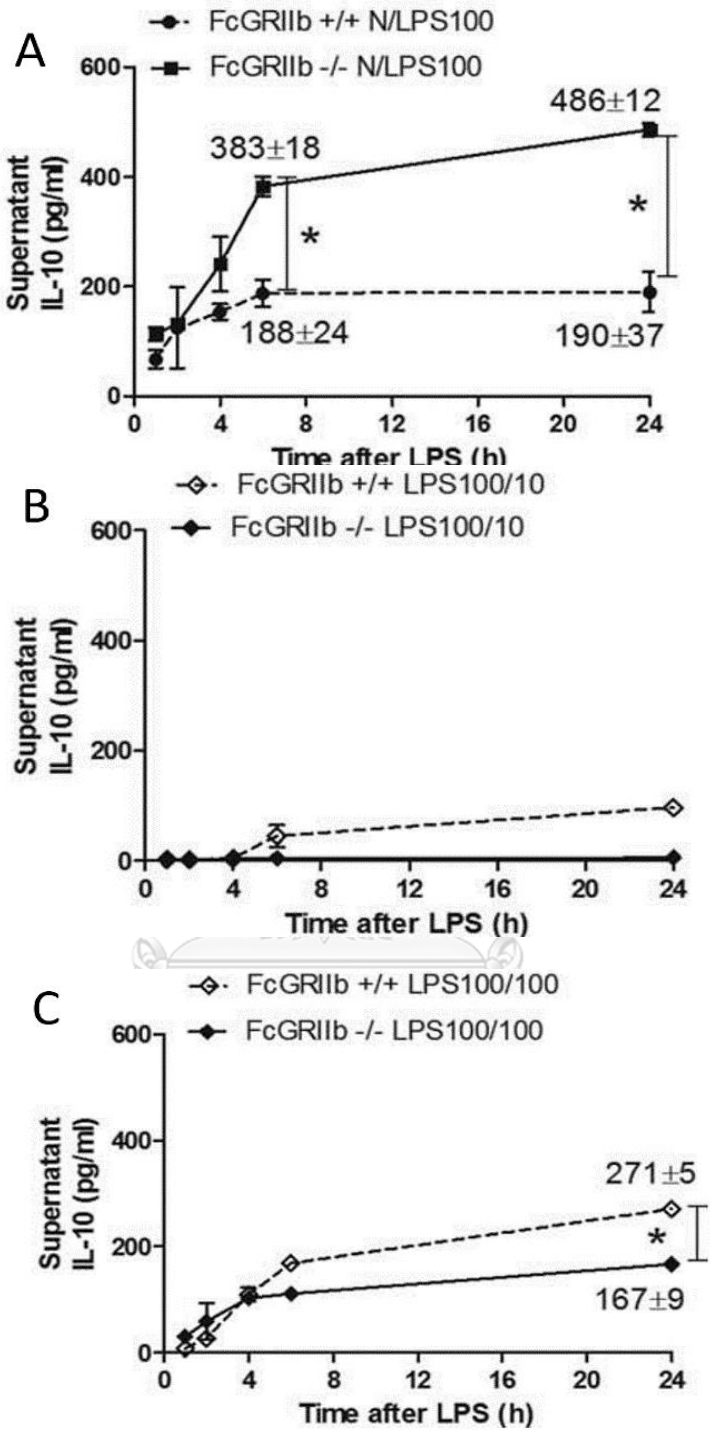
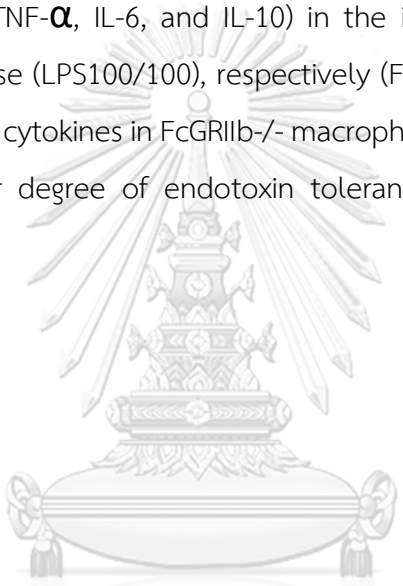


Figure 22. Cytokine responses of IL-10 in supernatant media from macrophages. FcGRIIb-/- or wild-type (FcGRIIb+/+) after activation with a single high-dose LPS (N/LPS100), IL-10 (A) and sequential LPS doses, low and high-dose LPS (LPS100/10 (B) and LPS100/100 (C), respectively). (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, $P<0.05$.

Macrophage endotoxin tolerance was indicated by lower cytokine production (TNF-a, IL-6, and IL-10) after the second LPS stimulation compared with the responses to the initial dose (Figure 23-25). Endotoxin tolerance could be demonstrated in FcGRIIb -/- macrophages with all cytokines in all LPS stimulation regimens. In contrast, wild-type cells showed robust tolerance only with TNF-**α**. In addition, the degree of macrophage endotoxin tolerance was determined from the ratio of AUC-CR after two separated LPS (LPS/LPS)/AUC-CR after a single LPS (N/LPS) (see the Methods). High degree of the tolerance could be demonstrated by two-thirds of selected cytokines and by all cytokines (TNF-**α**, IL-6, and IL-10) in the induction by LPS in low dose (LPS10/10) and high dose (LPS100/100), respectively (Figure 9). Moreover, the ratio of AUC-CR was lower in all cytokines in FcGRIIb-/- macrophages over wild-type cells which represented the higher degree of endotoxin tolerance of FcGRIIb-/- macrophages (Figure 19).



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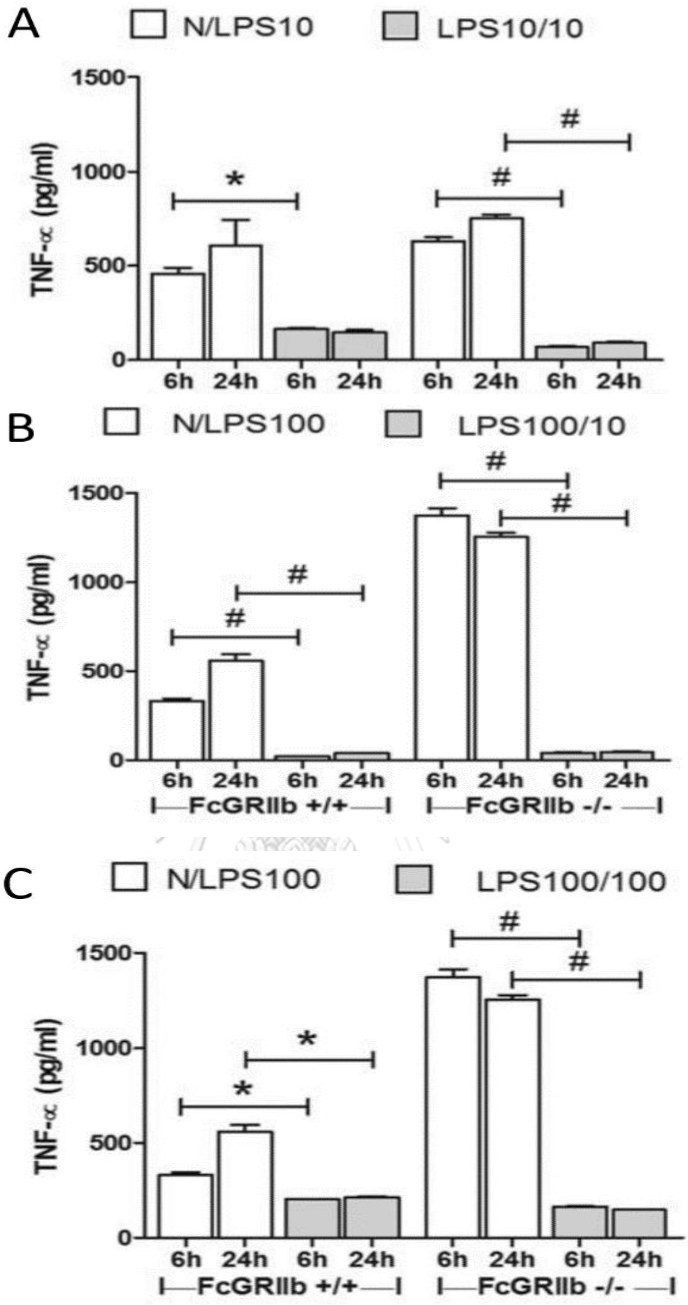

Figure 23. Macrophage endotoxin tolerance evaluated by the difference in cytokine responses of TNF-**α** in supernatant media from macrophages of FcGRIIb -/-or wild-type (FcGRIIb+/+). After activation with a single low-dose (N/LPS10) versus sequential low-dose LPS (LPS10/10) (A), and the difference between a single high-dose (N/LPS100) versus sequential high and sequential high/low dose of LPS (LPS 100/10) (B) versus twice high dose of LPS (LPS100/100) (C) were demonstrated. (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, $P<0.05$; #, $P<0.01$.

