Endotoxemia from NSAIDs-induced gut-leakage enhanced lupus characteristics in Fc gamma receptor IIb deficient lupus mice

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เอนโดท็อกซินที่รั่วซึมจากลำไส้เนื่องจากการใช้ยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์เร่งการแสดงออกของลูปัสในหนูที่ขาด Fc gamma receptor IIb

น.ส.ธันย์สิตา บุณยกาญจนารัตน์

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ธันย์สิตา บุณยกาญจนารัตน์ : เอนโดท็อกซินที่รั่วซึมจากลำไส้เนื่องจากการใช้ยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์เร่งการแสดงออกของลูปัสในหนูที่ขาด Fc gamma receptor IIb. (Endotoxemia from NSAIDs-induced gut-leakage enhanced lupus characteristics in Fc gamma receptor IIb deficient lupus mice) อ.ที่ปรึกษาหลัก : รศ. ดร.อัษฎาศ์ ลีฬหวนิชกุล

ยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์ (NSAIDs) เป็นกลุ่มยาต้านอักเสบที่นิยมใช้เพื่อบรรเทาอาการปวด การใช้ยากลุ่มนี้ในขนาดสูงอาจทำให้เกิดความรุนแรงของโรคลูปัสมากขึ้นได้ เนื่องจากยาในกลุ่มนี้สามารถทำให้เกิดการรั่วซึมของ endotoxin (ภาวะลำไส้รั่ว) ที่อยู่ในลำไส้เข้าสู่กระแสโลหิต (endotoxemia) ผู้วิจัยให้ยาอินโดเมทาซิน (25 มก./วัน) เป็นเวลา 7 วัน ในหนูลูปัสที่เกิดจากการขาด Fc gamma receptor IIb (FcgRIIb-/-) ที่อายุ 24 สัปดาห์ เทียบกับหนูปกติ (wild-type) ที่มีอายุเท่ากัน พบว่ายาอินโดเมทาซินที่เหนี่ยวนำให้เกิดภาวะลำไส้รั่วมีความรุนแรงสูงกว่าในหนู FcgRIIb-/- ซึ่งแสดงให้เห็นโดยการบาดเจ็บในลำไส้ (ลักษณะทางพยาธิวิทยา immune-deposition และการหลั่งไซโตไคน์ในเนื้อเยื่อลำไส้) ความรุนแรงของภาวะลำไส้รั่ว (FITC-dextran assay และระดับ endotoxin ในเลือด) และระดับไซโตไคน์ในเลือด นอกจากนี้การตอบสนองต่อระดับ endotoxin ที่สูงขึ้นในหนู FcgRIIb-/- ที่ได้รับยาอินโดเมทาซินยังอาจจะมากกว่าหนูปกติ เนื่องจากเซลล์แมคโครฟาจที่ได้จากไขกระดูก (bone marrow-derived macrophages) ของหนู FcgRIIb-/- ไวต่อ endotoxin มากกว่าเซลล์แมคโครฟาจจากหนูปกติ โดย endotoxin ชักนำให้เกิดการแสดงออกของ activating-*FcgRs* (*FcgRIII* และ *FcgRIV*) และ inhibitory-*FcgRIIb* ในเซลล์แมคโครฟาจจากหนูปกติ ในขณะที่เพิ่มการแสดงออกของ activating-*FcgRs* เพียงอย่างเดียว ในเซลล์แมคโครฟาจจากหนู FcgRIIb-/- สรุปได้ว่า endotoxemia ที่เกิดจากการรั่วของลำไส้มีความรุนแรงมากกว่าในหนู FcgRIIb-/- ที่ได้รับยา indomethacin เมื่อเทียบกับหนูปกติ เนื่องจากการผลิตไซโตไคน์ของเซลล์แมคโครฟาจจากหนู FcgRIIb-/- เมื่อกระตุ้นด้วย endotoxin มีความรุนแรงมากกว่าเซลล์แมคโครฟาจจากหนูปกติ ซึ่งอาจเนื่องมาจากการขาดการแสดงออกของ inhibitory-*FcgRIIb* ดังนั้นเป็นไปได้ว่าภาวะลำไส้รั่วจากการใช้ยา NSAIDs ในขนาดสูงอาจทำให้เกิดความรุนแรงของโรคลูปัสที่มากขึ้นได้

สาขาวิชา	จุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต .....
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Thansita Bhunyakarnjanarat : Endotoxemia from NSAIDs-induced gut-leakage enhanced lupus characteristics in Fc gamma receptor IIb deficient lupus mice. Advisor: Assoc. Prof. ASADA LEELAHAVANICHKUL, M.D., Ph.D.

