Effects of lipopolysaccharide on mandibular and tibial bone regeneration

in a murine model of SLE induced by *FcγRIIB* deficiency

Miss Sirikanda Jantaboon

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ผลของไลโปโพลิแซกคาไรด์ต่อการสร้างใหม่ของกระดูกขากรรไกรล่างและกระดูกหน้าแข้ง
ในโมเดลหนูเมาส์ที่เป็นโรคเอสแอลอีจากการขาดยีนเอฟซีแกมมารีเซพเตอร์ทูบี

น.ส.สิริกานดา จันทบูรณ์

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By	Miss Sirikanda Jantaboon
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Thesis Advisor	Associate Professor SUTADA LOTINUN, Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.... Dean of the GRADUATE SCHOOL

(Associate Professor YOOTTHANA CHUPPUNNARAT, Ph.D.)

THESIS COMMITTEE

.... Chairman

(Assistant Professor SUKANYA JAROENPORN, Ph.D.)

.... Thesis Advisor

(Associate Professor SUTADA LOTINUN, Ph.D.)

.... Examiner

(Associate Professor Thongchai Sooksawate, Ph.D.)

.... Examiner

(Associate Professor CHALIDA LIMJEERAJARUS, D.D.S., Ph.D..)

.... External Examiner

(Associate Professor Prapaporn Pisitkun, M.D.)

สิริกานดา จันทบูรณ์ : ผลของไลโปโพลิแซกคาไรด์ต่อการสร้างใหม่ของกระดูกขากรรไกร
ล่างและกระดูกหน้าแข้งในโมเดลหนูเมาส์ที่เป็นโรคเอสแอลอีจากการขาดยีนเอฟซี
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แม้ว่าโรคแพ้ภูมิตัวเอง (systemic lupus erythematosus: SLE) จะเป็นที่รู้จักว่าคือ
โรคภูมิคุ้มกันต้านตนเองที่กระตุ้นการอักเสบและความบกพร่องของกระดูก แต่ความรู้เกี่ยวกับกลไก
ที่นำไปสู่การลดลงของการสร้างกระดูกใหม่ยังคงมีไม่เพียงพอ โดยพบว่าผู้ป่วยที่เป็นโรค SLE มี
อุบัติการสูญเสียกระดูกสูงอันเนื่องมาจากการตอบสนองทางภูมิคุ้มกันของโฮสต์ที่มีการชักนำให้เกิด
สภาวะต่อต้านเชื้อโรค งานวิจัยนี้ใช้ไลโปโพลิแซกคาไรด์ (lipopolysaccharide: LPS) ทั้งแบบ
ออกฤทธิ์เฉพาะจุดและแบบฉีดเข้าสู่กระแสเลือดทั่วร่างกายเพื่อจำลองการติดเชื้อในหนูโมเดลโรค
SLE หรือ *FcγRIIB*$^{-/-}$ จากผลการวิจัยพบว่า การขาดยีนส์ *FcγRIIB* ในหนูเมาส์ที่ได้รับ LPS แบบ
เฉพาะที่และทั่วร่างกาย ส่งผลให้ปริมาณกระดูกบริเวณที่เจาะรูของกระดูกขากรรไกรล่างและ
กระดูกหน้าแข้งลดลงเมื่อเปรียบเทียบกับกลุ่ม WT, *FcγRIIB*$^{-/-}$ และ WT+LPS สอดคล้องกับผล
การย้อมสีอะนิลีนบลูที่พบว่ามีการลดลงของอัตราส่วนแร่ธาตุ/คอลลาเจน (mineralization to
collagen ratio) ลดลงเช่นกัน ซึ่งจากผลการศึกษาโดยวิธี qPCR พบว่า การขาดยีนส์ *FcγRIIB*
ร่วมกับการให้ LPS ส่งผลให้มีการเพิ่มการแสดงออกของยีน osteoclast แต่ลดการแสดงออกของ
ยีน osteoblast ซึ่งบ่งชี้ถึงการลดลงของการสร้างกระดูกใหม่ นอกจากนั้นระดับ TNF-α, IL-6
และ MCP-1 ในซีรั่มยังสูงในหนู *FcγRIIB*$^{-/-}$ + LPS บ่งบอกถึงผลของ proinflammatory
cytokine ที่ถูกกระตุ้นด้วย LPS ทำให้เกิดการลดลงของเซลล์สร้างกระดูกที่มีบทบาทสำคัญในการ
สร้างคอลลาเจน ดังนั้นจึงสรุปได้ว่า LPS สามารถยับยั้งการสร้างกระดูกใหม่และการสะสมคอลลา
เจนในกระดูกขากรรไกรล่างและกระดูกหน้าแข้งของหนูเมาส์ที่ขาดยีนส์ *FcγRIIB* เนื่องจากการ
เพิ่มปริมาณของ proinflammatory cytokine ในซีรั่ม

สาขาวิชา	สรีรวิทยา (สหสาขาวิชา)	ลายมือชื่อนิสิต ....
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Although systemic lupus erythematosus (SLE) is known as the autoimmune disease that triggers inflammation and bone impairment, the mechanisms leading to the decrease in bone regeneration remain inadequately characterized. Patients with SLE have been observed high prevalence in bone loss due to host immune responses that may induce host antimicrobial activities. Here, this study used systemic and local lipopolysaccharide (LPS) administration for mimicking infectious condition in $Fc\gamma RIIB^{-/-}$ mice as a lupus murine model. The results showed $Fc\gamma RIIB$ lacking $Fc\gamma RIIB^{-/-}$ in mice with local and systemic LPS led to decreased cancellous bone volume in their drilled hole region of tibial and mandibular bone compared to WT, $Fc\gamma RIIB^{-/-}$, and WT+LPS groups. Likewise, the data of aniline blue staining in histological analysis showed a decrease of mineralization/collagen ratio in $Fc\gamma RIIB$-deficient activated by local and systemic LPS compared to others. Deleting $Fc\gamma RIIB$ with LPS administration increased osteoclast gene expression but decreased osteoblast gene expression indicating the reduction of bone regeneration. Moreover, serum TNF-α, IL-6, and MCP-1 level was high in $Fc\gamma RIIB^{-/-}$+ LPS mice. The induction of proinflammatory cytokines may cause LPS-induced osteoclast formation and a decrease in osteoblasts that have an important role in collagen production. Herein, LPS administration could

Field of Study: Physiology   Student's Signature ....

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LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
BCR	B cell antigen receptor
BMD	Bone mineral density
BMPs	Bone morphogenic proteins
BMUs	Bone multicellular units
BV/TV	Cancellous bone volume
Conn.D,	Connectivity density
DCs	Dendritic cells
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
FcγR	Fc gamma receptor
FcγRI	Fc gamma receptor I
FcγRIIA	Fc gamma receptor IIA
FcγRIIB	Fc gamma receptor IIB
FcγRIIC	Fc gamma receptor IIC
FcγRIII	Fc gamma receptor III
FcγRIIIA	Fc gamma receptor IIIA
FcγRIIIB	Fc gamma receptor IIIB
FcγRIV	Fc gamma receptor IV
GM-CSF	Granulocyte macrophage colony stimulating factor

IACUC	Institutional Animal Care and Use Committee
ICs	Immune complexes
IgG	Immunoglobulin G
IFN-α	Interferon alpha
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-15	Interleukin-15
IL-12	Interleukin-12
IL-12p70	Interleukin-12p70
IL-17	Interleukin-17
IL-17A	Interleukin-17A
IL-23	Interleukin-23
IL-27	Interleukin-27
i.p.	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif

ITIM	Immunoreceptor tyrosine-based inhibition motif
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MSCs	Mesenchymal stem cells
NBF	Neutral buffered formalin
NETs	Neutrophil extracellular traps
NFATc1	Nuclear factor of activated T cells 1
NF-κB	Nuclear factor kappa B
OCN	Osteocalcin
OPN	Osteopontin
pDCs	Plasmacytoid dendritic cells
PI3K	Phosphatidylinositol-3-kinase
qPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SHIP	SH2-containing inositol 5-phosphatase
SLE	Systemic lupus erythematosus
SMI	Structural model index
Tb.N	Trabecular number
Tb.Th	Trabecular thickness

Tb.Sp	Trabecular separation
TGF-β	Transforming growth factor-beta
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase
V-ATPase	Vacuolar H+ adenosine triphosphatase
WT	Wild type
μCT	Micro-computed tomography

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Fc gamma receptor IIB (FcγRIIB) is an only inhibitory receptor of Fc receptor (FcγR) family. It plays important roles in immune and inflammatory responses, negatively regulates immune cells to clear the accumulation of immunoglobulin G (IgG) immune complexes (ICs) and (1, 2) and maintains B cell tolerance. Changes in B cell expression and function may contribute to the etiology of autoimmunity. Systemic lupus erythematosus (SLE) is one of the autoimmune diseases with excessive deposition of ICs and B cell hyperactivity resulting in the increased accumulation of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-17 (3). These proinflammatory cytokines could increase receptor activator of nuclear factor kappa-B ligand (RANKL), which is the part of TNF superfamily member. Their major role is to stimulate osteoclast differentiation and bone resorption, although their role in inflammatory disorders is questionable. Patients with SLE have osteoporotic fracture and around 50% of SLE patients have a lower density of bone with over 10% of them experience osteoporosis early (4). In murine model, previous studies indicated that male mice with *FcγRIIB* deletion at 6 months old had cancellous bone loss in long bones and mandibles (5, 6).

Bone regeneration is a process of bone formation that occurs during normal fracture healing and continues throughout life. The process of bone healing

is considerably based on the inflammatory condition, which is driven by immune cells and bone progenitor cells, the primary regulators of bone repair. This process may negatively influence bone formation due to the accumulation of inflammatory cytokines (7).

Endotoxin called lipopolysaccharides (LPS) can trigger the substantial number of proinflammatory cytokines to enhance inflammatory process (8). Both male and female SLE patients have significantly greater levels of sCD14, a co-receptor for LPS produced by monocytes/macrophages. Earlier studies in rodent models showed higher circulating proinflammatory markers (IL-1, IL-6, and TNF-α) and more severe impaired bone formation in C57BL/6J mice given a varied dose of slow release LPS pellets (9).

However, the effect of *FcγRIIB* deficiency with local and systemic LPS administration on mandibular and tibial bone regeneration had not yet been investigated. Therefore, this present study aims to investigate the mechanism of LPS on bone regeneration of the mandible and tibia in *FcγRIIB*$^{-/-}$ mice compared to their WT littermate.

1.2 Research question

1.2.1 What are the effects of LPS on mandibular and tibial bone regeneration in a murine model of SLE induced by *FcγRIIB* deficiency?

1.2.2 What is the mechanism of LPS on mandibular and tibial bone regeneration in a murine model of SLE?

1.3 Research objectives

1.3.1 To investigate the effects of LPS on mandibular and tibial bone regeneration in a murine model of SLE induced by *FcγRIIB* deficiency.

1.3.2 To investigate the effects of LPS on pro-inflammatory cytokine in a murine model of SLE induced by *FcγRIIB* deficiency.

1.4 Research hypothesis

1.4.1 LPS decreases mandibular and tibial bone regeneration in a murine model of SLE induced by *FcγRIIB* deficiency.

1.4.2 LPS decreases mandibular and tibial bone regeneration via increasing pro-inflammatory cytokine to increase bone resorption in a murine model of SLE induced by *FcγRIIB* deficiency.