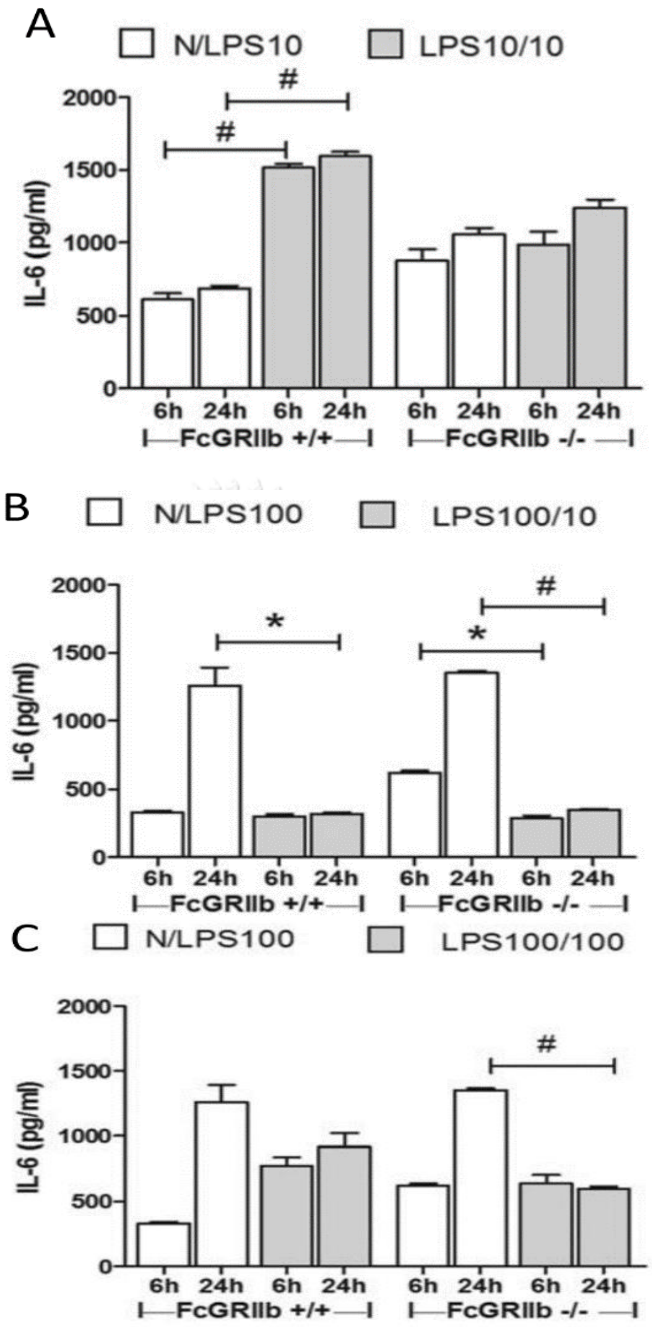

Figure 24. Macrophage endotoxin tolerance evaluated of IL-6 from supernatant macrophages. The difference in cytokine responses of IL-6 in supernatant media from macrophages of FcGRIIb-/- or wild-type (FcGRIIb+/+) after activation with a single low-dose (N/LPS10) versus sequential low-dose LPS (LPS10/10) (A), and the difference between a single high-dose (N/LPS100) versus sequential high and sequential high/low dose of LPS (LPS 100/10) (B) versus twice high dose of LPS (LPS100/100) (C) were demonstrated. (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, $P<0.05$; #, $P<0.01$.

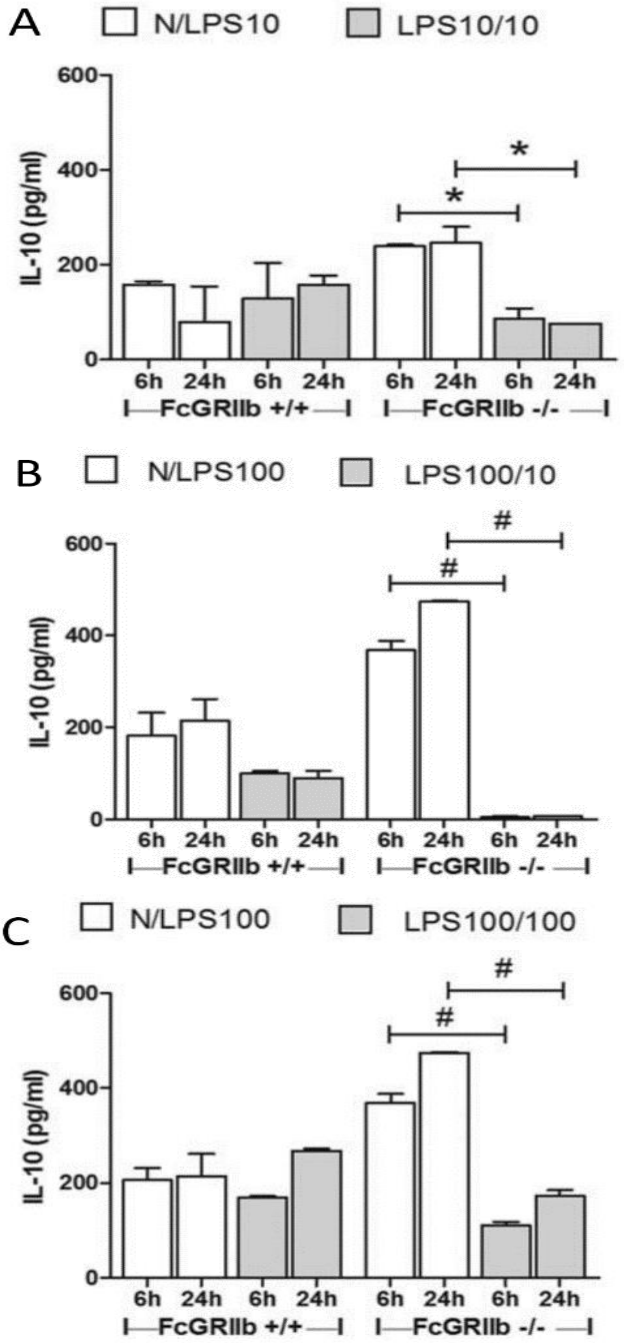

Figure 25. Macrophage endotoxin tolerance evaluated by the difference in cytokine responses of IL-10 in supernatant media from macrophages of FcGRIIb-/-or wild-type (FcGRIIb+/+) after activation with a single low-dose (N/LPS10) versus sequential low-dose LPS (LPS10/10) (A), and the difference between a single high-dose (N/LPS100) versus sequential high and sequential high/low dose of LPS (LPS 100/10) (B) versus twice high dose of LPS (LPS100/100) (C) were demonstrated. (Separate, triplicate experiments were performed; repeated measures ANOVA with Bonferroni post hoc analysis was performed), *, $P<0.05$; #, $P<0.01$.

These results indicate that endotoxin tolerance is more readily inducible in FcGRIIb-/- macrophages relative to wild-type. Of note, cell viability was not decreased by these LPS regimens and was not different between wild-type and FcGRIIb-/- cells even with 2 days of LPS100/100 (data not shown). Interestingly, the killing activity of FcGRIIb-/- macrophages (inversely associated with blood bacterial burdens) declined relative to wild-type in a LPS dose-dependent manner (Figure 26).

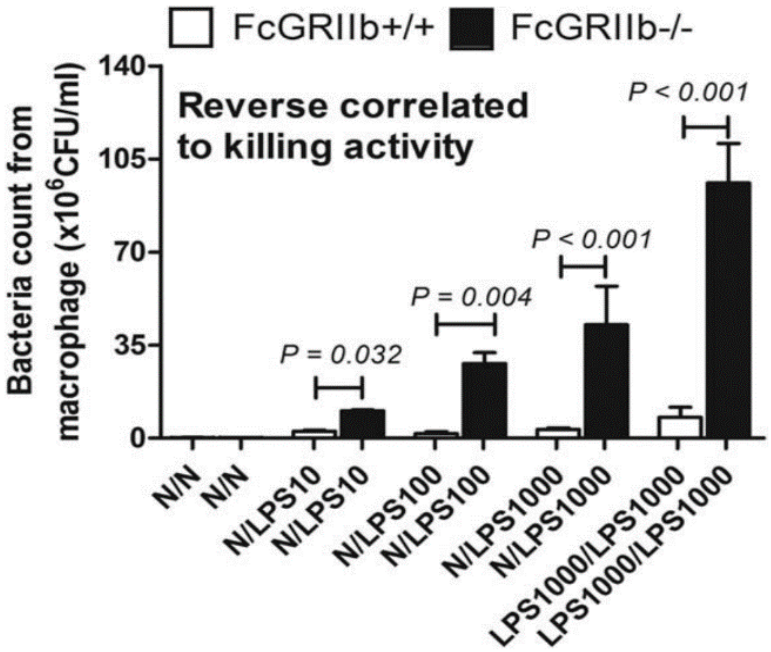

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Figure 26. Macrophage killing activity, inversely correlated with bacteria count from cell lysate, was shown. (Separate, triplicate experiments were performed; the unpaired Student t test was used).