High dose of non-steroidal anti-inflammatory drugs (NSAIDs), the common analgesia, might induce lupus activity through NSAIDs-induced gastrointestinal permeability defect (gut leakage) that causes endotoxemia. Indomethacin (25 mg/day) was orally administered for 7 days in 24-week-old Fc gamma receptor IIb deficient (FcgRIIb-/-) mice, an asymptomatic lupus model, and age-matched wild-type (WT) mice. The severity of indomethacin-induced enteropathy in FcgRIIb-/- mice was higher than WT mice as demonstrated by intestinal injury (histology, immune-deposition and intestinal cytokines), gut leakage (FITC-dextran assay and endotoxemia) and serum cytokines. In addition, higher responses against endotoxemia in indomethacin-administered FcgRIIb-/- mice was also supported by the prominent responses of FcgRIIb-/- bone marrow-derived macrophages toward lipopolysaccharide (LPS) compared to WT macrophages. LPS induces the expression of both activating-*FcgRs* (*FcgRIII* and *FcgRIV*) and inhibitory-*FcgRIIb* in WT macrophages, while enhanced only activating *FcgRs* in FcgRIIb-/- mice cells. In conclusion, gut leakage-induced endotoxemia is more severe in NSAIDs-administered FcgRIIb-/- mice when compared with WT. Due to a lack of inhibitory *FcgRIIb* expression, cytokine production from FcgRIIb-/- macrophages were more prominent than the WT cells. Hence, lupus disease activation from NSAIDs-induced gut leakage is possible through NSAIDs enteropathy.

Field of Study:	Medical Microbiology	Student's Signature .....
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Thansita Bhunyakarnjanarat

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CHAPTER I

INTRODUCTION

Lupus, known as Systemic Lupus Erythematosus (SLE) is a common chronic autoimmune disease that damages several parts of the body. The pathogenesis of lupus correlates with many factors, including age, gender, environmental factors and multiple genetic defects. Polymorphisms of Fc gamma receptors (FcgRs) are one of the genetic defects that are responsible for the development of lupus (1). FcgRs are receptors for recognition of Fc portion of immunoglobulin that are classified into the activating and inhibitory receptors. FcgRIIb is the only inhibitory FcgR, expressing on B-cells and on myeloid lineage effector cells. FcgRIIb induces an inhibitory signaling via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (2, 3). There was a high prevalence of a dysfunctional polymorphism of FcgRIIb in Asian populations (4-7). Mice with FcgRIIb deficiency (FcgRIIb-/-) have been used in several studies as a representative lupus model. FcgRIIb-/- mice spontaneously develop lupus after 16-24 weeks old (detectable of anti-dsDNA) and develops full-blown lupus after 32-40 weeks old (detectable of both anti-dsDNA and lupus nephropathy) (8-11). Therefore, FcgRIIb-/- mice older than 24 weeks are a representative for models of auto-antibody positive lupus. The deficiency of inhibitory signaling in FcgRIIb-/- mice not only causes lupus, but also results in the hyper-responsiveness against pathogen molecules, including

lipopolysaccharide (LPS). This is perhaps due to the crosstalk between TLR-4 (LPS receptor) and FcgRs (9, 12-15)

In addition to LPS (a major cell wall component of Gram-negative bacteria), TLR-4 also recognizes other pathogen associated molecular patterns (PAMPs) from other organisms and damage associated molecular pattern (DAMPs) from of the damaged host cells (16, 17). Hence, the loss of inhibitory FcgRIIb might enhance the reaction against either molecules from pathogens or host cells due to the possible crosstalk between TLR-4 and the enhanced activating FcgRs (18). Although gastrointestinal (GI) symptoms in lupus are not prominent in either patients or mice (9, 19), the immune complex deposition induces mucosal permeability defect (gut leakage) and endotoxemia in the active lupus FcgRIIb-/- mice is demonstrated (9). Without the inhibitory FcgRIIb, TLR-4 cross-links only to the activate FcgRs (9, 20, 21). In addition, macrophages, a major type of immune cells, recognizes LPS through TLR-4 (22-24) and FcgRs (4-6), the innate- and adaptive- immune receptors, respectively. Hence, the physiologic alteration of the receptors between wild-type (WT) and FcgRIIb-/- cells may be different.