1.5 Conceptual framework
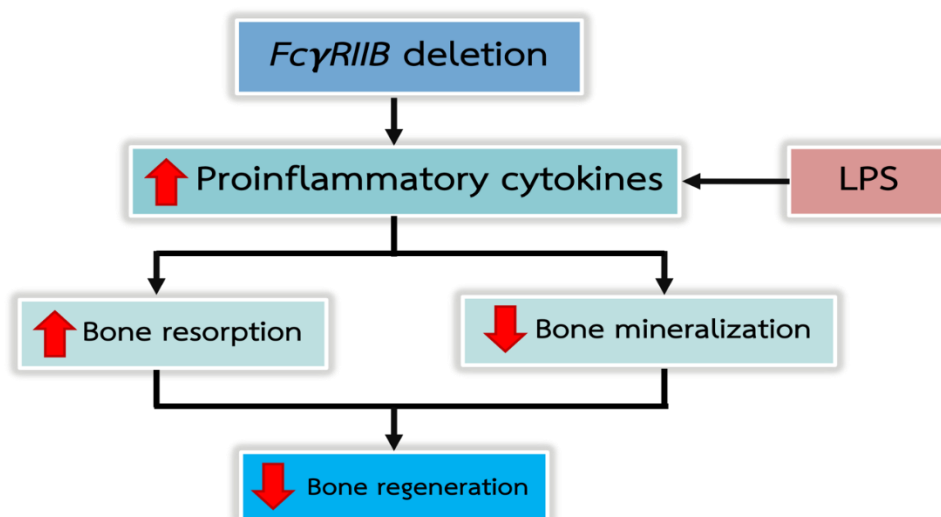
CHAPTER II

LITERATURE REVIEWS

2.1 Basic physiology of bone regeneration

Complicated and well-coordinated physiologic processes of bone formation are involved in bone regeneration process, and bone remodeling. Bone regeneration is required for bone defect caused by trauma, malignant tumor resection and infection as well as in clinical process including orthopedic surgery and maxillofacial intervention. There are several clinical techniques to increase bone regeneration that used to regenerate and repair long bone defects, namely, bone-graft. Using autologous bone is regarded as the 'gold standard' since it has all the features necessary in a bone-graft material including osteogenesis by mesenchymal stem cells; MSCs or osteoprogenitors of osteoblasts, osteoinduction by bone morphogenic proteins; BMPs or other growth factors e.g. transforming growth factor-beta; TGF-β, and osteoconduction or scaffold (10). Osteoconduction capabilities are commonly applied in clinical practice. One of the most important grafting scaffold is collagen, which can extend bone grafts and provide modest structural support. Additionally, the grafting material also enhances the development of bone cells that are necessary for bone regeneration. Research is continuous to develop biomaterial structure of scaffold to stimulate osteoblast adhesion, as well as to allow for vascular growth and bone tissue formation. Several col lagen scaffolds are used in skeleton tissue engineering including Avitene™

UltraFoam™ sponge, or microfibrillar bovine collagen-based hemostat is necessary for adjunctive hemostasis during various types of surgery (11).

2.2 Bone remodeling

2.2.1 Basic knowledge of bone remodeling

The human bone undergoes ongoing remodeling throughout life. Bone remodeling is a dynamic and continuous process, which is regulated by a coordinated coupling of resorption and formation by removing damaged bone and replacing it with new bone. Bone remodeling is important for controlling mineral homeostasis or keeping blood calcium levels stable. It also helps to repair microarchitectural defects that can cause small amounts of bone damage. A change in the balance of bone remodeling cause metabolic bone disorders including osteoporosis, which is shown to be higher in osteoclastic bone resorption than osteoblastic bone formation (12).

2.2.2 Cells involved in bone remodeling

Bone multicellular units (BMUs) are a group of bone cells that play a role in bone remodeling. They consist of osteoblasts, bone-lining cells, osteocytes, and osteoclasts. Osteoblasts and osteoclasts mediate the dynamic balance between bone synthesis and resorption in order to keep bone homeostasis. However, other BMUs have particular activity, and also influence the recruitment of collaborating cells (13).

2.3 Bone remodeling cycle

Bone remodeling cycle consists of activation, resorption, reversal, formation, and termination phase.

2.3.1 Activation phase

After being exposed to bone microcracks, local apoptotic osteocytes cause the generation of signaling molecules, such as sclerostin which suppresses osteoblast formation. This inhibition, BMUs are not activated in areas that bone remodeling is not required. In this initial stage, a raised-form continuous layer called a canopy is produced over the spot that will be resorbed by osteoclasts in the following step. In addition, the lining cells release collagenase, which digests the bone matrix layer, allowing the bone surface to be revealed and exposed (14).

2.3.2 Resorption phase

In the canopy area, osteoclasts migrate in order to resorb old bone matrix. They attach tightly to the bone matrix by integrin, which is a dynamic ring composed of tight cluster podosomes under the ruffled border to attach osteoclasts onto the bone matrix. Osteoclasts begin to produce digestive enzymes and transport ions into lacunae to acidify the environment with a pH of 4.5, which is performed by the function of the vacuolar H+ adenosine triphosphatase (V-ATPase) proton pump in the ruffled border (15). These ions are necessary for degrading bone mineral and increasing the enzymes capacity to digest the organic components of bone,

such as cathepsin K, which functions as a collagenase, matrix metalloproteinases, and phosphatases (tartrate-resistant acid phosphatase; TRAP). This phase ends when osteoclasts in Howship's lacunae, the small resorption cavities, undergo apoptosis.

2.3.3 Reversal phase

Following the aftermath of osteoclast activity, the Howship's lacunae are covered with undigested demineralized collagen matrix, which can cause scarring. Osteoblasts are recruited to form the new bone matrix after bone resorption occurs, and bone-lining cells migrate to the lacunae in order to remove any remaining collagen fragments in the bottom of the cavities. Osteoblasts synthesize cement line with non-collagenous mineralized layer enriched (16) in osteopontin (OPN) (17). As a result, OPN has come to be known as one of osteoblastic cell differentiation markers. the phosphorylated glycoprotein that plays critical roles in embedding osteoblasts stimulated by the regulatory cytokines and TGF-β. (18).

2.3.4 Formation phase

This is the most time-consuming stage, with a duration of around 90 days. Osteoblasts are formed through the storage of type I collagen fibers by mesenchymal stem cells. Then these osteoblasts form osteoid that serves as a supportive structure for further mineralization. Bone mineralization involves depositing hydroxyapatite

crystals between collagen fibrils, and controls calcium and phosphate ratio inside extracellular matrix vesicles (19).

2.3.5 Termination phase

When an equivalent amount of resorbed bone has been restored. Then after mineralization, mature osteoblasts disappear, and return to a bone-lining cell, bury in the mineralized matrix, and undergo terminal differentiation into osteocytes. Osteocytes play a critical function in generating signaling to terminate the remodeling process by releasing of sclerostin that is an antagonists of osteogenesis signaling. Finally, the resting bone surface environment is restored and maintained before the next remodeling cycle (19).

2.4 FcγRIIB
2.4.1 FcγRIIB and its family

FcγRIIB is the part of FcγRs family. It is the receptor for subclasses of IgG region by representing in IgG-ICs or on IgG-coated cells. It plays the most important roles among other FcγRs, which generally serve as the link between humoral and cellular immune responses by creating a connection between antibodies and effector cells. The FcγRs are heterogeneous group of transmembrane glycoproteins expressed in several immune cells including B cells, T cells, monocytes/macrophages and dendritic cells (DCs) of both mice and humans. The classical FcγRs family contains 4 subtypes conserved in mice: FcγRI, FcγRIIB, FcγRIII, and FcγRIV, and conserved

6 subtypes in humans: FcγRI, FcγRIIA, FcγRIIB, FcγRIIC FcγRIIIA, FcγRIIIB (20). FcγRs have two different types of receptors with opposite role, both types control the balance of cytokine releasing. They consist of activating and inhibitory receptors. The activating FcγRs contain an immunoreceptor tyrosine-based activation motif (ITAM) on FcγRI, FcγRIIA, FcγRIIC FcγRIII and FcγRIV. On the other hand, the only inhibitory receptor is FcγRIIB, and plays an important role in transmitting signals by immunoreceptor tyrosine-based inhibition motif (ITIM) (21). The subtypes of FcγRs, especially FcγRIIA, FcγRIIB, FcγRIIIA, FcγRIIIB has been identified as a genetic factor causing the risk to SLE, which is the most common autoimmune disorder (22).

2.4.2 FcγRIIB signaling pathway in autoimmune diseases

FcγRIIB is unique among other FcγRs due to their role in containing an inhibitory ITIM. FcγRIIB is a potent immunological response inhibitor that plays a part in autoimmune diseases. The B cell antigen receptor (BCR) signaling pathway is responsible for downregulating FcγRIIB in order to modulate the autoimmune response by reducing B cell antibody production. Previous studies indicated autoimmune symptoms are more severe in mice with *FcγRIIB* deletion (6, 23).

FcγRIIB interacts with activating receptors. The FcγRIIB receptor is activated via ITAM signaling, then a glycosylphosphatidylinositol anchor binds FcγRs to the cell membrane (24). After that, the inositol 5-phosphatase (SHIP), the primary enzyme triggered by FcγRIIB and BCR crosslinking, is recruited by attaching to an SH2 site

on the FcγRIIB ITIM. When the ITIM binds to an ITAM-bearing receptor resulting in the transphosphorylation leading to the inhibition of B cell in autoimmune activity that would be generated by mature B cells in SLE (25).

2.4.3 Signaling pathway on the regulation of bone remodeling process

Bone homeostasis is regulated by osteoblastic bone formation and osteoclastic bone resorption. An imbalance in osteoclast activity can happen in the potent autoimmune disease as SLE subsequently leading to severe bone loss. FcγRIIB is an indirect receptor that regulates bone microstructure. The osteoporosis and osteopenia phenotypes in *FcγRIIB*$^{-/-}$ mice was recently reported by increasing of osteoclastogenesis, which is caused by *FcγRIIB* deficiency response to deposition of IgG-ICs (36). Moreover, the elevation of TNF-α serum level leads to cortical and trabecular bone loss of long bones in *FcγRIIB*$^{-/-}$ murine model (5, 6).

2.4.4 *FcγRIIB*-deficient model of SLE

2.4.4.1 Origin

This *FcγRIIB*-deficient strain mice were generated by Dr. Silvia Bolland in the Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York. They initially obtained on a hybrid Sv129/C57BL/6 background and mice exhibited no anomalies in growth, lifespan and immunity. After that, the constitutive knockout strain was performed in C57BL/BALB/c mice and the process was repeated for a total of 12 generations until they found the *FcγRIIB*$^{-/-}$B6 were dead due to renal

failure with high levels of proteinuria at 5 months old (24). After developing completed $Fc\gamma RIIB^{-/-}$ model, this murine model has been used in several studies.

2.4.4.2 Role of *FcγRIIB* gene and its genetic interactions in *FcγRIIB*-deficient mice

FcγRIIB gene is located on chromosome 1, and it is closed to *Sle1* gene and other SLE-related genes loci implying its interaction with others. Bolland et al. illustrated that *FcγRIIB* and *Sle1* genes have an identical pathway that link up to trigger a loss of tolerance to nuclear antigens and subsequent development of antinuclear antibodies. Likewise, genes including *Yaa* and *Lpr* affect the susceptibility of these antibodies. Additionally, *Yaa* worsens the disease by modifying the affinities of autoantibodies (26).

2.4.4.3 Phenotypes

$Fc\gamma RIIB^{-/-}$ at 10 months of age has positive antinuclear antibodies, high spleen weight, and high proteinuria phenotypes (26). Moreover, previous evidence suggested that the lymphocyte phenotype is altered in 8-month-old $Fc\gamma RIIB^{-/-}$ mice. The percentage of $IgM^{low}IgD^{low}CD24^{null}$ B cell population has increased. CD4+ and CD8+ cells in the T cell compartment were higher in these mice. Furthermore, the activation marker, CD69, were also highly expressed on CD4 cells in $Fc\gamma RIIB^{-/-}$ spleens (24).

2.5 SLE

2.5.1 Pathogenesis

2.5.1.1 Innate immune cells in SLE and FcγRIIB

2.5.1.1.1 Neutrophils

Neutrophils are the most plentiful and short-lived type of granulocytes. Neutrophils in SLE pathogenesis are impaired phagocytosis ability causing an increase of apoptotic debris (27), enhanced oxidative activity along with reactive oxygen species (ROS) generation that disturbs apoptosis resulting in a higher apoptosis activity and a lag in the clearing of apoptotic debris. (28), and increased the production of neutrophil extracellular traps (NETs), a kind of cell death that involves the discharge of DNA-histone complexes and microbial enzyme (29). NETs causes pDCs to make type I IFN to increases the amount of NETs in the feedback cycle and promotes diseases spread (30). The role of FcγRIIB in preventing autoimmunity to granular neutrophil by suppressing neutrophil activity (31).