Prominent responses with profound exhaustion after LPS stimulations in the FcGRIIb−/− macrophage

To demonstrate the different responses to LPS of FcGRIIb−/− and wild-type cells, supernatant cytokine concentrations after LPS stimulation were measured. Increased cytokine production after a single LPS stimulation (N/100) with obvious LPS-tolerance (cytokine-level difference between N/100 and 100/100) of FcGRIIb−/− macrophages over wild-type cells was demonstrated by all cytokines (Figure 27; A–C), highlighting the immune inhibition defect of FcGRIIb−/− (68). In parallel, increased phagocytosis with limited microbicidal activity (due to excessive phagocytosis) of N/100 FcGRIIb−/− macrophages compared with N/100 wild-type cells was evident (Figure 28). In addition, LPS tolerant (100/100) FcGRIIb−/− cells exhibited less phagocytic activity (1h post incubation) than LPS-tolerant wild-type cells and N/100 FcGRIIb−/− cells (Figure 29;A). Moreover, the microbicidal activity of LPS-tolerant FcGRIIb−/− cells was lower than that of N/100 FcGRIIb−/− cells (Figure 28), despite the smaller number of phagocytosed bacteria in LPS-tolerant cells (Figure29). Hence, the LPS tolerance affected several macrophage functions, including cytokine production, phagocytosis, and microbicidal activity, predominantly in FcGRIIb−/− cells over the wild-type cells.

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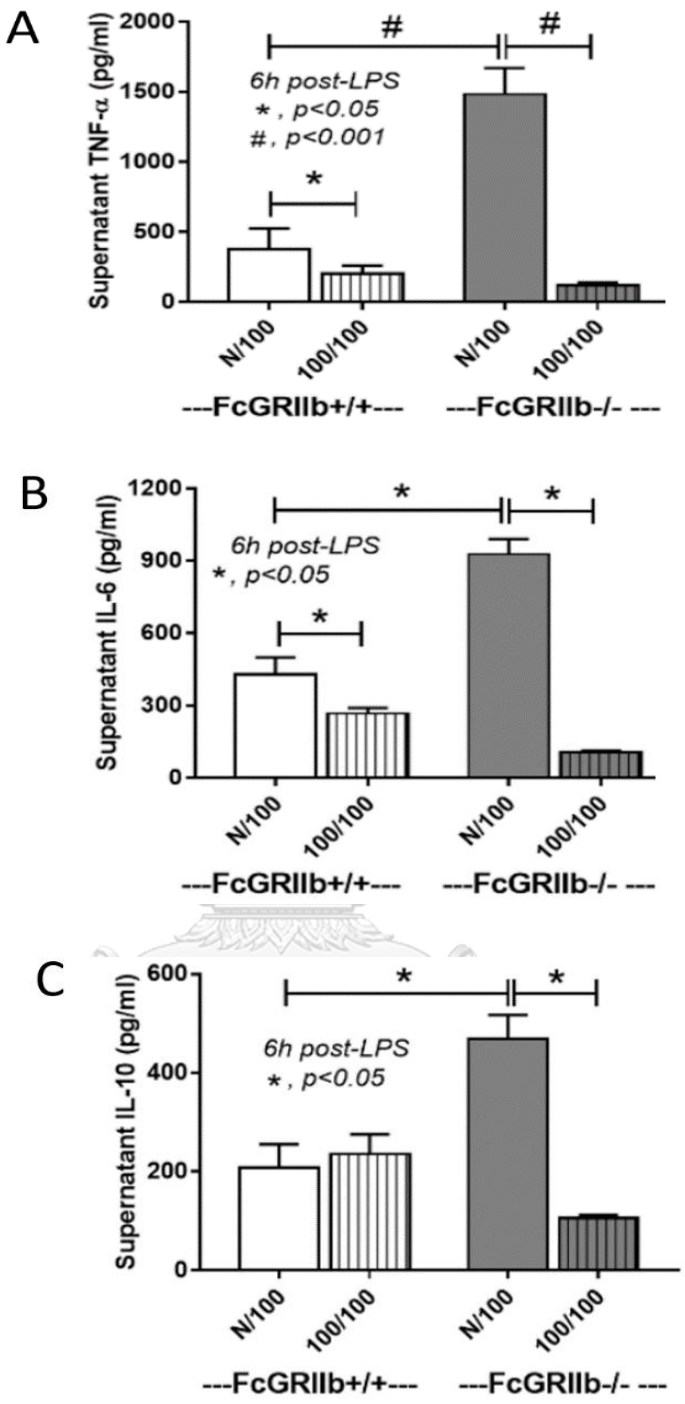

Figure 27. The characteristics of FcGRIIb+/+ versus FcGRIIb−/− macrophages as cytokine activation. FcGRIIb+/+ versus FcGRIIb−/− macrophages after a single LPS stimulation (N/100) and sequential LPS activation (100/100; LPS tolerance) shown as cytokine activation (A–C). Independent experiments were performed in triplicate.

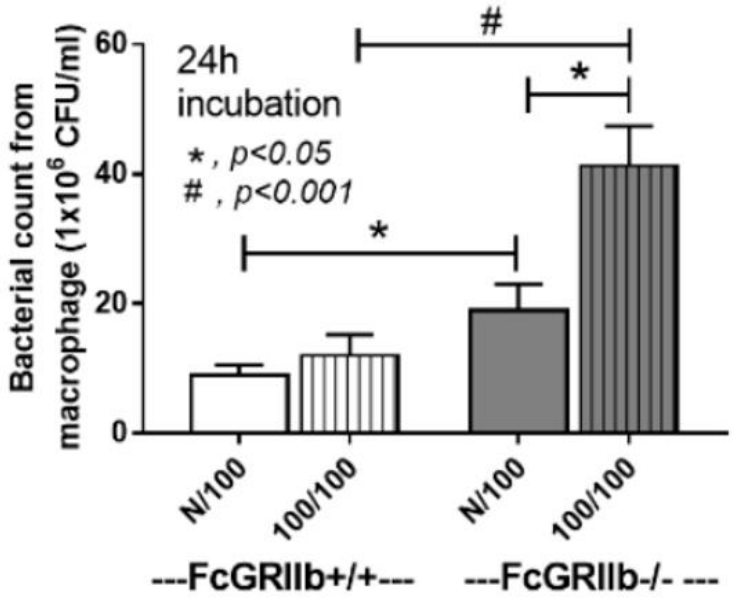

Figure 28. The characteristics of macrophages as microbicidal activity.

FcGRIIb+/+ (WT) versus FcGRIIb−/− macrophages (KO) after a single LPS stimulation (N/100) and sequential LPS activation (100/100; LPS tolerance) shown as microbicidal activity. Independent experiments were performed in triplicate.