Non-steroidal anti-inflammatory drugs (NSAIDs; indomethacin, diclofenac, aspirin, ibuprofen etc.) are commonly used to relieve several symptoms (musculoskeletal pain and arthritis) in patients with active autoimmune diseases. The anti-inflammatory property of NSAIDs bases on the blockage of cyclooxygenase (COX), also referred to as prostaglandin-endoperoxide synthase, to prevent prostaglandins

(PGs) conversion from arachidonic acid, a cell membrane polyunsaturated phospholipid (25). COX exists in two isoforms, constitutively expressed COX-1 and inducible COX-2. COX-1 and COX-2, are responsible for initiating PGE2 synthesis. COX-1 is a housekeeping enzyme for protecting the stomach and maintaining several functions including lung function (airway smooth muscle), blood flow in several organs (kidney and gut), platelet activity, and intestinal mucosa (25). Meanwhile, COX-2 is an inducible enzyme for the synthesis of several proinflammatory-PGs including in macrophages/ monocytes (26, 27). Hence, the blockage of COX enzymes results in several NSAIDs adverse effects, mainly through the smooth muscle contraction (vasospasm and bronchospasm) and the mucosal injury (28, 29). The inflammatory reaction is an important part of the wound healing process (30) that could be severe enough for the induction of systemic inflammation (31). With NSAIDs intestinal side effect, the mucositis from NSAIDs could be severe enough to cause gut translocation of endotoxemia that enhanced further inflammatory responses (cytokines and cell apoptosis) (25). Hence, it is very surprising that the increased inflammatory activity is possibly caused by an anti-inflammatory drug (NSAIDs).

Among all of the NSAIDs side effects, intestinal injury and nephropathy are the most common complications (32). Although gastritis is the most common NSAIDs-induced enteropathy, NSAIDs actually damage gut mucosal throughout the GI tract (32) and causing gut permeability defect (gut leakage or leaky gut syndrome) (32, 33). Additionally, NSAIDs cause nephropathy (34, 35) that induces systemic inflammation

through gut-renal axis, partly from uremia-induced gut leakage and endotoxemia (36). Since inflammation and gut leakage induce lupus flare-up and lupus activity (11, 37, 38), it is possible that NSAIDs might activate lupus disease activity through NSAIDs-induced gut leakage. Despite an availability of the selective COX-2 inhibitory NSAIDs with a lower GI side effect, the short-acting non-selective COX-1 and COX-2 inhibitory NSAIDs are still currently used in several situations (39-41). Indomethacin, a potent NSAIDs with a high likelihood of a GI side effect, continues to be administered to patients and is frequently employed in animal models (42, 43). Here, our study performed both in vitro and in vivo investigations to determine the impact of indomethacin, a representative NSAIDs, against lupus using FcgRIIb-/- asymptomatic lupus mice at 24-week-old.

CHAPTER II

OBJECTIVE

- To demonstrate NSAIDs enteropathy induced endotoxemia (gut leakage) in FcgRIIb-/- mice and wild-type mice.
- To determine the expression of different types of Fc gamma receptors on FcgRIIb-/- and wild-type macrophages after endotoxin activation.

CHAPTER III

LITERATURE REVIEW

Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) also known as Lupus is an autoimmune disease caused by a complex mixture of genetic and environmental factors, particularly in females of childbearing age. The history of SLE dates back all the way to 400 BC. The first patient suffered from SLE with cutaneous ulcerous skin was described by Hippocrates (44, 45). The signs and symptoms of SLE vary among affected individuals, and can involve many organs and systems, including mouth, lungs, heart, skin, joints, kidneys and other organs (Fig.1). The effects of lupus on the body are as following (46):

1) Heart: If inflammation affects the heart, it can result in myocarditis and endocarditis. It can also affect the membrane that surrounds the heart, causing pericarditis. Chest pain or other symptoms may present. Endocarditis can damage the heart valves, causing the thickening valvular surface and developing heart failure.
2) Kidneys: Around 1 in 3 people who have systemic lupus erythematosus develop some form of kidney inflammation, called lupus nephritis. Inflammation of the kidneys (nephritis) can make it difficult for the body to effectively remove waste products and other toxins.