2.5.1.1.2 Macrophages

Macrophages have ability to remove debris left behind by apoptotic cells, allowing adaptive immune cells to be exposed to more possible auto-antigens. The M1 phenotype of macrophages promotes in the inflammatory response by secreting cytokines including TNF-α and IL-12. In contrast, shifting towards an M2 phenotype boosts the immune-modulatory response and stimulates

the release of anti-inflammatory cytokines like IL-10 and IL-4 (32). According to previous studies, FcγRIIB inhibits macrophage phagocytosis in mice (33). THP-1 cells administered IL-4 had their phagocytic capacity reduced by the effects of FcγRIIB (34).

2.5.1.1.3 Dendritic cells (DCs)

DCs link innate and adaptive defense through the production of cytokines. IFN-α release promotes pDCs and T-cell activity, suggesting a connection between adaptive and innate immunity in the pathological process of SLE (35). DCs can generate immunological tolerance and T-cell hyperresponsiveness. By keeping DCs non-proliferative and tolerogenic, FcγRIIB-mediated ITIM signaling helps to prevent the development of autoreactive T cells and the production of autoantibodies (36).

2.5.1.2 Adaptive immune cells in SLE and FcγRIIB

2.5.1.2.1 B cells

In SLE, B lymphocytes play a key part as Antigen-presenting cells (APCs) and the progenitors cells that make autoantibodies. Patients with SLE had a higher rate of class switching from naïve to late memory B cells by BCR activity. Furthermore, SLE patients undergo an impairment in the clearance of autoreactive B cells, even in the inactive stage of the disease (37). An increased number of circulating plasma cells is the hallmark of SLE. Those with SLE

have impaired kidneys function due to an abundance of plasma cells. FcγRIIB controls the activity of B cells by enhancing BCR activation and inhibiting B cell as APCs to T cells causing a decrease in autoimmunity (38). As mentioned above, FcγRIIB ITIM crosslinks with BCR ITAM leading to the negative control of B cell proliferation that causes proinflammatory cytokine release (25).

2.5.1.2.2 T cells

Disruption in Tfh cell activity appears to have a significant role in the aetiology and progression of SLE disease by contributing to the production of pathogenic autoantibodies (39). SLE pathogenesis is influenced by the presence of Th17 cells, a type of T cell which change immune response by the production of IL-17 (40). FcRIIB plays a role in restricting the proliferation of memory CD8+ T cells and reducing their cytotoxicity. FcγRIIB play a role in restricting the proliferation of memory CD8+ T cells and reducing their cytotoxicity (41).

2.5.2 Epidemiology

SLE has been found to present differently in people of varying origin. The incidence and prevalence of SLE worldwide depending on the segregation of region and ethnicity. The prevalence rate is 72.1-74.4 cases per 100,000 individual people per year and incidence rate is 5.6 per 100,000 individual people per years (42). The majority of SLE patients are African-Americans when compared to Caucasians, Asian

and Hispanic (43). Moreover, 90% of SLE patients are women according to 9:1 in a ratio of female to male in the age of reproductive years (44).

2.5.3 Symptoms and complications

SLE is characterized by a dysfunctional immunological response of the immune systems which results in tissue destruction in several organs. SLE typically manifests in these three common symptoms including arthritis that take 85% in musculoskeletal system among other tissues, skin rashes or butterfly rash and fatigue (45). For other tissue such as kidneys: those who suffer from lupus their kidneys are unable to filter blood and eliminate waste products. It is commonly called lupus nephritis. For instance, cardiovascular system are caused by myocarditis. Gastrointestinal system are caused by lupus enteritis.

Moreover, one of the most common chronic inflammation diseases, known as periodontitis, which is caused by a wide range of bacteria species in dental plaque. According to previous research, SLE patients are more prone to develop periodontitis, which can cause a number of oral problems, such as tooth loss and a severe gum infection. Periodontitis is a disease in the periodontal tissues, such as the gingiva, root cementum. In addition, it causes destroyed periodontal ligament and eroded alveolar bone, ultimately leading to tooth loss (46). Periodontitis is normally caused by the most common pathogen, *Porphyromonas gingivalis*, and its pathogenicity factor called LPS (47). LPS also caused bone loss

in alveolar bone by decreasing bone volume of the posterior maxilla (48). Earlier study showed that sCD14 (LPS co-receptor) levels are elevated in bacterial infections, particularly periodontitis. Likewise, a systemic reaction to microbial infection is shown in increasing the levels of CD14 in serum (49).

2.5.4 Risk factors

2.5.4.1 Genetics: SLE may be enhanced by various genes including *FcγRIIB* (50).

2.5.4.2 Female sex and hormonal influence: Estrogens and prolactin enhance B-cell activation factor synthesis and alter lymphocyte and pDCs activation.

2.5.4.3 Drugs: Sulfa drugs and estrogen-containing hormone therapy for menopause are known to be the trigger of flares in SLE patients (51).

2.5.4.4 Environment: Ultraviolet light and sunlight can cause erythema and cell apoptosis. Moreover, cigarette smoking also triggers the development of SLE (52).

2.6 Bone regeneration in SLE

Patients with SLE have problem with their bones including osteoporosis and bone fracture with reduced bone mineral density (BMD). Inflammatory condition affects bone healing due to the increasing of proinflammatory including TNF-α, IL-1, IL-6, IL-8, IL-11, IL-15 IL-17 and IL-23, which are identified as osteoclastogenic cytokines (53). However, TNF-α can strongly stimulate RANKL by triggering TRAP+ multinucleated osteoclasts via the activation of nuclear factor kappa B (NF-κB) signaling (54).

Furthermore, RANKL can enhance TNF-α induced bone resorption through a tumor necrosis factor receptor-associated factor 6 (TRAF6)-independent signaling pathway (55).

The dysregulation of proinflammatory cytokines is a significant factor in osteoporosis. Previous study reported that mice have a similar physiological mechanism of the skeletal and immune systems even in the different genetically modified knockout murine model. Prior evidence demonstrated bone loss is generally seen in autoimmune diseases (54). Moreover, the results have also shown an increase in bone loss and fractures with reduced bone mineral density in SLE patients (56). The inflammation disrupted the balance of BMUs causing bone loss by promoting osteoclast, and inhibiting osteoblast to promote bone growth (57).

2.7 LPS

2.7.1 Structure and function

LPS is a structural component anchored in gram-negative bacteria outer membrane such as *Escherichia coli* and *Porphyromonas gingivalis gingivalis* (48). Its structure contains polysaccharide and domains of lipid: the core oligosaccharide or lipid A, and an O-side chain or O-antigen polysaccharide that attached to the core. It is a potent endotoxin that plays an essential role in the induction of innate immunity and proinflammatory cytokines. Even low dose of LPS can cause proinflammatory cytokines that activate the neuroimmune and neuroendocrine systems leading to systemic inflammation and sepsis (58). TLR4 are the main receptor of LPS that are found widely

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on the membranes of various immune cells such as lymphocytes, monocyte/macrophages and DCs (59). The signaling of LPS/TLR4 is begin when LPS is bound with TLR4, and the downstream pathway causes release of IFNs, proinflammatory cytokines including IL-1, IL-6, and TNF-α, and chemotactic cytokines including IL-8 and IL-10 (60). Likewise, systemic or circulating LPS has been linked to low-graded systemic inflammation or chronic inflammation, which is the prolonged development of proinflammatory components related to obesity, type 2 diabetes, and systemic arterial hypertension in human (61-63).

2.7.2 LPS in SLE

SLE patients have elevated blood levels of soluble CD14 or sCD14, a protein produced by monocytes in response to LPS, indicating LPS may play a role in the development of SLE (64). Moreover, continuous administrations of LPS into SLE animals model leading to a high autoantibody production (65). Previous study indicated Ig-ICs can inhibit TLR4-induced inflammatory process in macrophages by the FcγRIIB receptor signaling pathway (66). In addition, follow-up study investigated *FcγRIIB-*overexpressing DCs (DC- FcγRIIB) with the accumulation of ICs, and they stimulate DCs expression by intraperitoneal (i.p.) administration of LPS. The result showed that in *FcγRIIB* overexpression can more substantially downregulate immunological response, both *in vitro* and *in vivo* (66).

2.7.3 LPS in bone regeneration

LPS is a widely used in the stimulation of the immune system and inflammation response (67). LPS stimulates the NF-κB pathway to release proinflammatory cytokines such as TNF-α and IL-1. Following RANKL activation, mitogen-activated protein kinase (MAPKs) and phosphatidylinositol-3-kinase (PI3K)– protein kinase B (AKT) pathways induced c-Fos and nuclear factor of activated T cells 1 (NFATc1), transcriptional regulators of osteoclastogenesis, which are essential for bone resorption (68). Several studies indicate that LPS can induce bone loss via stimulating RANKL/RANK-related osteoclast formation (69-72). Previous studies apply LPS in varieties of method including circulating or systemic injection, slow-release pellet transplant (9) and osmotic pump. However, the half-life of LPS is estimated to be 12 hours, therefore, several injections are not ideal for delivering LPS (73). The slow-release pellet transplant and osmotic pumps administration are more consistent in releasing rate, which closed to human LPS circulating (9), and also mimic of chronic proinflammatory condition related with aging (74).

To date, the comparison of local and systemic administration of LPS on bone regeneration has not been investigated. However, there are few studies use local administration of LPS. These studies investigate the early stages of inflammation, and bone formation in response to various LPS release behaviors from gelatin sponges *in vivo*. They implanted LPS sustained-release gelatin sponge and LPS rapid-release

gelatin sponge into the circular defect of rat calvarial bone. The results showed that both type of local LPS release caused bone loss in the calvaria (75, 76).

CHAPTER III

MATERIALS AND METHODS

3.1 Research design

3.1.1 Research design

Six-month-old mice were divided into 4 groups WT, WT+LPS, $Fc\gamma RIIB^{-/-}$ and $Fc\gamma RIIB^{-/-}$ + LPS in both local and systemic LPS experiments. I created a hole in mandible and tibia using a round burr attached to a dental drill (Krafit, Daegu, Korea). Collagen hemostat sponge (Avitene UltraFoam, RI, USA) filled with LPS (500 µg/mouse) was placed over the drilled hole in local LPS experiment. For systemic LPS experiment, mice were subcutaneously injected with 25 mg/kg body weight LPS.