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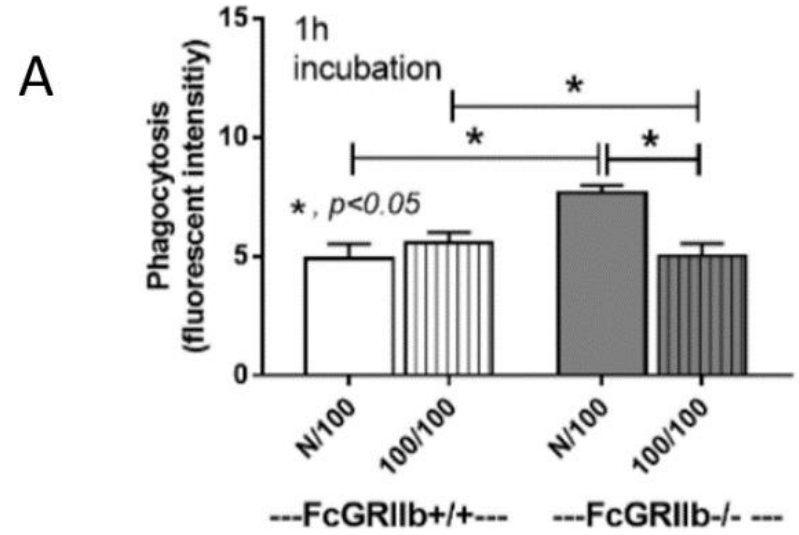

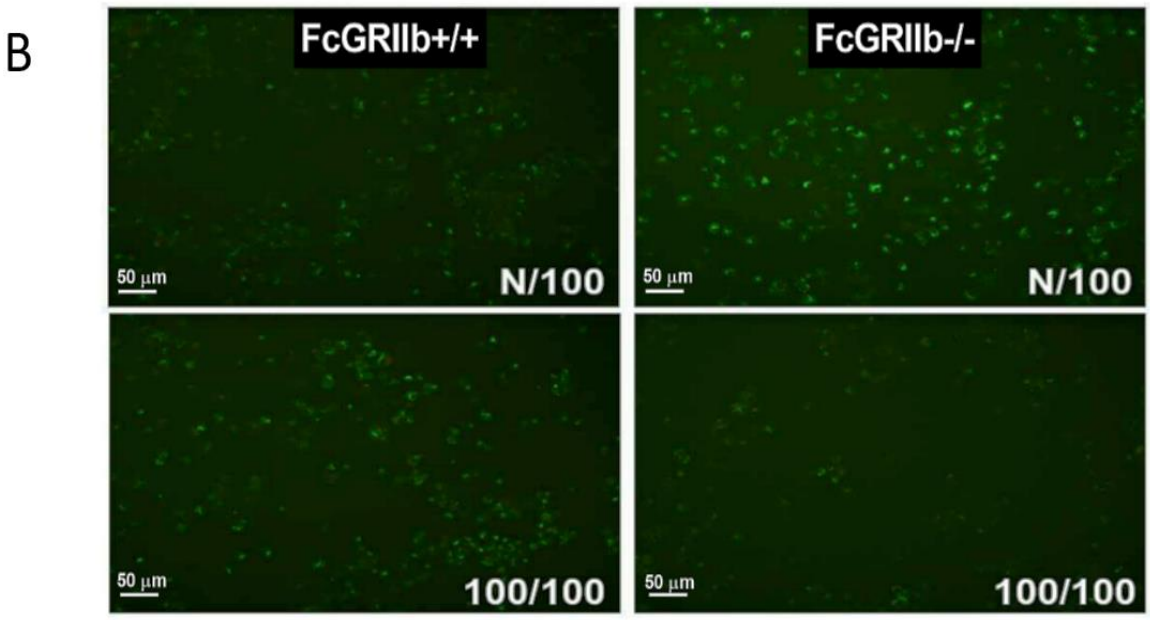

Figure 29. The characteristics of macrophages as phagocytosis.
FcGRIIb+/+ (WT) versus FcGRIIb−/− macrophages (KO) after a single LPS stimulation (N/100) and sequential LPS activation (100/100; LPS tolerance) shown as phagocytosis with mean fluorescent intensity (A) and the representative of phagocytosis by a fluorescent microscope (green dots were phagocytosed FITC-dextran conjugated zymosan) (B). Independent experiments were performed in triplicate.

Significantly decreased protein Kinase C-β Type II (PKCB) in FcGRIIb−/− macrophage with LPS tolerance by the phosphoproteomic analysis

To explore the different protein responses of FcGRIIb−/− and wild-type cells, phosphoproteomic analysis was conducted. A total of 21, 37, and 781 phosphopeptides on 21, 37, and 499 proteins were identified in control (N/N), single LPS stimulation (N/100), and sequential LPS induction (100/100) samples, respectively (Figure 30). Interestingly, of the 781 differentially quantified phosphoproteins, 381 phosphoproteins, including protein kinase C-β type II (PKCβ), were significantly down-regulated in LPS-tolerant FcGRIIb−/− macrophages, as illustrated by the volcano plot analysis (Figure 31). Using the DAVID Functional Annotation Clustering Tool, the functional annotation clustering identified PKCB mediated signaling downstream of a phagocytosis pathway (Figure32). Indeed, the downregulation of phosphoproteins in the FcGR-mediated phagocytosis pathway might be associated with the induction of LPS-tolerance in FcGRIIb−/− macrophages. Then, the 122 differently expressed proteins were subjected to Gene ontology (GO) analysis using the PANTHER tool. The functional classifications and the number percentages of the proteins we found are shown in Figure 33. Among several phosphoproteins in this group, the serine-threonine protein kinase AKT1 (Akt1), sphingosine kinase (SPHK), P21-Activated Kinase 1 (PAK1), and protein kinase C-β type II (PKCB) (Figure 34) are important mediators for phagocytosis, as determined by KEGG pathway analysis. While there are numerous studies of Akt signaling in macrophages, focusing on several cell activities (128, 140), some studies mention protein kinase C as the association mediator of TLR signaling (95, 128). Thus, the defect of a specific isoform of protein kinase C (-β type II) might be responsible for the extensive LPS tolerance in FcGRIIb−/− macrophages. To explore whether PRKCB could enhance macrophage functions, (Figure 35 and 36). Indeed, the abundance of protein kinase C-β type II (PKCB) shown by western blot analysis from a bone marrow devide macrophage cell in sequential LPS activation (100/100; LPS-tolerance), (A) and spleed in mice (B).

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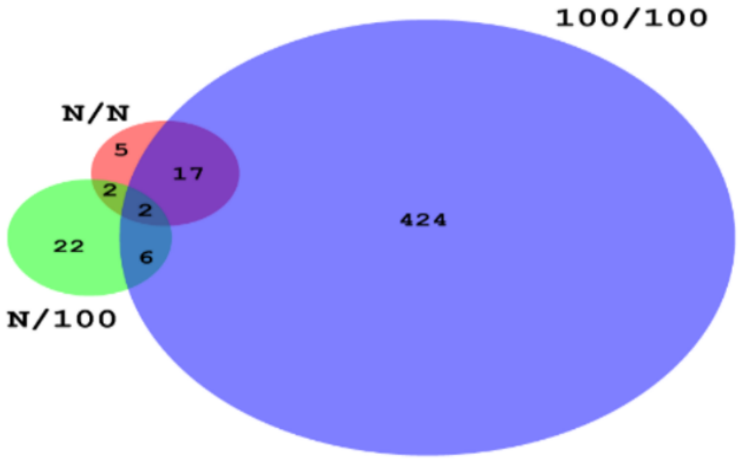

Figure 30. Venn diagram demonstrating the different number of phosphoproteins. FcGRIIb+/+ macrophages in comparison with FcGRIIb−/− after a single LPS stimulation (N/100), sequential LPS activation (100/100; LPS-tolerance), and the control (N/N) (n = 4/each group).

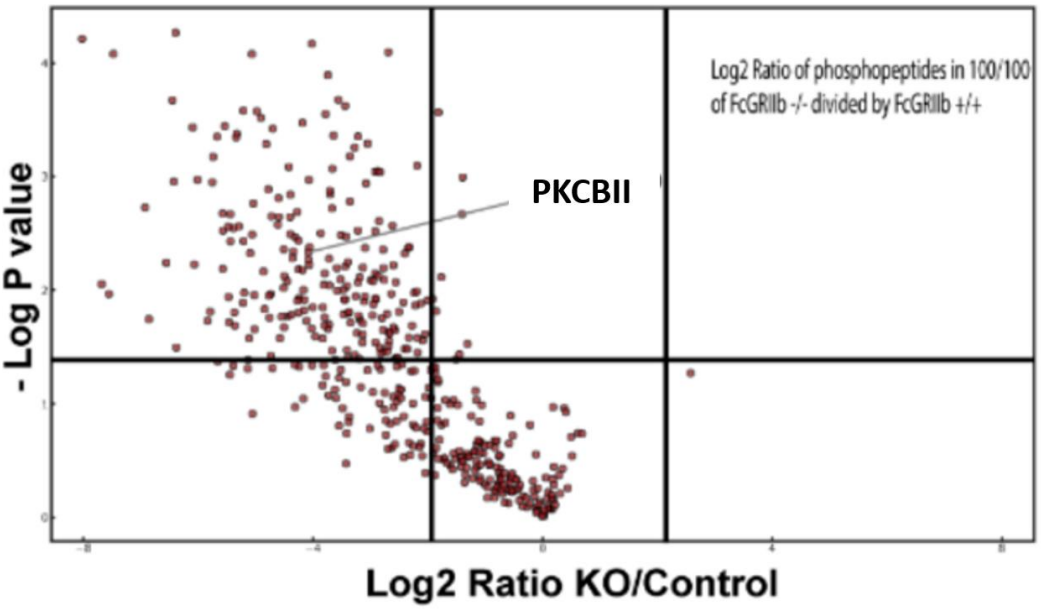

Figure 31. The volcano plot analysis of downregulated phosphoproteins from the sequential LPS activation of FcGRIIb−/− compared with FcGRIIb+/+ macrophages.