3) Lungs: Some people develop pleuritis, an inflammation of the lining of the chest cavity that causes chest pain, particularly with breathing. Pneumonia may also develop.

4) Blood: Lupus can cause anemia, leukopenia (a decreased number of white blood cells) or thrombocytopenia (a decrease in the number of platelets in the blood, which assist in clotting).

Some people experience an occasional period of active SLE symptoms. Nevertheless, SLE is characterized by immune complex deposition and the development of several autoantibodies which anti-dsDNA is a specific auto-antibody of lupus. The loss of tolerance to various self-antigens is an important pathogenesis of SLE.

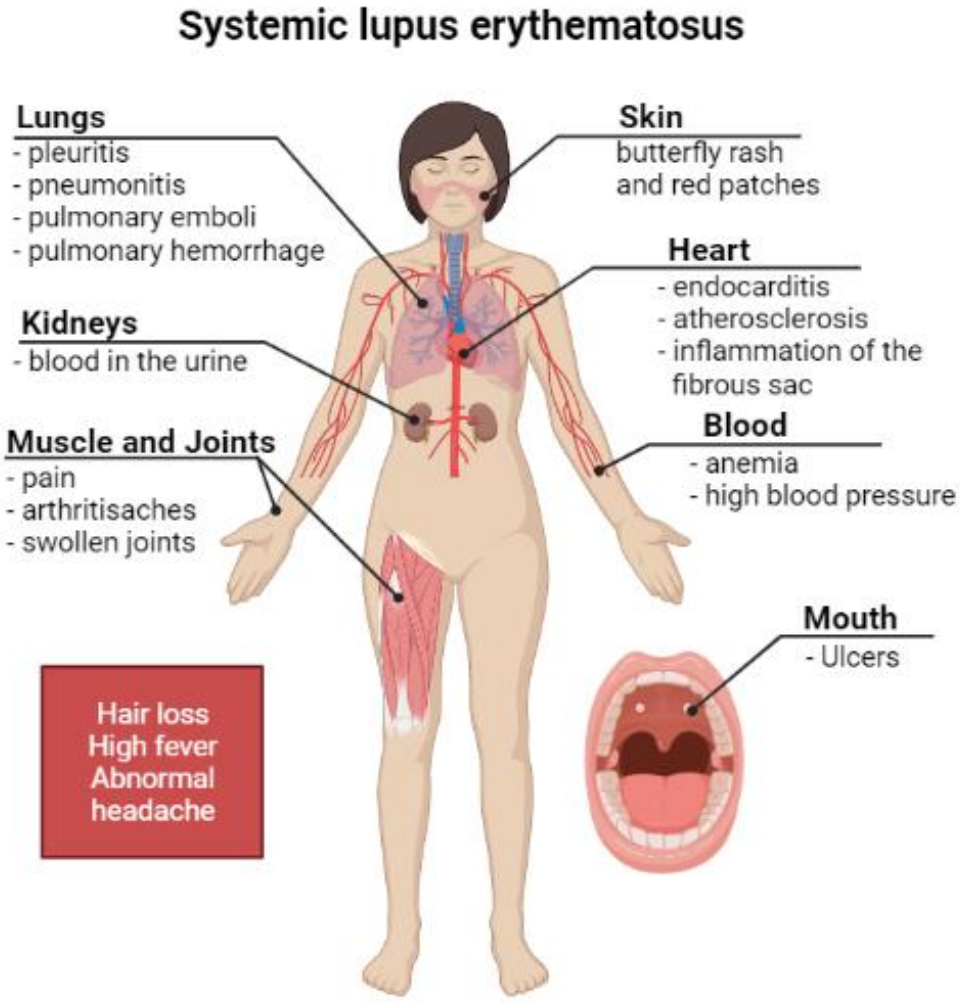


Figure 1. SLE effects on other body systems and affects people in different ways. Symptoms can occur in many parts of the body. (Image, using BioRender.com, is modified from reference number 46; Vincent J. Tavella and Yvette Brazier, 2020)

The innate immunity

The human body has an immune response to foreign pathogens. The immune system is a complex network of biological processes that defends the body against infection and can be activated by a lot of different stimuli that the body doesn't recognize as its own. These are called antigens (47, 48). There are two main parts of the immune system: the innate immune system (non-specific) and the adaptive immune system (specific). Both of these two systems work together when germs or pathogens trigger the immune responses (48).