After that, right mandibles and right tibiae were collected for micro-computed tomography (µCT) analysis and histology for collagen accumulation analysis. Femurs were used for quantitative PCR (qPCR) analysis of osteoblast and osteoclast marker genes. Sera were collected for proinflammatory cytokines analysis using bead-based immunoassay by flow cytometry and colorimetric assay including blood urea nitrogen (BUN), creatinine, calcium and phosphorus (Figure 1).
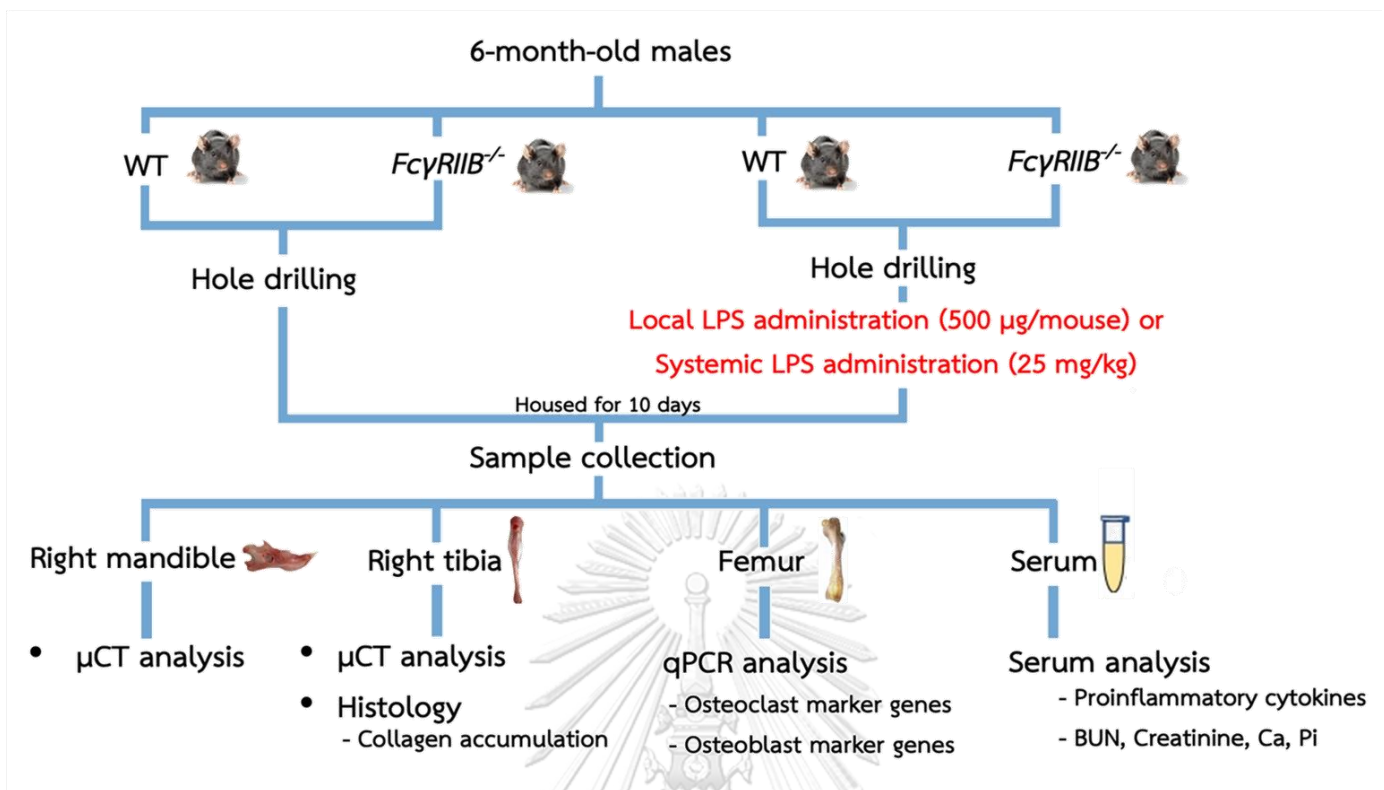
Figure 1 Research design of local and systemic LPS administrations

3.1.2 Local LPS experiment

In the operation day, mandibular and tibial holes were covered with sponge filled with 500 µg LPS per mouse. On day 10, mice were sacrificed for sample collection. The bone sample including tibiae and mandibles were fixed in 10% neutral-buffered formalin (NBF) and kept in 4 °C for µCT analysis or histology analysis. The femurs were fixed in 70% Ethanol and kept in -80°C for qPCR analysis. The sera were also kept in -80°C for proinflammatory cytokines and colorimetric assay (Figure 2).

Figure 2 The timeline of local LPS experiment from day 0 to day 10

3.1.3 Systemic LPS experiment

Mice were subcutaneously injected with 25 mg/kg LPS on days 0, 3, 6, and 9. After that, mice were sacrificed on day 10 and the sample were fixed and kept in the identical manner as local LPS experiment (Figure 3).
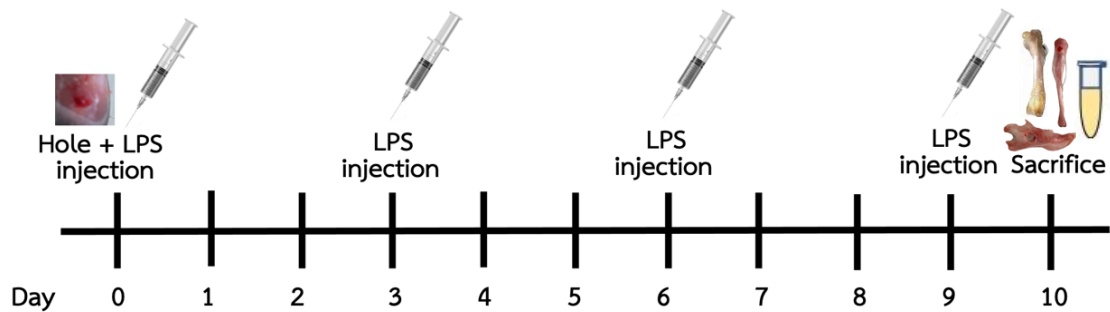

Figure 3 The timeline of systemic LPS experiment from day 0 to day 10

3.2 Declaration of approval for animal experiments

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine Chulalongkorn University in accordance with the Guide for the Care and Use of Laboratory Animals (eight edition), National Research Council (approval number: 027/2564).

3.3 Animals

Male $Fc\gamma RIIB^{-/-}$ mice on a C57BL/6 background were housed at Faculty of Medicine, Chulalongkorn University. All mice received mouse diet (C.P. Mice Feed, Perfect Companion Group Co., Ltd., Thailand) and drinking water *ad libitum*. The mice were kept at room temperature in a 12-hour light-dark cycle. Male and female heterozygous mice were crossed to generate $Fc\gamma RIIB^{-/-}$ mice and their wild type (WT) littermates as controls. Mice were separated in groups of 6 mice maximum per cage. They were separated into male and female cage on 3-4 weeks of age after they were born. Their ear was label by punching with ear punch applicator. The labels consist of 0; no notch on the ear, 1; one upper notch on left ear, 3; one lower notch on left ear, 4; two notches on left ear, 10; one upper notch on right ear, 0*; one lower notch on right ear. After labelling, their tails were cut for further genotyping. When they turn 6 months old, mice were randomly divided into 4 groups, WT, $Fc\gamma RII^{-/-}$, WT+LPS, and $Fc\gamma RII^{-/-}$+LPS.

3.4 DNA extraction

DNA of tail snips were isolated by adding 500 μl of tail lysis buffer with proteinase K. Then the tail samples were incubated in incubator for 2 hours at 55 °C. After that, the samples were added 700 μl of isopropanol and centrifuged at 14,000 rpm for 20 minutes. Supernatant from the tube were removed before adding 300 μl of distilled water and incubated for 15 minutes at 55 °C.

3.5 Genotyping identification

The genomics DNA from tail samples were isolated and genotyped using PCR. Denaturation at 95 °C for 15 s, 60 °C for 30 s, extension at 72 °C for 30 s, and repeat for 35 cycles were performed. The samples were kept until loading in agar gel for gel electrophoresis at 100 V, 3A, 300W for 1 h. Genotype was identified using 3 primers: FcREC1 (5′AAGGCTGTGGTCAAACTCGAGCC-3′), OL4143 (5′-CTCGTGCTTTACGGTATCGCC-3′), and OL4080 (5′-TTGACTGTGGCCTTAAACGTGTAG-3′) which formed a ～190-basepairs for WT ($Fc\gamma RIIB^{+/+}$) fragment, a ～300-bp for knockout ($Fc\gamma RIIB^{-/-}$) fragment and both of the fragments for heterozygous ($Fc\gamma RIIB^{+/-}$).

3.6 Serum analysis

Serum IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL17A, IL-23, IL-27, MCP-1, IFNβ, IFN-γ, TNF-α and GM-CSF were analyzed using LEGENDplex™ bead-based immunoassay (BioLegend, San Diego, CA, USA). The urea nitrogen, creatinine, calcium, and phosphorus levels in the serum were measured following the protocols according to the manufacturer guidelines (Standbio Laboratory, Boerne, TX),

3.7 LPS preparation

3.7.1 Local LPS preparation

LPS for the local experiment was prepared by dissolving 25 mg LPS (Sigma, St. Louis, MO, USA) in distilled water (DW) 300 μl for the stock concentration of 83.33 μg/μl. Six μl LPS (500 μg) was used. The LPS was filled in the holed with the 2x2 mm sponge.

3.7.2 Systemic LPS preparation

25 mg of LPS was dissolved in 10 ml distilled water for the stock concentration 2.5 mg/ml. LPS was used at the dosage of 25 mg/kg body weight.

3.8 Hole drilling technique and dose regimen

After adequate anesthesia with isoflurane vaporizer, the hole was created by dental drill attached with round burr with a tip diameter of 1.6 mm on angular process region of right mandible, and 1.0 mm diameter on anterior aspect of right proximal tibia. After that, the drilled hole was covered with sponge filled with LPS (500 µg) for local LPS experiment and sacrificed on day 10 after operation. In systemic LPS experiment, mice were subcutaneously injected with LPS (25 mg/kg) on day 0, 3, 6 and 9 after operation. Then mice were sacrificed afterwards on postoperative day 10 for sample collection. Right mandibles and tibiae were fixed with NBF for further µCT analysis and histology.

3.9 µCT analysis

After being harvested and fixed, the samples were scanned by µCT (Scanco Medical AG, Bassersdorf, Switzerland) at 7 µm isotropic voxel size, 73 kVp, and 113 µA for analyzing bone microarchitecture of cancellous bone which provides in 200 transverse slices in mandible and 150 transverse slices in tibia. Cancellous bone volume (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, /mm), trabecular separation (Tb.Sp, mm), Connectivity density (Conn.D, /mm^3),

Structure model index, (SMI, (-)) and bone mineral density (BMD, mgHA/cm^3) were analyzed at threshold of 270 and 220 for mandibles and tibiae, respectively.

3.10 Histology

The tibiae were decalcified in 10% ethylenediaminetetraacetic acid (EDTA), pH 7.4 and embedded in paraffin. Longitudinal sections with 5-µm-thick paraffin blocks were cut with a microtome (Leica 2065, Wetzlar, Germany). A section was stained with aniline blue for the determination of collagen accumulation.

The collagen accumulation in the hole region by the aniline-blue-stained area was selected by magic wand tool in Adobe photoshop program version 23.1.1. The histological pictures of each slide were imported into digital image files with 10^6 pixels. The mineralization to collagen ratio (%) were calculated as BV/TV (µCT parameter) divided by aniline blue–positive area.

3.11 Sample size

The number of mice per group (sample size) for comparison of 4 groups was analyzed using G*Power program version 3.1. The µCT results from preliminary study determined BV/TV, (%) that is the common µCT parameter in bone microarchitecture analysis. The data showed in each group were mean ± SEM as following; group 1: control (n=8) 20.18±1.95%, group 2: *FcγRIIB$^{-/-}$* (n=7) 11.54±1.88%, group 3: WT+ LPS administration (n=7) 10.61±2.27%, and group 4: *FcγRIIB$^{-/-}$*+LPS administration (n=6) 3.29±0.68%. The SEM is calculated to be SD by multiplying square

root of each group number with SEM before using in the estimation of the effect size in

G* power program. The result showed total sample size of 20 mice, which divided into

5 mice per group (Figure 4).

Figure 4 Sample size calculation by G*Power program

3.12 Statistical analysis

The data was expressed as mean ± SEM. The significance of differences between 4 groups was analyzed using one-way ANOVA followed by Fisher's protected least significant difference test. Differences were defined as significance at $p<0.05$.