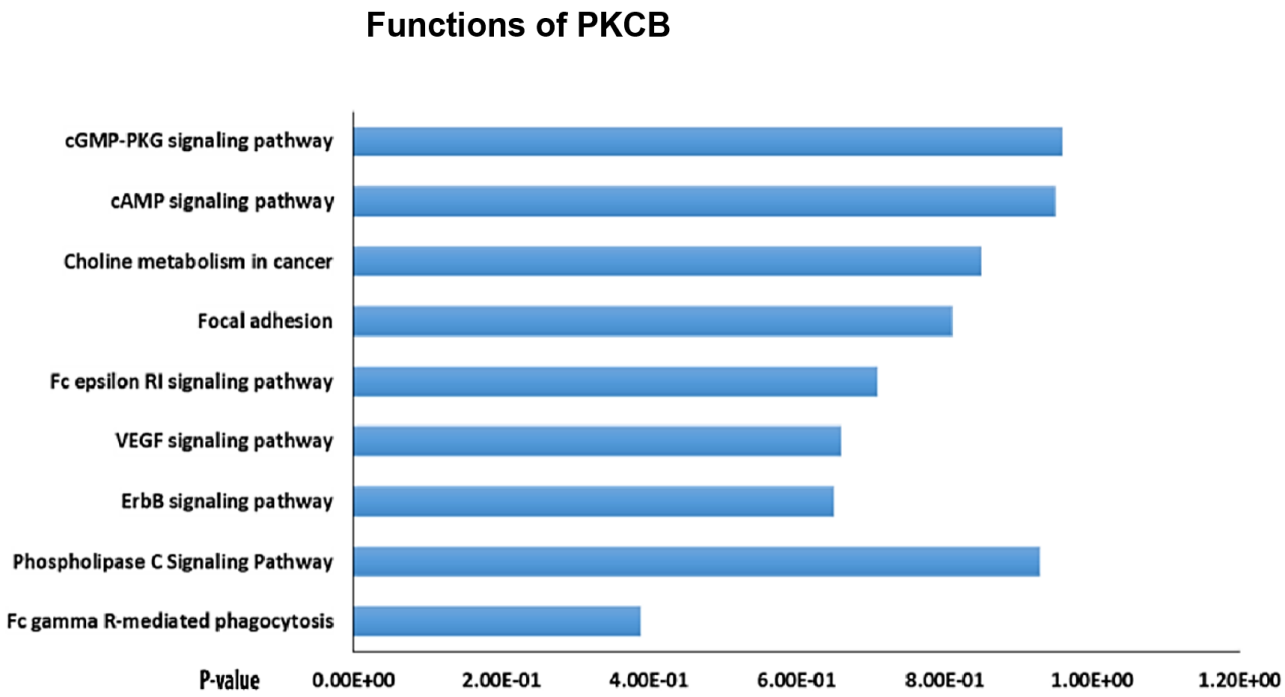

Figure 32. Pathway analysis clusters (DAVID) of the significantly altered phosphopeptides. in FcGRIIb+/+ compared with FcGRIIb−/− in LPS-tolerance (100/100).

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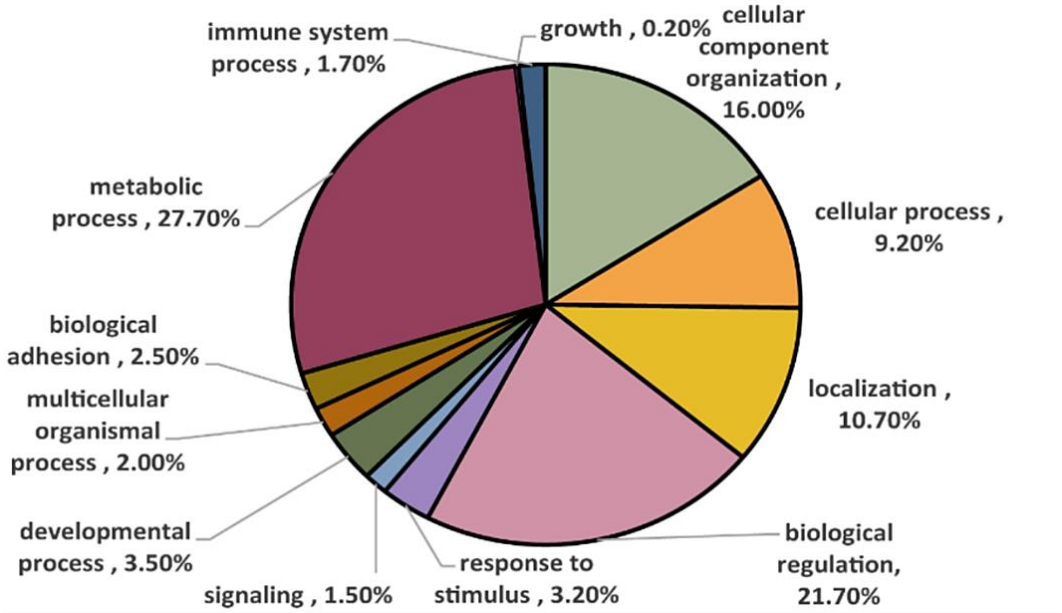

Figure 33. GO annotation of differentially expressed proteins (FcGRIIb+/+ compared with FcGRIIb−/−) in biological processes.

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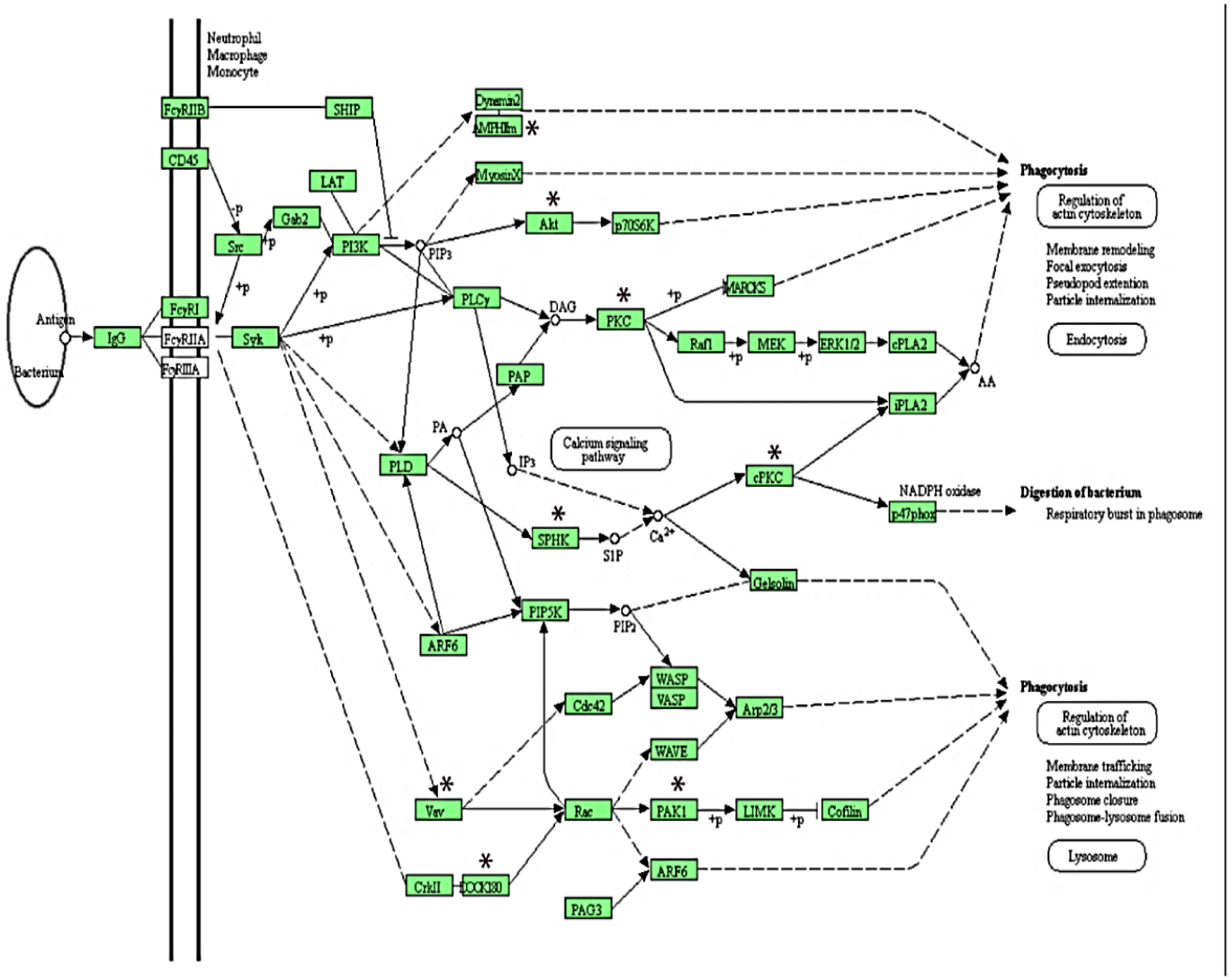

Figure 34. The enrich pathway of the phosphoproteome.of macrophages.