The innate immune system is the first line defenses against non-self-pathogens that can be activated immediately in order to prevent the spreading of pathogens. Components of innate immune response are divided in 2 parts:

1) Barriers (Anatomical barrier and Physiological barrier) is the first line of defense in preventing and destroying germs, including skin, gastrointestinal tract and respiratory tract which are covered with epithelial cells to prevent pathogens from entering the body. In addition, there are also substances that help preventing the invasion of pathogens such as secretions, mucous, normal flora, gastric acid, saliva, tears, and sweat.
2) Cellular components and humoral factors play an important role in destroy pathogens that enter the body through various processes, by intracellular killing and releasing substances to destroy pathogens (47) (Fig.2).

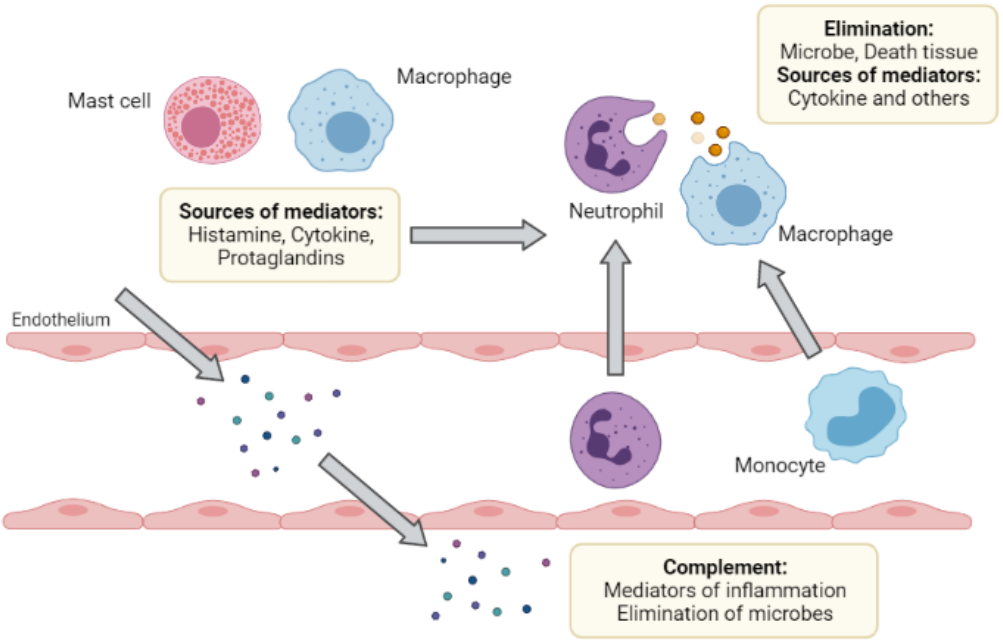


Figure 2. Functions of innate immune cells. Mast cells secrete histamine that dilates the nearby capillaries. Neutrophils and monocytes migrate from the capillaries into the infection site. Some monocytes in blood also alter into macrophages in the tissue. Neutrophils and macrophages release several chemicals to stimulate the inflammatory responses. Neutrophils and macrophages also consume the invading bacteria by phagocytosis. (Image, using BioRender.com, is modified from reference number 47; กาญจนา อู่สุวรรณทิม, 2560)

A key component of innate immunity is white blood cells (WBCs), also called leukocytes. They work to defend and protect the human body against infection. In order to patrol the entire body, leukocytes travel by way of the circulatory system. Most leukocytes are able to move freely and capture cellular debris, foreign particles, and invading microorganisms. These cells have antigen receptors which are molecule expressed on phagocytes such as neutrophil, monocyte and macrophage, which are able to recognize components and molecules from the structure of pathogens, called pathogen-associated molecular patterns (PAMPs) (48). They are recognized by pattern

recognition receptors (PRRs) such as toll-like receptors (TLRs), scavenger receptors, C-type lectin-like receptors (CLRs), mannose receptor etc. (Fig.3) (4 7) . When PAMPs/DAMPs binds to PRRs, phagocytes engulf the particle and digest through the phagocytosis process.