CHAPTER IV
RESULTS

4.1 Decrease in serum calcium and phosphorus but increase urea nitrogen levels in 6-month-old *FcγRIIB$^{-/-}$* mice with systemic and local LPS administration

To investigate whether lack of *FcγRIIB* with LPS administration affected BUN, creatinine, calcium and phosphorus levels, I measured serum chemistries in male mice at 6 month of age. Serum urea nitrogen of *FcγRIIB$^{-/-}$* mice was significantly higher than WT control. Likewise, creatinine level in *FcγRIIB$^{-/-}$* +LPS mice significantly increased when compared to WT control and WT+LPS. These finding suggested that lupus mice with LPS administration underwent renal flare which is commonly seen in SLE animal model. The data showed a significant decrease in serum calcium and phosphorus in the lupus mice with LPS injection as compared with healthy control. A significant decrease in serum phosphorus was also presented in mice lacking *FcγRIIB* with systemic LPS administration compared with *FcγRIIB$^{-/-}$* mice and WT with LPS injection groups indicating imbalance calcium and phosphorus homeostasis which may affect bone mineralization and bone regeneration (Figure 5a). Correspondingly, mice lacking *FcγRIIB$^{-/-}$* with local and systemic LPS administration had significantly higher levels of serum urea nitrogen and creatinine but lower levels of calcium than WT control, *FcγRIIB$^{-/-}$* and WT+LPS (Figure 5b).
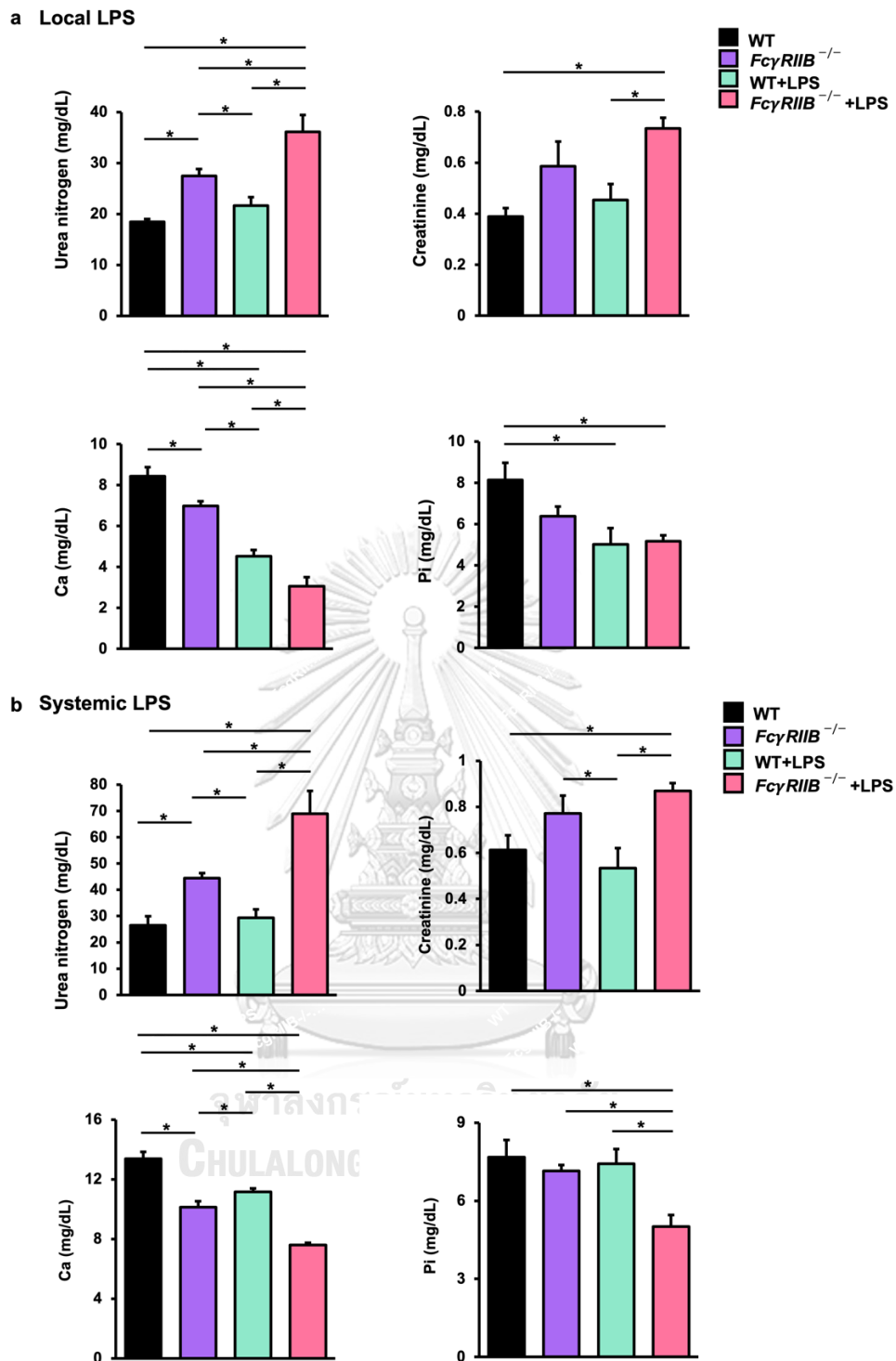
Figure 5 The absence of *FcγRIIB* in lupus mice with local and systemic LPS administration caused an alteration in serum chemistry.

(a) local and (b) systemic LPS administration caused a decrease in serum calcium and phosphorus but increase in urea nitrogen and creatinine in 6-month-old mice. Data are mean ± SEM (n =5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at *p*<0.05.

4.2 Local LPS administration induced impaired bone regeneration in mandibular bone defects in *FcγRIIB*$^{-/-}$ mice

To determine whether LPS affected the newly-formed bone at the defect area in the mandible. I administrated local LPS in 6-month-old *FcγRIIB* and their control mice. The data showed that *FcγRIIB*$^{-/-}$ mice had significantly reduced cancellous bone volume compared with WT control. *FcγRIIB*$^{-/-}$+LPS mice had lower connectivity density than WT littermate controls and WT+LPS. BMD were significantly decreased compared with WT control (Figure 6). These results indicated that *FcγRIIB*$^{-/-}$ mice given LPS had lower mandibular cancellous bone regeneration at 6 months.

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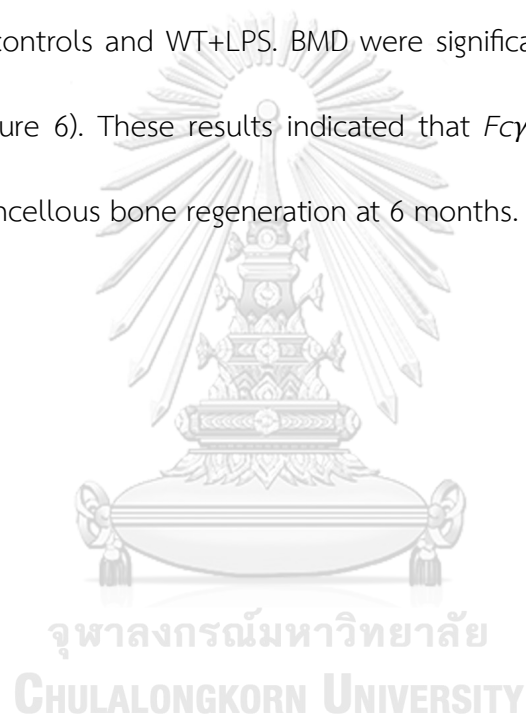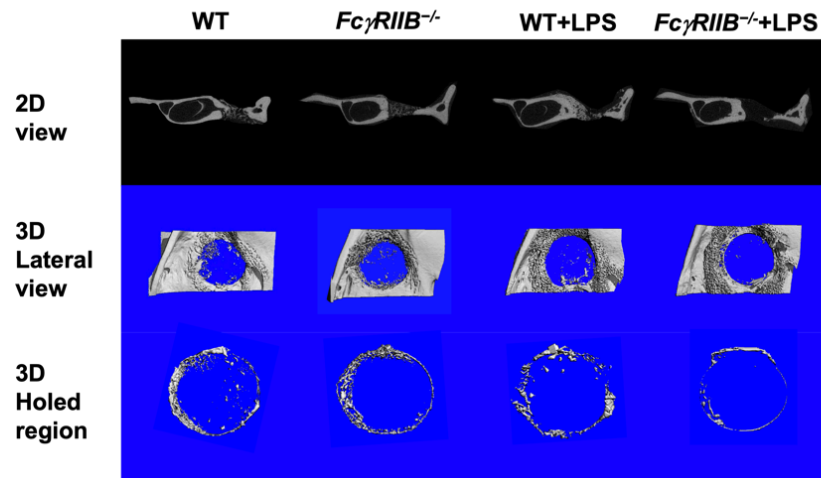
Figure 6 *FcγRIIB* deletion with local LPS administration impaired bone regeneration in mandibular bone defects.

(**a**) Representative 3D images of μCT analysis and (**b**) μCT analysis of mandibular cancellous bone from 6-mounth-old WT control, *FcγRIIB*$^{-/-}$, WT+LPS and *FcγRIIB*$^{-/-}$ +LPS. Data are mean ± SEM (n =5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at $p<0.05$.

4.3 Local LPS administration impaired bone regeneration in tibial bone defects in *FcγRIIB$^{-/-}$* mice

To investigate the effect of local LPS on tibial bone regeneration observed in 6-month-old male mice with lacking *FcγRIIB,* we performed μCT analysis and found that *FcγRIIB$^{-/-}$* +LPS mice had a significant reduction in cancellous bone volume, connectivity density and BMD compared with WT control, *FcγRIIB$^{-/-}$* and WT with local LPS administration group (Figure 3). Moreover, trabecular number significantly decreased in knockout mice with LPS administration, but trabecular separation significantly increased compared to controls and *FcγRIIB$^{-/-}$* mice, indicative of decreased bone regeneration (Figure 7).