FcGRIIb+/+ compared with FcGRIIb−/− was related to the phagocytosis pathway that MPHIm, Akt, PKC, SPHK, PAK1, Vav, and DOCK180 (in black star) were the proteins involved in this pathway.

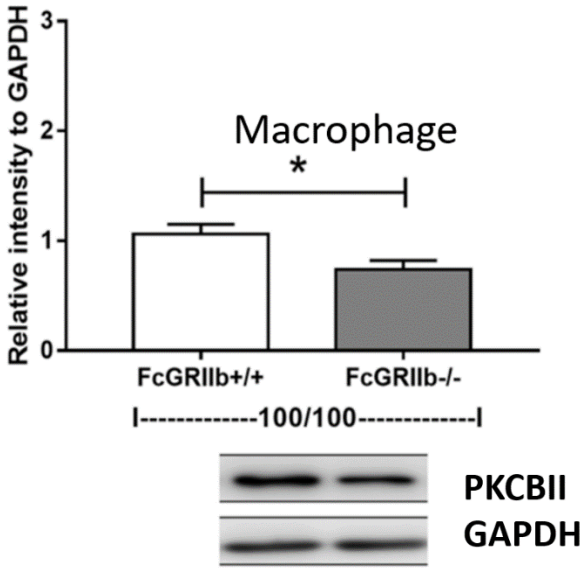

Figure 35. The abundance of protein kinase C-β type II (PKCB) shown by Western blot analysis from a bone marrow derived macrophage cell in sequential LPS activation (100/100; LPS-tolerance).

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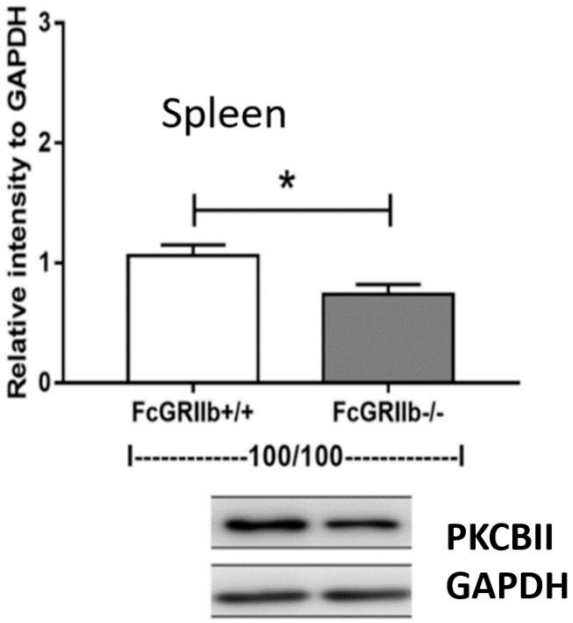

Figure 36. The abundance of protein kinase C-β type II (PKCB) shown by Western blot analysis from a spleen in mice cell in sequential LPS activation (100/100; LPS-tolerance).

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CHAPTER VI
DISCUSSION

The immune response of FcGRIIb-/-mice is enhanced due to inhibitory signaling defects and the effective eradication of several organisms has been reported (7, 8, 68). We hypothesized that enhanced tolerance might occur with enhanced LPS stimulus. We therefore tested the immune response utilizing sequential LPS stimulation in FcGRIIb-/ -mice and macrophages in comparison to wild-type. Endotoxin tolerance is characterized by measuring cytokine responses after repeated LPS stimulation (16, 76). Accordingly, a panel of pro- and anti-inflammatory cytokines was measured in mouse sera after LPS administrations using Luminex-based multiplex analysis. Robust cytokine responses were observed after the first LPS stimulation in FcGRIIb-/-mice compared with wild-type. This corresponded to previously reported immune response enhancement (7). With a second LPS exposure, at a five times higher dose than the first one, all of these cytokines were not higher but, in contrast, were lower than the first response in both groups of mice. This demonstrated endotoxin tolerance. Despite knock-out and wild-type mice producing similar serum cytokines after the second LPS stimulus, the differences between cytokine levels after the first and the second LPS stimulations and the degree of the tolerance determined by the ratio of the ratio of AUC-CR (Figure 9) were more prominent in FcGRIIb-/-mice. Subsequently, surgically induced sepsis (CLP) was used to model a clinical context in which endotoxin tolerance could be evaluated. Although sepsis was more severe in mice with LPS preconditioning in both FcGRIIb-/ -and wild-type, FcGRIIb-/- mice demonstrated a higher mortality rate and worse blood marker levels (bacterial count, cytokines, Scr, and ALT). Interestingly, FcGRIIb-/- mice seems to be more tolerable to the repeated LPS stimulations, a non-bacteremia model, but more susceptible to bacteremia model (CLP). Our data indicate that repeated-LPS stimulation induced endotoxin tolerance and worsened polymicrobial infection and its consequences. More severe sepsis in CLP after sequential LPS administration demonstrated the adverse effect of endotoxin tolerance

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which might be due to the reduced serum cytokines at the early phase of CLP (3 and/or 6 h). With adequate organism eradication, endotoxin tolerance limits the over-expression of cytokines and attenuates sepsis (141, 142). Without antibiotic for microorganism control, as in our model, low cytokine concentrations in the early phase of sepsis (a manifestation of endotoxin tolerance *in vivo*) may reduce blood bacterial control. This supports the necessity of innate immune cytokine elevation to control microorganisms in the early phase of infection. However, in the late phase of sepsis (18 h), cytokines were higher in FcGRIIb-/-mice. This may be due to more severe organ injury-induced cytokine production. The *in vitro* data was consistent with the *in vivo* data. Endotoxin tolerance was more prominent in FcGRIIb-/ -macrophages and might also occur in other cell types. IL-5 and IFN-γ that are mainly produced by mast cell and NK cell, respectively, demonstrated tolerance responses. Indeed, functional FcGRIIb effects have been reported in all of these cells (7, 68, 143). FcGRIIb-/-macrophages demonstrated endotoxin tolerance in all LPS regimens with nearly all cytokines (Figure 8) in the higher degree than wild-type cells (Figure 19). In contrast, wild-type macrophages showed tolerance only with TNF-α and IL-6 expression with only a few LPS-conditions. Interestingly, the higher dose of LPS stimulation (LPS100/ 100), but not the lower dose (LPS10/10) induced the tolerance of all cytokine responses in FcGRIIb-/ -macrophages (Table 5). Hence, the proper doses of LPS were necessary to demonstrate endotoxin tolerance. This implied that there might be a cut-point level of endotoxin to induce a clinical significant endotoxin tolerance in patients. More studies are needed.

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Because of the high incidence of FcGRIIb dysfunction polymorphism in Asian populations (144), FcGRIIb–/– mice might be one of the good representative models of lupus in Asian populations. The activity of the endotoxin against FcGRIIb–/– macrophages supported the idea of cross-talk between Fc gamma receptors and Toll-like receptor-4, as previously described (145). The characteristics of prominent endotoxin exhaustion of FcGRIIb–/– macrophage. Although reduced cytokine production after the second LPS stimulation is a main characteristic of macrophage endotoxin-tolerance (16), the data on other macrophage functions after LPS tolerance

induction is still lacking. Indeed, in wild-type macrophages, LPS tolerance reduced cytokine. production without the influence on phagocytosis and microbicidal activity. In contrast, LPS tolerance in FcGRIIb−/− macrophages reduced all these functions, at least in part, supporting the notion of increased susceptibility to infection in FcGRIIb−/− mice over the wild-type mice, as we reported previously (20). While LPS tolerance affected only TNF-α and IL-6 production in wild-type macrophages, LPS tolerance also impaired other pathogen control mechanisms (phagocytosis and microbicidal activity) in FcGRIIb−/− cells as a mechanism possibly responsible for the significant LPS exhaustion in lupus. Indeed, the importance of LPS tolerance in bacterial sepsis has been described (16) and infection with Gram negative bacteria (the source of LPS) is one of the leading causes of death in lupus (146). Likewise, enhanced endotoxin tolerance might be, at least in part, responsible for the increased mortality rate of patients with lupus, which is a major cause of death of young females in the US (147). Hence, the mechanistic study of LPS tolerance might improve the infection control in patients with lupus.