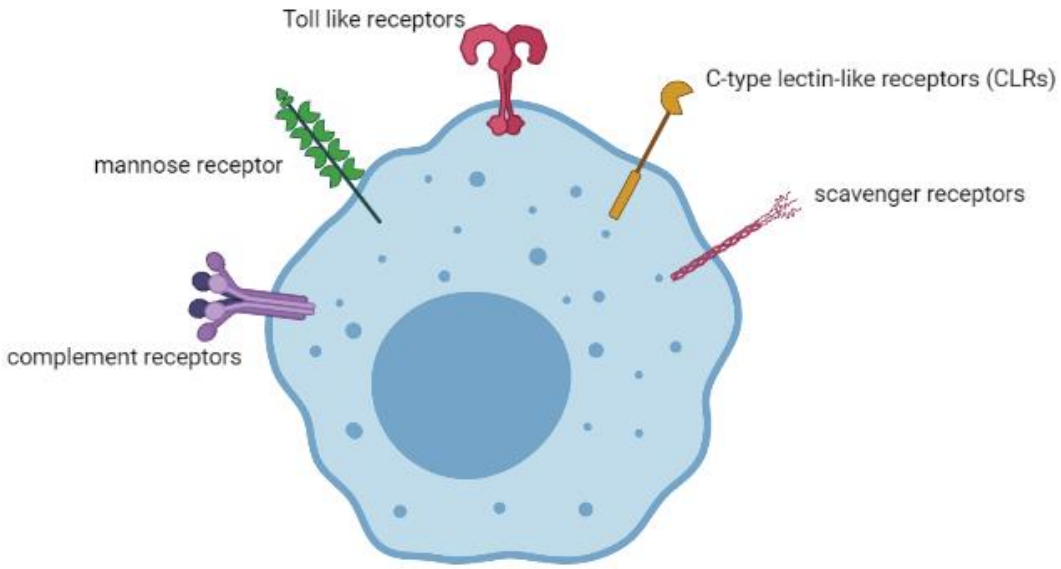


Figure 3. The pattern recognition receptors on macrophage.
(Image is generated by BioRender.com)

Macrophage, commonly abbreviated as "Mϕ", is a type of phagocyte, which is a cell responsible for detecting, engulfing and destroying pathogens. Macrophages are efficient phagocytic cells that can leave the circulatory system by moving across the walls of capillary vessels. The capacity of migration beyond blood circulation is essential for macrophage as it facilitates macrophages to recognize and capture pathogens. Macrophages can also release chemokines in order to recruit other inflammatory cells to the site of infection (47, 48).

Fc gamma receptors and lupus mouse model

Fc gamma receptors (FcgRs) are molecules expressed on the surface of a variety of immune cells that recognize the Fc portion of immunoglobulin (IgG) and are important in both promotion and regulation of immune cell responses, including the degranulation of mast cells, phagocytosis by macrophages, and proliferation of B cells (49, 50). FcgR family comprises three members (FcgRI, FcgRII and FcgRIII) in human (51) (Fig.4) and four members (FcgRI, FcgRII, FcgRIII and FcgRIV) in mouse (52) (Fig.5). FcgRs are classified in two types, the activating and inhibitory receptors. The activating receptors are the high-affinity receptor FcgRI and a family of low-affinity receptors FcgRIIa, FcgRIIc, FcgRIIIa and FcgRIIIb and FcgRIV in mice (53), which directs an activating signaling via immunoreceptor tyrosine-based activation motifs (ITAMs). FcgRIIb is an inhibitory receptor that is expressed on all innate immune cells and B cells, which directs an inhibitory signaling *via* immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (3).