a **Tibia, Local LPS**

b **Tibia, Local LPS**

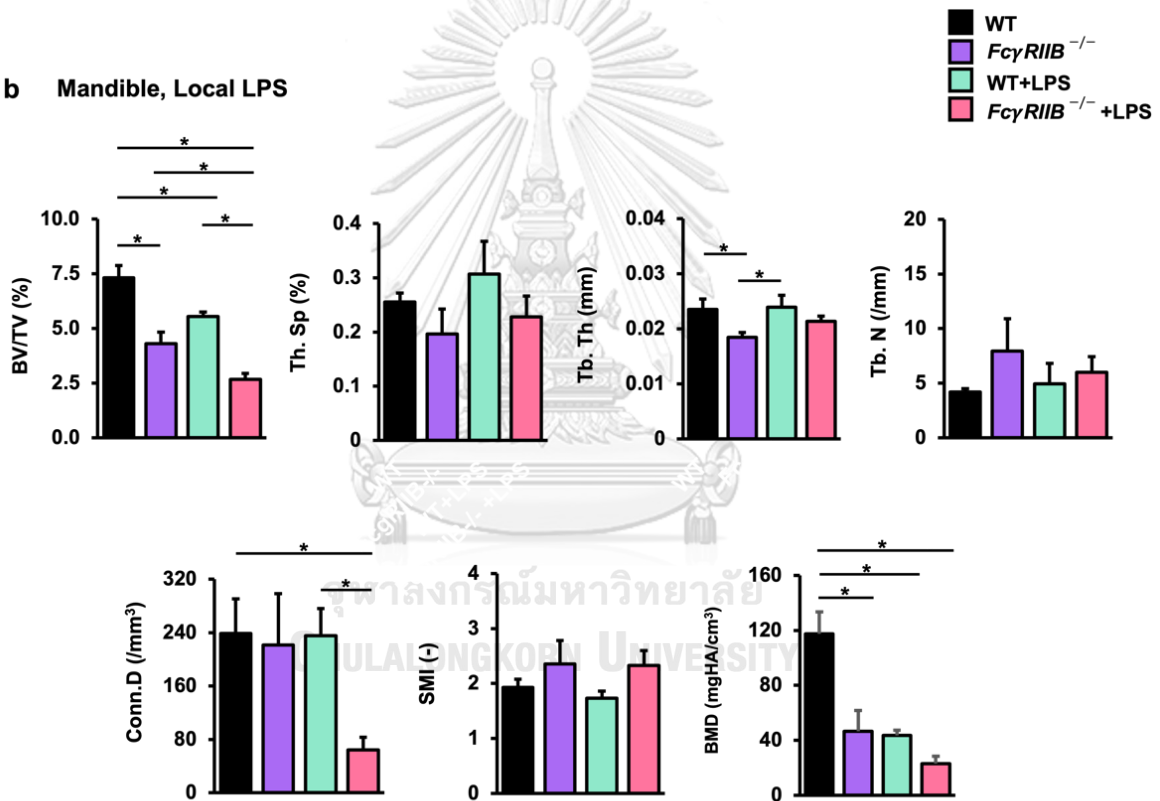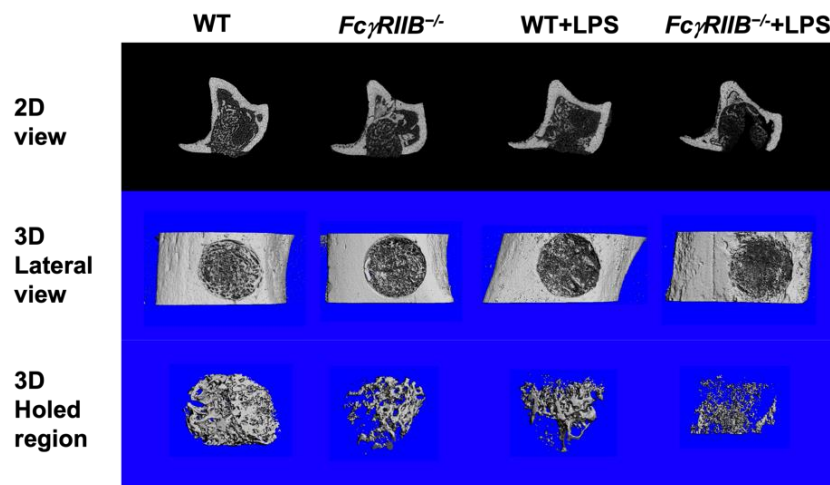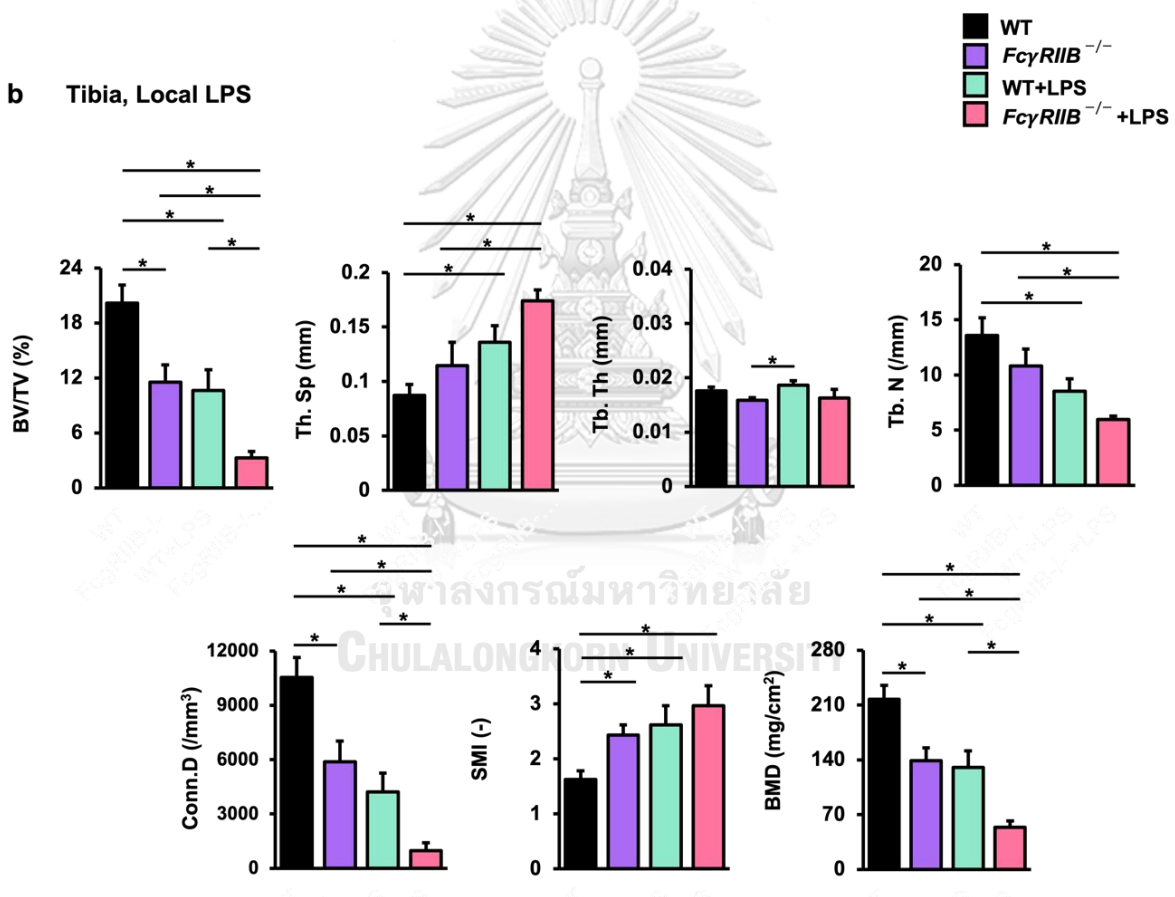**Figure 7** *FcγRIIB* deletion with local LPS administration impaired bone regeneration in tibial bone defects.

(a) Representative 3D images of μCT analysis and (b) μCT analysis of cancellous bone of tibial shaft from 6-mounth-old WT *control, FcγRIIB⁻ᐟ⁻,* WT+LPS and *FcγRIIB⁻ᐟ⁻+*LPS. Data are mean ± SEM (n =5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at $p<0.05$.

4.4 Systemic LPS administration caused decreased bone regeneration in tibial bone of mouse with *FcγRIIB* deletion at 6 months old

Previous study indicated *FcγRIIB* deletion caused reduced cancellous bone volume. To examine whether systemic LPS administration affected cancellous bone regeneration in mice with *FcγRIIB* deletion, I analyzed μCT parameter in 6-month-old *FcγRIIB*$^{-/-}$ mice. The results indicated a significant decrease in cancellous bone volume of *FcγRIIB*$^{-/-}$ with LPS injection group compared to WT control, *FcγRIIB*$^{-/-}$ and WT with systemic LPS injection group. Moreover, trabecular number and BMD of *FcγRIIB*$^{-/-}$, WT+LPS and *FcγRIIB*$^{-/-}$ +LPS were significantly decreased but trabecular separation was significantly increased compared to WT control. Therefore, these results indicated that mice lacking *FcγRIIB* with systemic LPS decreased bone regeneration (Figure 8).

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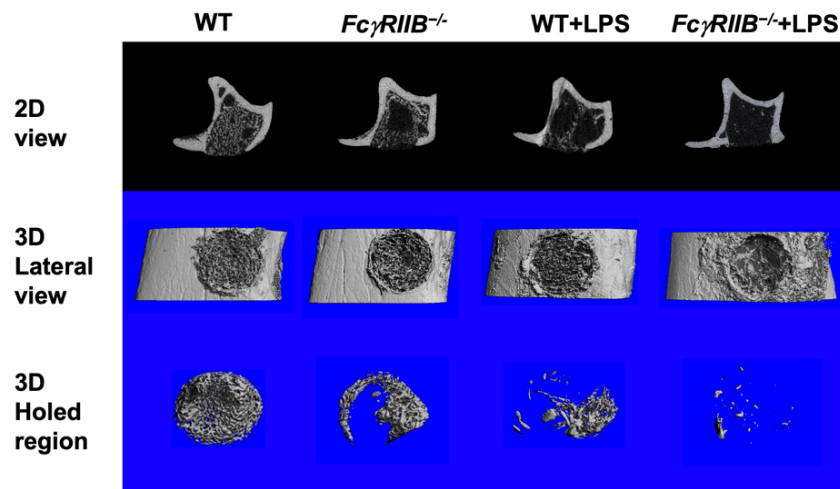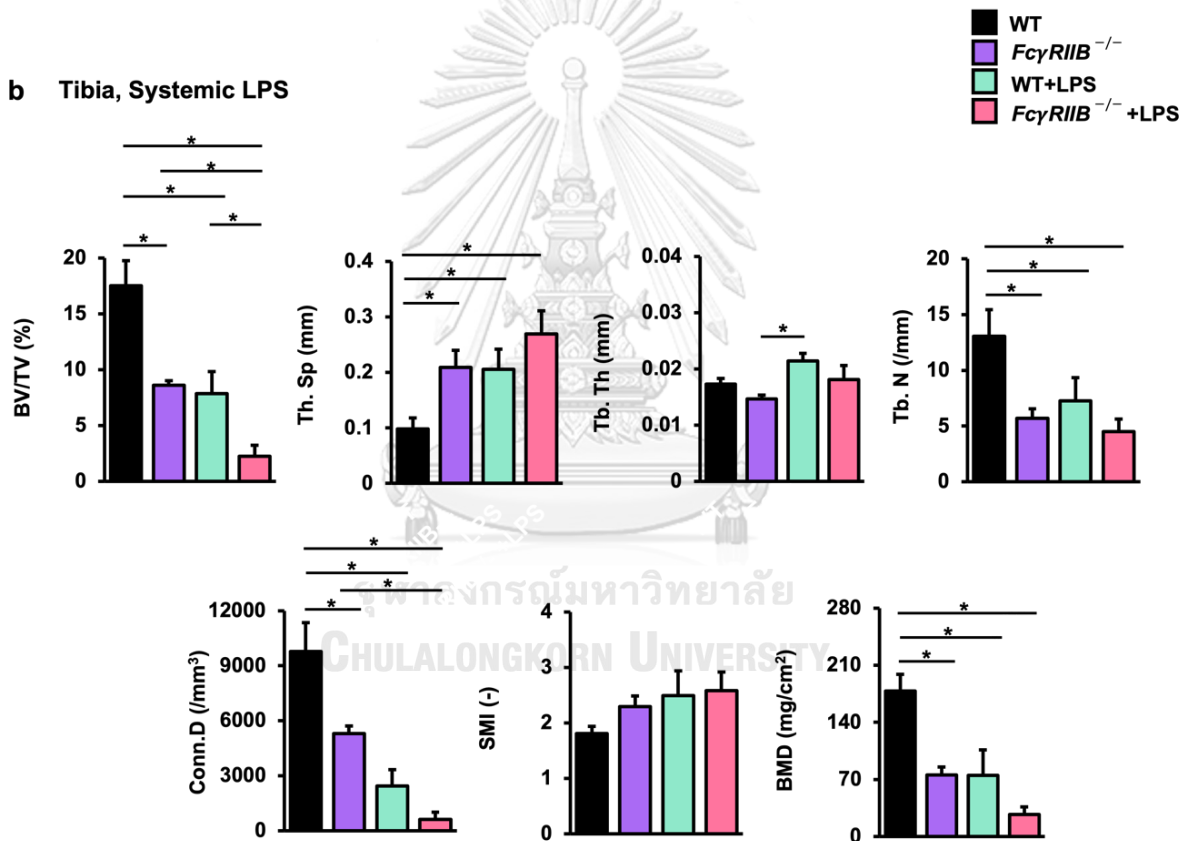
Figure 8 *FcγRIIB* deletion with systemic LPS administration induced bone loss in 6-month-old mice.

(**a**) Representative 3D images and (**b**) μCT analysis of cancellous bone at right tibiae from 6-mounth-old WT control, *FcγRIIB* [−/−], WT+LPS and *FcγRIIB* [−/−]+LPS. Data are mean ± SEM (n =5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at *p*<0.05.