Protein Kinase C-β Type II (PKCB), One of the responsible mediators of the prominent LPS tolerance in FcGRIIb−/−.Our phosphoproteomic analysis demonstrated a lower abundance of several phosphoproteins in the FcGRIIb−/− macrophages over wild-type ones. Among them, PRKCB was an intriguing protein responsible for several signaling cascades (148). In addition, PKCB also decreased in LPS-tolerant FcGRIIb−/−macrophages and in the spleens of FcGRIIb−/− mice with sequential LPS administration. Moreover, LPS can stimulate ET as determined by decreased PKCB2, cytokine levels, phagocytosis and bacterial killing activity in FcGRIIb−/− more predominantly than wild-type macrophage (Figure 37). Indeed, several subtypes of protein kinase C are associated with immune responses (149). The defect of protein kinase C-β type I and -β type II in mice induced a marked immunodeficiency (150). However, the research involving this specific type of protein kinase C (-β type II: PKCB) in macrophages is still limited. The KEGG pathway analysis demonstrated that the PKCB pathway was downstream of FcGR signaling (Figure 30). The data suggest the influence of PKCB downstream of LPS stimulation in macrophages.

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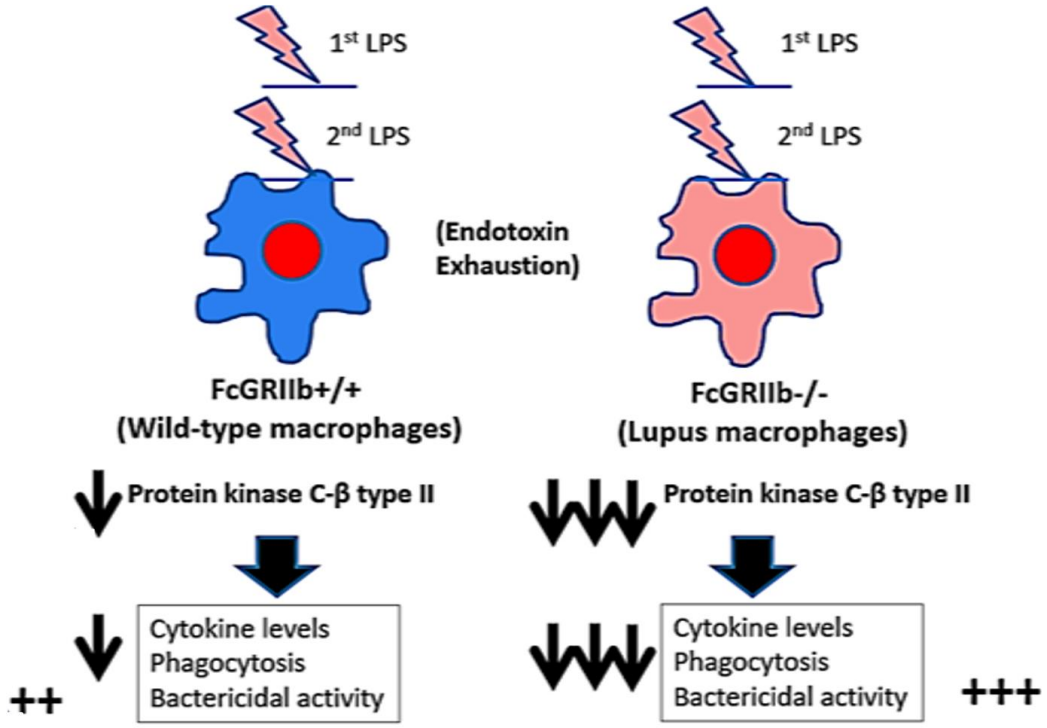

Figure 37. Effect on LPS stimulated exhaustion in macrophage from 100/100 ng/ml in the both *in vitro* and *in vivo*.

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CHAPTER VII
CONCLUSION

Endotoxin tolerance in FcGRIIb-/ -macrophages showed in several functional defects, cytokine responses and killing activity. In contrast, tolerance in wild-type cells was demonstrabled only by cytokine responses. Endotoxin tolerance of FcGRIIb-/-and wild-type cells may be mediated by different pathways. Studies to explore the mechanistic differences of endotoxin tolerance in these two backgrounds are warranted. In the translational aspect, we demonstrated a potential model of the impact of the repeated infections/stimulations in patients with sepsis that easily progress into more severe infections. Indeed, increased circulating endotoxin in patients with sepsis was demonstrated and lupus-monocytes expressed the transcriptome that could be replicated in control monocytes by LPS stimulation (77). Their study implied chronic LPS exposure in patients with sepsis. Together with an increased susceptibility to infections in sepsis patients (5), high bacterial susceptibility due to LPS induced immune-response exhaustion was possible, at least, in some patients. As such, repeated LPS exposures might be more harmful in patients with lupus than in healthy persons. However, our results possibly only apply to infectious conditions in certain patients with bacterial infections in which LPS is released and might be limited to polymicrobial sepsis. More studies are needed. In short, the greater prominence of endotoxin tolerance in FcGRIIb/ mice over wild-types is possibly due to macrophage-exhaustion induced the defects in micro-organism-control and severe sepsis. Perhaps chronic exposure of LPS in patients with loss-of-function polymorphisms in FcGRIIb should be concerned.

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In conclusion, the prominent LPS-tolerance of FcGRIIb–/– macrophages over wild-type ones was, in part, due to the significantly depleted PKCBII. The levels of PKCBII were reduced in LPS-tolerant macrophages and in the spleens of LPS-tolerant mice. There is a more severe in LPS tolerance of FcGRIIb –/– macrophage than wild-type cell. The reduced PKCBII is demonstrated in LPS tolerance of FcGRIIb-/- macrophage and spleen. PKCBII may be an interesting target for patients with sepsis-

induced LPS tolerance. The results may be useful for sepsis treatment in these patients in the future. PKCBII might be considered a novel target for the treatment of LPS-tolerance in lupus.

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APPENDIX

APPENDIX A

CHEMICAL AGENTS AND INSTRUMENTS

General Reagents

0.25% Trypsin in 1 mM EDTA (Hyclone, USA)

Absolute alcohol (Merck, Germany)

anti-F4/80 and anti-CD11b (Biolegend, USA)

Ammonium chloride (NH_4Cl) (Sigma-Aldrich, USA)

Beta-mercaptone (Sigma-Aldrich, USA)

Bovine serum albumin (BSA) (Sigma-Aldrich, USA)

DMEM Free serum medium (Hyclone, USA)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)

EASY-nLC1000 1100 system coupled to Q Exactive Orbitrap Plus mass spectrometer (Thermo scientific, CA)

EDTA (Merck, Germany)

Fetal bovine serum (FBS) (Hyclone, USA)

Formic acid (FA,Sigma)

Fractionated by high pH reversed phase peptide fractionation kit (Thermo Scientific, USA.)

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Glycerol (Merck, Germany)

HEPES (Hyclone, USA)

Horse serum (Hyclone, USA)

Mouse TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ ELISA kit (eBioscience, USA)

Lipopolysaccharide (LPS) (Sigma-Aldrich)

Microcentrifuge tube 0.2,0.7 and 1.5 ml (Eppendorf, USA)

MTS assay kit (Promega Corporation, USA)

Pierce BCA Protein assay kit (BSA assay kit)(Thermo Scientific,USA.)

Phospho-PKCBII (Thr 641) primary antibodies (Thermo scientific Rockford,IL,USA)

Protease inhibitor (Sigma-Aldrich, USA)

Phosphatase inhibitor (Sigma-Aldrich, USA)

Rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnoogy)

Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA)

Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Germany)

Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, Germany)

Sodium chloride (NaCl) (Sigma-Aldrich, Germany)

Sodium dihydrogen phosphate (NaH_2PO_4) (Sigma-Aldrich, Germany)

Sodium Pyruvate (Hyclone, USA)

Steptomycin/Penicillin G (Hyclone, USA)

Sulfuric acid (H_2SO_4) (Sigma-Aldrich, Germany)

Trypan blue solution (Hyclone, USA)

Tris (Sigma-Aldrich, Germany)

Tween20 (Merck, Germany)

Research instruments

-20°C and -80 °C Freezer (Sanyo, Japan)

0.22 µm Surfactant-free cellulose acetate membrane filters (Sartorius Stedim Biotech GmbH, Germany)

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96-Well Flat-bottom tissue culture plates (Nunclon D, Denmark)

Autoclave (Hirayama, Japan)

Auto pipette: P-10, P20, P-100, P-200 and P-1000 (Gilson, France)

Auto pipette: P-10, P20 and P-1000 (Socorex, Switzerland)

Barrier tips; 10, 20, 200, and 1,000 µl (Neptune, Mexigo)

Biological safety cabinet Class II (Astec-Microflow, Bioquell UK Ltd, UK)

Chemiluminescence detection system (Thermo-scientific Rockford,IL,USA)

CO_2 incubator (BINDER GmbH, Germany)

Conical centrifuge tube; 2, 15 and 50 mL (Nunc, USA)

Cryovial (Nunc, Denmark)

CytoSpin chamber (Thermo Scientific, USA)

Electrophoresis (Wealtec, Taiwan)

Equipment gel electrophoresis apparatus (Bio-Rad Laboratories)

Heat block (Scientific Industries, Inc, USA)

Hemacytometer (Bright line) (BOECO, Germany)

Hemocytometer (Boeco, Germany)

Hotplate (Stuart, Germany)

Incubator (Memmert GmbH, Germany)

Inverted microscope (Olympus, Japan)

LightCycler 2.0 Instrument (Roche, Germany)

Micropipettes (Gilson, France)

Multi channel Auto pipette: 10, 20 and 300 ul (Eppendorf, USA)

Nano DropTM 1000 Spectrophotometer (Thermo Fisher Scientific, Inc, USA)

pH meter (Thermo Fisher Scientific, Inc, USA)

Refrigerated centrifuge (Sanyo, Japan)

Serological pipettes; 2, 5, 10, 25 and 50 mL (Corning, USA)

Spectrophotometer (Bio-Rad Smart SpecTM Plus, Bio-Rad Laboratories, Inc,USA)

Syringe (Nipro, Thailand)

Tissue culture flask; 25, 75 and 125 cm2 (Nunc, Denmark)

Tissue culture plates; 6, 12, 24 and 96 wells (Nunc, Denmark)

Ultrasonic water bath (GEN-PROBE, Germany)

UV Transilluminator (Bio-Rad, USA)

Vertical Laminar Flow workstation (Microflow, UK)

Water bath (MemMert GmbH, Germany)

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APPENDIX B

BONE MARROW DERIVED MACROPHAGE

1. Media preparation

1.1 DMEM completed media

DMEM serum free media (high glucose)	100 ml
Fetal bovine serum (FBS)	10 ml
HEPES	1 ml
Sodium Pyruvate	1 ml
Steptomycin/Penicillin G	1 ml

1.2 BMM media

DMEM completed media	80 ml
Horse serum	5 ml
L929 cell culture supernatant	20 ml

1.3 Preparation of freezing media

DMEM (without serum) + 20% FBS	500 ul
DMEM (without serum) + 20% FBS+ 20%DMSO	500 ul

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2. L929 culture supernatant preparation

NCTC clone 929 [L cell, L929, derivative of Strain L] (ATCC® CCL-1™)

(1) Thaw NCTC clone 929 c ells and washing by DMEM serum free media, centrifuge 1000 rpm, 10 minute at 4°C.

(2) Add DMEM completed media 8 ml, culture cells in 5% CO2, 37 °C.

(3) Cell growth at 80% confluent before passage or collection of supernatant

(4) Filtrate supernatant using 0.2 um Surfactant-free cellulose acetate membrane filters.

(5) Collection and Freeze cells by using freezing media, keep in -80 °C for overnight before transferring to liquid N_2 for long storage.

3. Red Blood Cell lysis buffer (Ammonium chloride lysis)

EDTA	0.018	g
$KHCO_3$	0.5	g
NH_4Cl	4.15	g

Made up to 500 ml in distilled (DI) water

Autoclave before use.

Resuspend cell in 1 ml media, add to 9 ml lysis buffer (ratio 1:10)

Incubation on ice 3 min.

4. Protocol

(1) FcgRIIb -/- mice on C57BL/6 background and wild type mice were sacrified and collected femur bone.

(2) Bone were suspended in DMEM serum free (with Steptomycin/Penicillin G) on sterile petri dish.

(3) Bone were resuspended in DMEM serum free (with Steptomycin/Penicillin G) in 15 ml centrifuge tube on ice to transfer to tissue culture room

(4) Pour bones and media on steriled petri dish in laminar flow

(5) Prepare 0.5 ml steriled microcentrifuge tube with a pore at the bottom of tube and then put on 1.5 ml steriled microcentrifuge tube and on ice.

(6) Cut the bone marrow by using scissors at header and footer of bone and put a piece of bones in steriled microcentrifuge tube with pore.

(7) Centrifuge 6,000 rpm for 10 min at 4 °C. Bone marrow cells were pelleted on bottom of 1.5 ml steriled microcentrifuge tube.

(8) Add 1 ml of DMEM serum free in cell pellet and dispersed by pipette up and down.

(9) To eliminate red blood cell, prepare 9ml Red Blood Cell lysis (RBC lysis or NH4Cl solution) in 15 ml centrifuge tube

(10) Add 1 ml of cell suspension in 9ml RBC lysis (ratio 1:10) and incubate for 5 min on ice

(11) Centrifuge 1,000 rpm, 10min, 4 °C and discard supernatant

(12) Add BMM media 1ml/pellet and dispersed by pipette up and down and pool cell suspension in 50 ml centrifuge tube on ice

(13) Count bone marrow cells with 0.4% trypan blue.

(14) Plate cell on petri dish at $5x10^6$ cells/plate in 8 ml BMM media

(15) Incubate at 37 °C, 5%CO_2

(16) Add fresh BMM media 3 ml at day 4 and incubation until 7 day

(17) Harvest BMM cells at day7 of incubation by using cold-PBS

(18) Count BMM cell and feed cells into tissue culture well plate following our experimental design.

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APPENDIX C

FLOW CYTOMETRY

1. Media preparation

1.1 FACS staining buffer

PBS

1% (v/v) Fetal Calf Serum or FBS

1.2 Permeabilization Buffer (fix cells)

PBS

1% FCS

0.1% Saponin

2. Bone marrow derived macrophage marker by Flow cytometer

1. Wash cells in cold 1x PBS once
2. Resuspend BMM cells in 200ul staining buffer
3. Add fluorochrome-conjugated antibody to cell suspension (1ul CD11b PE (FL2) or 1 ul F4/80 Biotin or Alexa Fluor® 488 + 10 ul Strepavidin ECD (FL3)/sample) in dark room
4. Incubate on ice 15 min
5. Wash cells 3x in staining buffer

Note: This step can fix cells with 4% paraformaldehyde on 15 minutes, wash and store in 1xPBS at 4 °C until use.

6. Directly analysis on Flow cytometer

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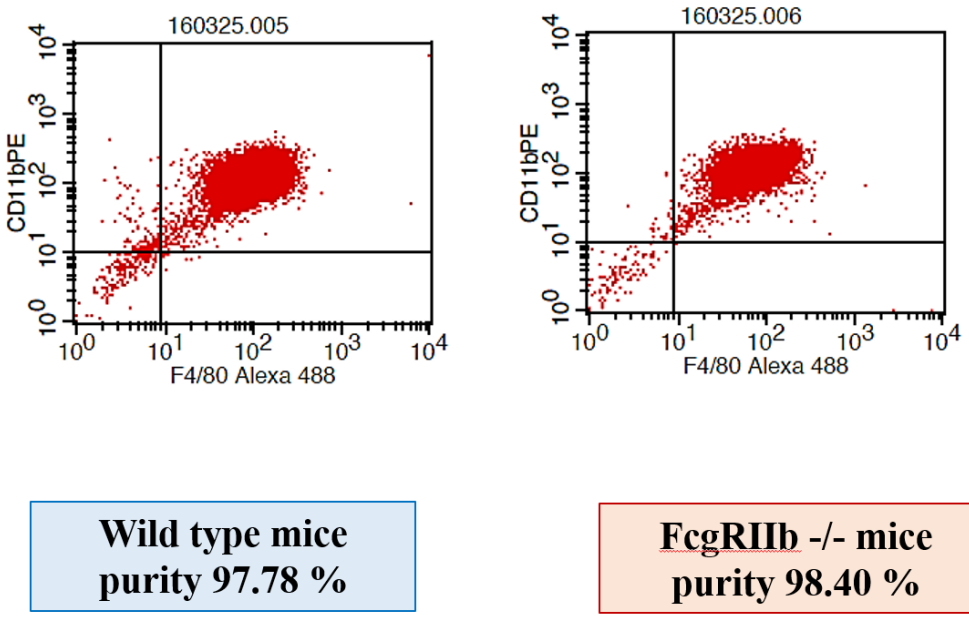

Figure 38. Flow cytometry show bone marrow derived macrophage marker by using the F4/80 and CD11b Ab For FcgRIIb -/- and wild type mice.

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