4.5 Local LPS administration decrease collagen formation during tibial bone regeneration

To further evaluate the effect of LPS on collagen formation and mineralization, I stained mouse tibiae with aniline blue resulting in a reduction of the newly collagen accumulation in the defect hole region in $Fc\gamma RIIB^{-/-}$ mice with local LPS administration (Figure 5). The data showed lower density of aniline blue staining in hole region of $Fc\gamma RIIB^{-/-}$+LPS mice compared to WT controls, $Fc\gamma RIIB^{-/-}$ and WT+LPS mice. I found that mineralization/collagen ratio was significantly decreased in $Fc\gamma RIIB^{-/-}$, WT+LPS and $Fc\gamma RIIB^{-/-}$ with LPS administration compared to controls, likely resulting from the decreased density of aniline blue staining in hole region. These data indicated that administering LPS inhibits collagen production and subsequently worsened bone regeneration in mice lacking $Fc\gamma RIIB$ (Figure 9).
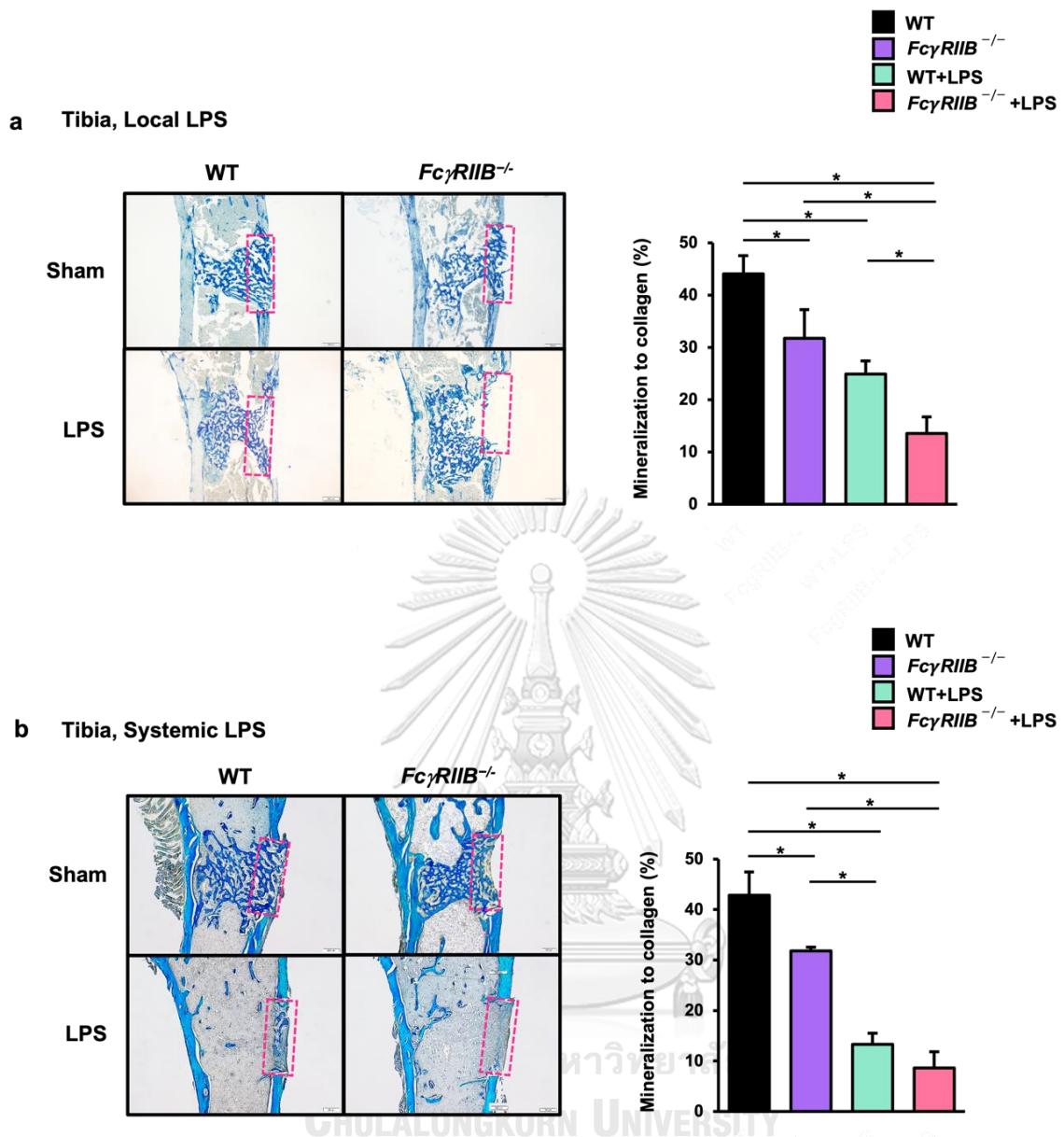
Figure 9 *FcγRIIB⁻/⁻* with local and systemic LPS administration decreased collagen accumulation in tibial bone defects in mice.

(**a**) Aniline blue staining showing the mineralization to collagen ratio in WT and *FcγRIIB⁻/⁻* mice treated with local LPS. (**b**) Aniline blue staining showing the mineralization to collagen ratio in WT and *FcγRIIB⁻/⁻* mice treated with systemic LPS. Stripped pink rectangles show the area of mineralization to collagen ratio. Scale bars: 200 μm. Data are mean ± SEM (n=5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at $p<0.05$.

4.6 High level of TNF-α, IL-6 and MCP-1 in serum of 6-month-old *FcγRIIB⁻/⁻* mice with systemic LPS administration

Bone regeneration can be triggered by inflammation, potentially because the bone repair requires several cytokines to achieve bone healing process. Thus, to confirm whether LPS could affect bone cytokines secretion, serum IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL17A, IL-23, IL-27, MCP-1, IFNβ, IFN-γ, TNF-α and GM-CSF were measured. I observed a significant elevation in TNF-α, IL-6 and MCP-1 in *FcγRIIB⁻/⁻* mice with systemic LPS administration group compared to WT controls and *FcγRIIB⁻/⁻* mice, suggesting that systemic LPS administration enhanced proinflammatory cytokines levels in lupus mice. Other cytokines did not alter (Figure 10).

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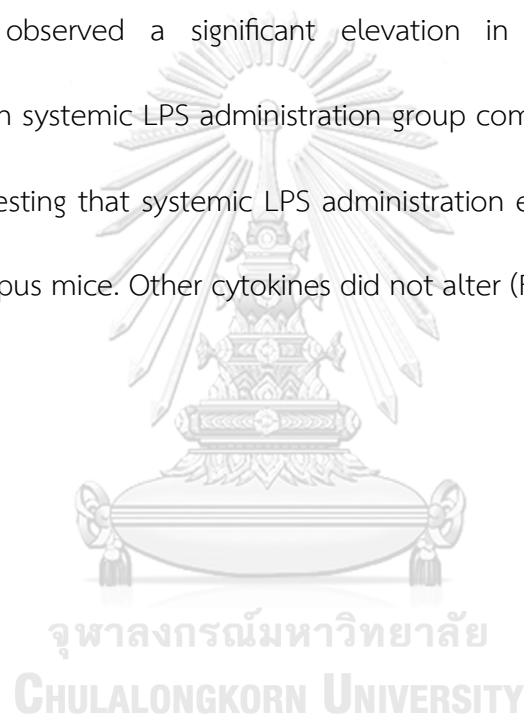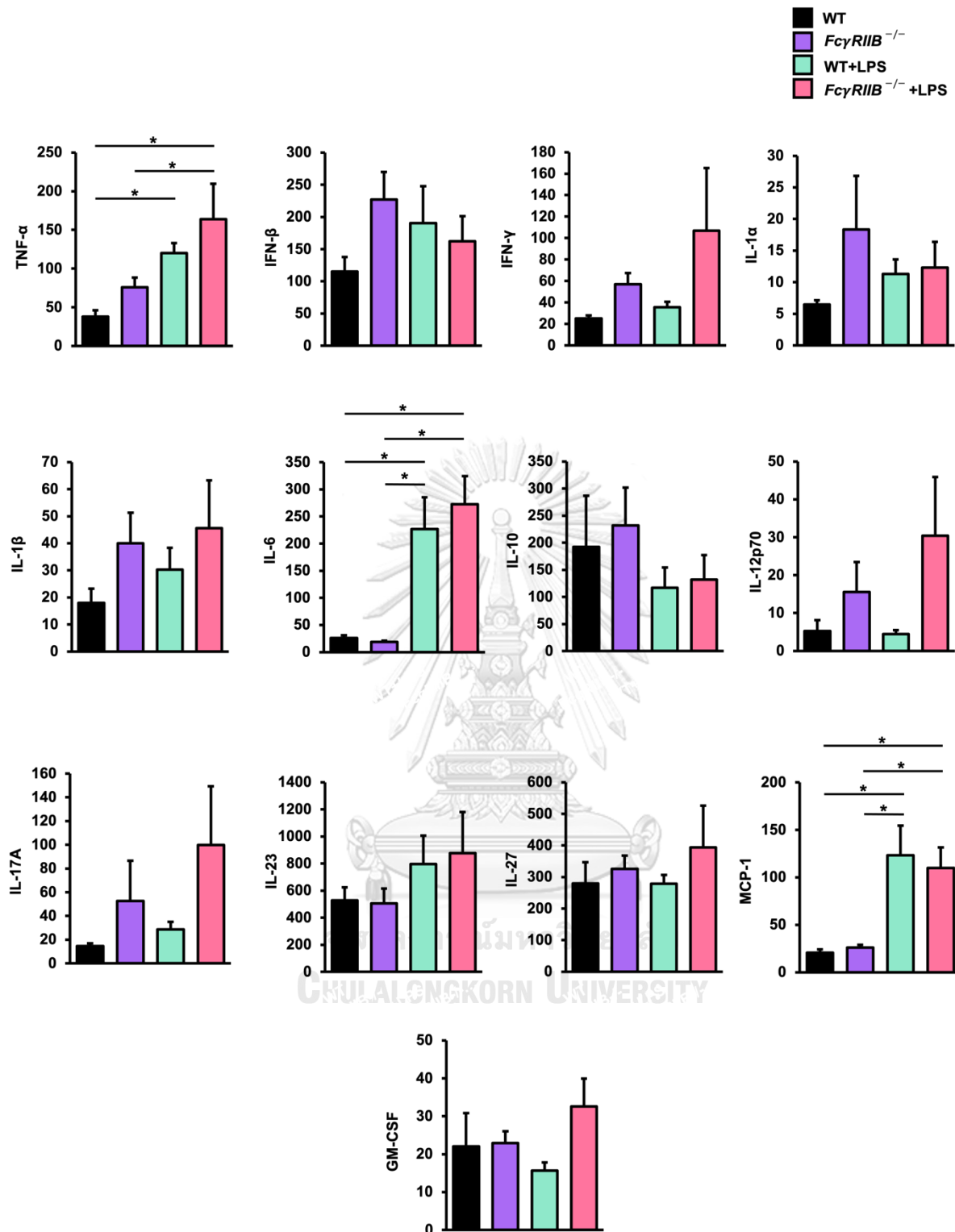
Figure 10 Six-month-old *FcγRIIB⁻/⁻* mice had increased TNF-α, IL-6 and MCP-1 serum levels while there are no changes in others.

Data are mean ± SEM (n =5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at p<0.05.

4.7 Increased osteoclast markers genes while decreased in osteoblast marker genes in mice lacking *FcγRIIB* with systemic LPS administration

In order to assess osteoclast and osteoblast marker gene expression, mRNA expression in the distal femur metaphysis of *FcγRIIB*$^{-/-}$ mice was analyzed by qPCR. Significantly, *FcγRIIB*$^{-/-}$ mice with systemic LPS administration displayed significantly higher *Tnfsf11/Tnfrsf11b* ratio compared to controls and *FcγRIIB*$^{-/-}$ mice, indicating high bone resorption (Figure 7). *FcγRIIB*$^{-/-}$+LPS mice had downregulated expression of *Sp7*, *Alpl* and *Col1a1* compared to WT controls. *Col1a1* also decreased in *FcγRIIB*$^{-/-}$+LPS mice compared to WT+LPS. Osteoblast marker genes including *Bglap, Hhip* and *Creb5* in *FcγRIIB*$^{-/-}$ + LPS mice had decreased significantly compared to *FcγRIIB*$^{-/-}$ mice (Figure 11).

Figure 11 Osteoblast and osteoclast gene expression in mice lacking *FcγRIIB* treated with systemic LPS administration.

(a) Osteoblast marker gene expression and (b) Osteoclast marker gene expression. Data are mean ± SEM (n = 5-7). This graph illustrates asterisk symbol to indicate statistically significant differences between groups at $p<0.05$.

CHAPTER V

DISSCUSSION

SLE is an autoimmune disease characterized by chronic inflammation. The high risk profile for bone loss in SLE is caused by genetic-related factor. Eliminated 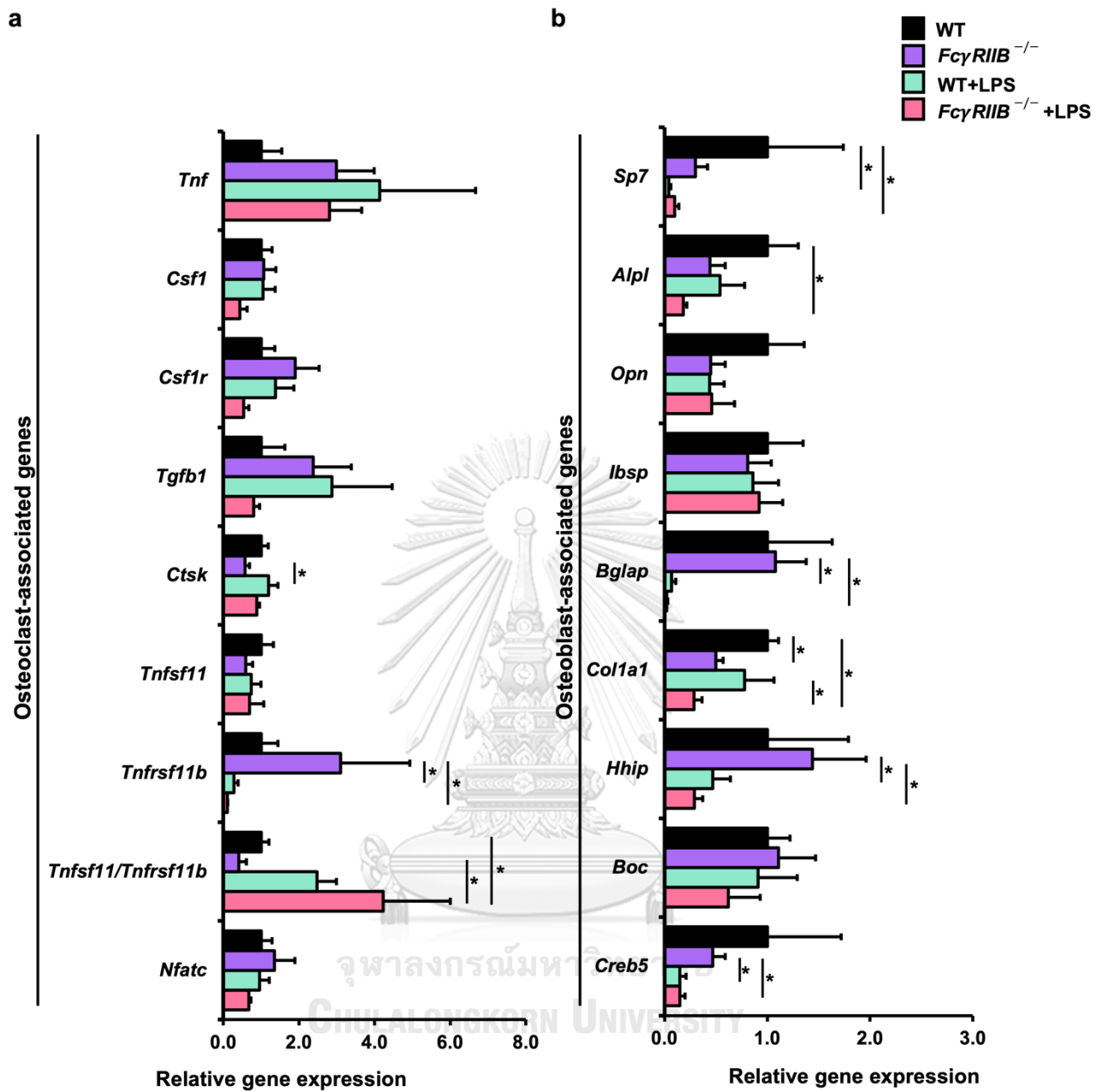*FcγRIIB* expression in mice exacerbates lupus-like condition due to the increase of serum proinflammatory cytokines. Since LPS is a potent stimulator of innate immune responses and the potent trigger of several proinflammatory cytokines. In this study, I analyzed the mice lacking *FcγRIIB* model of SLE to determine the effects of local and systemic administration on bone regeneration. The results indicated that *FcγRIIB* deficiency with LPS caused a reduction in mandibular and tibial bone regeneration, bone microarchitecture and collagen accumulation because of high level of proinflammatory cytokines including TNF-α, IL-6 and MCP-1. The relative gene expression of osteoclast marker gene, *Tnfsf11/Tnfrsf11b* were increased but osteoblast marker gene, *Sp7, Alp, Col1a1, Bglap, Hhip* and *Creb5* were decreased in *FcγRIIB*$^{-/-}$+LPS mice.

The gene *FcγRIIB* has been correlated to increased vulnerability to SLE in both mice and human. Insufficient inhibition of autoantigen-mediated BCR activation in genetic-modified mice with *FcγRIIB* deletion leads to the impaired immune complex clearance, decreased self-tolerance, and excessive inflammation. Apart from *FcγRIIB* knockout model, *FcγRIIB* has been shown to be the primary gene in regulation of an

inhibitory receptor in the murine SLE, MRL/1 (77) and BXSB Yaa (78) models. Moreover, some evidence suggests that the *FcγRIIB* may have low expression in NZB/W mice (16). Additionally, a prior study demonstrated that genetic variants in *FcγRIIB* are related to the phenotypes and susceptibility of SLE in Asians including Chinese, Japanese, Korean and Thai (79-82).

LPS drives systemic inflammation causing proteins and inflammatory cells to invade tissues. Serum TNF-α, IL-6 and MCP-1 in the knockout mice with systemic administration LPS were increased. Since these proinflammatory cytokines are the key players in the pathogenesis of systemic inflammatory disease including SLE and sepsis. Previous study showed that plasma concentration of these proinflammatory cytokines in patients with microbial sepsis were increased compared to healthy controls (83). The excessive production of anti-dsDNA autoantibodies and LPS-mediated TLR4 signaling, leading to severe lupus symptoms via the surge of IL-10 and IFN-γ, were seen in anti-dsDNA transgenic mice treated with LPS (84). As LPS activates TLR4, it increases Tnfsf11 levels and promotes osteoclast precursor cells, both of which worsen osteoclast capacities and activities (85).

The elevation of proinflammatory cytokines may alter Tnfsf11 secretion that leads to osteoclatogenesis process (56). Inflammatory process that causes bone loss is mediated by an increase in proinflammatory cytokine such TNF-α. Previous study demonstrated an increase in cancellous bone volume in 6-month-old *FcγRIIB* knockout

mice when using TNF-α blocker (5). TNF-α binds to its receptors coupled with the signaling of Tnfsf11 on osteoclast leading to TRAF6 activation resulting in stimulating NF-κB and Nfatc1 in the nucleus. This causes osteoclastogenesis and increases bone resorption leading to bone loss and decreased bone regeneration (86).

As described above, LPS relates with TLR4 that plays a role in stimulation of osteoclast functions and activities. Bone remodeling process requires *Tnfsf11* and *Tnfrsf11b* which belong to TNF receptor superfamilies and play a part in a crucial signaling molecule of bone macrophages maturation into osteoclasts and subsequent bone resorption trough TLR4/TNF-α signaling (86). While *Tnfrsf11b* exerts its effect by blocking *Tnfsf11* interaction. Previous research revealed that mice deficient in *Tnfrsf11b*^-/- had dramatic alveolar bone resorption in cortical regions. While there is no significantly change in *Tnfsf11* transgenic mice (87). Accordingly, this present study suggested that LPS caused the increase with significant difference of the relative expression of bone-resorption- related genes including *Tnfsf11/Tnfrsf11b and* TNF-α *in FcγRIIB*^-/- mice +LPS mice compared to *FcγRIIB*^-/- control. LPS activates TLR4 to increase *Tnfsf11* levels or promote osteoclast progenitor cells, impairing bone regeneration.

LPS downregulates *Runx2, Col1, Alpl,* and *Ibsp* expression by inhibiting BMP2-Smad1/5/8 signaling (88). However, other previous study exhibited LPS activated

the ERK1/2-JNK pathways to stimulate apoptosis in MC3T3-E1 cells which are one of osteoblastic cell lines *(89). Col1α1* is important in *forming* type I collagen by creating large molecule called *pro-alpha1 helical domain* with triple-stranded procollagen consisting of a pair of *alpha 1* chain and an alpha 2 chain*. Col1α1* expression *apparently related to decreased* mineralization to collagen ratio in *FcγRIIB* knockout with LPS administration. Previous study reported that local LPS administration affects osteolysis in murine model indicating that LPS influences bone mineralization (90). Moreover*, Creb5* regulates CREB protein that stimulates the expression of osteocalcin (OCN) that encoded by *Bglap* gene. The *Bglap* encodes great amount of bone protein released by osteoblasts. The protein is the part of inorganic bone matrix along with collagen fibrils which are essential in mineralization process during bone regeneration. *Hhip* have functions in osteoblast maturation, proliferation and differentiation which downregulated from *Runx2* that required in mature preosteoblastic cells from mesenchymal stem cells. Stimulation of *Hh* signalling, which is implicated in mineralization, takes place after osteoblastogenesis and alters the expression of *Col1α1, Sp7*, and *Bglap* (91).

Autoimmune glomerulonephritis considered as the lupus-like disease characteristic in *FcγRIIB* $^{-/-}$ mice. Serum urea nitrogen and creatinine is also strongly related to SLE patients with lupus nephritis condition. The previous study reported that serum creatinine was elevated due to renal function impairment leading to an increase

in creatinine concentration (92). As shown in systemic and local LPS administration, *FcγRIIB*$^{-/-}$+LPS mice in both experiments underwent higher serum urea nitrogen and creatinine. Since calcium and phosphorus homeostasis are regulated by calcium and phosphorus exchanges that proceed in the intestine, bone and kidney. In a similar manner, the renal processing of phosphorus can modify its capacity in order to fulfil the requirements of cells for phosphorus. As confirm with previous study, patients with SLE had decreased serum calcium compared to healthy controls (93). The result showed that serum calcium reduced in mice lacking *FcγRIIB* with and without exogenous LPS injection associated with vitamin D deficiency that was found widely in SLE patients (94). For serum phosphorus, SLE mice tended to have lower in phosphorus due to the possible cause from TNF-α and IL-6 (95). Moreover, serum phosphorus in patients with sepsis were found to be significantly higher in early sepsis and were related to both of the proinflammatory cytokines (96). The data suggested that those serum chemistries related with bone regeneration and mineralization by the effect of renal flare in lupus.

In addition, the timing of experiment and mouse generation affect serum chemistry. Since Local LPS experiment was held in 2020 while systemic was held in 2017, this may cause alteration that illustrated higher range in serum chemistry of local experiment than those in systemic experiment.

For comparing the effect of local and systemic LPS administration in lupus murine model, the data illustrated that local LPS causes more influent in lupus murine model due to its local administrative effects at the spot of contact and swiftly absorb after exposure. While systemic administration with subcutaneous injection has a typically disadvantage effect in slowly absorption rate due to decreased blood flow of adipose tissue beneath the skin. As shown in local LPS administration in tibial bone, the data exhibited the decrease of Conn.D, BMD and mineral to collagen ratio with difference significance in $Fc\gamma RIIB^{-/-}+$ local LPS mice compared to WT+ local LPS. However, there are no significance difference of these μCT and histology parameters in $Fc\gamma RIIB^{-/-}+$ systemic LPS mice compared to WT+ systemic LPS. Therefore, this comparison of local and systemic results of μCT and histology analysis suggested that because of the difference in absorptive time of local LPS administration in mice lacking $Fc\gamma RIIB$ cause more severe in bone regeneration and collagen synthesis than systemic LPS administration.

SUGGESTION AND RECCOMENDATION

This study exhibited the effect of LPS on bone regeneration in SLE murine model as the understanding for further research on the efficacy of cytokine blockers in suppressing inflammatory bone loss for novel therapy options for patients with SLE.

CONCLUSION

In summary, mice with *FcγRIIB$^{-/-}$* deletion exposed to LPS underwent the decrease in bone regeneration implying clinical management of bacterial infection and non-hygiene environment in SLE patients with impaired immune responses. The present findings may provide better understanding about LPS mechanism in SLE condition which is beneficial for patient health.

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VITA

NAME	Sirikanda Jantaboon
DATE OF BIRTH	16 July 1996
PLACE OF BIRTH	Samut Prakan province
INSTITUTIONS ATTENDED	Chulalongkorn university
HOME ADDRESS	271/63 Village No. 1, Samrong Tai sub-district, Phra Pradaeng district, Samut Prakan province, Thailand. 10130
PUBLICATION	-
AWARD RECEIVED	-