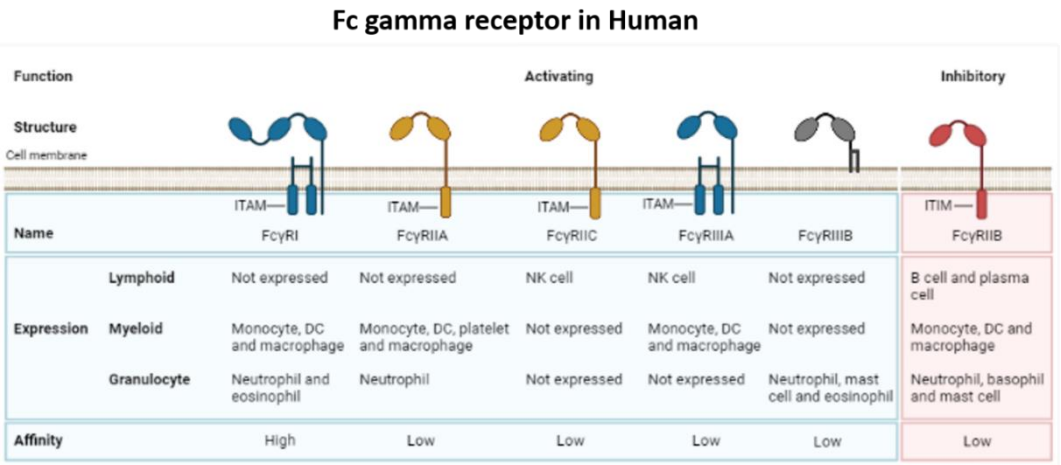


Figure 4. Structure, cellular distribution and affinity of human activating and inhibitory Fc gamma receptor family.

(Image, using BioRender.com, is modified from reference number 3; Smith and Clatworthy, 2010)

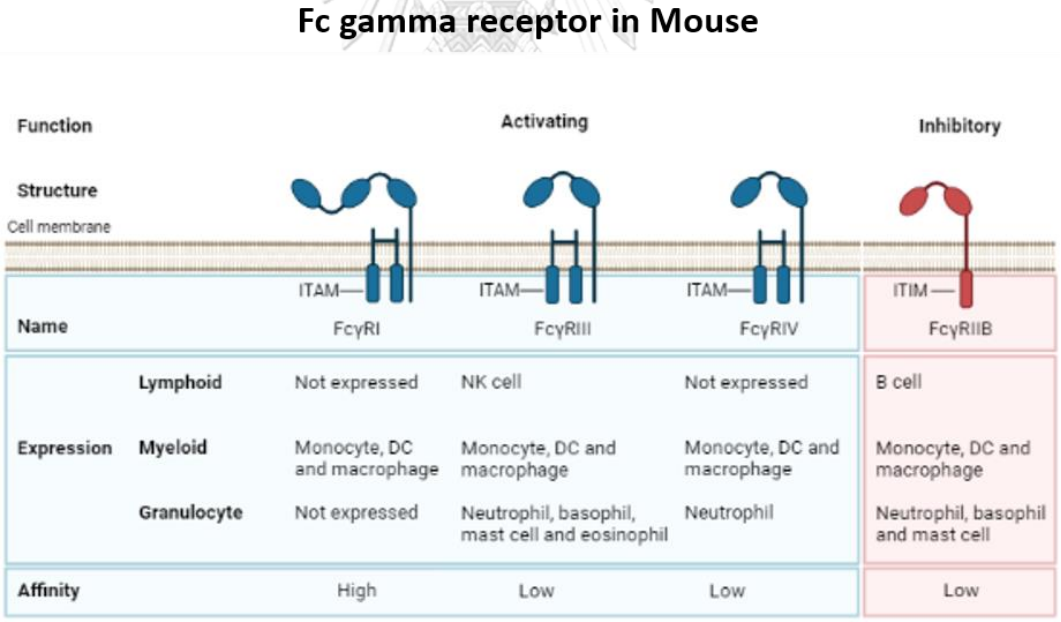


Figure 5. Structure, cellular distribution and affinity of mouse activating and inhibitory Fc gamma receptor family.

(Image, using BioRender.com, is modified from reference number 52; Bruhns, 2012)

The association between Fc gamma receptor IIb (FcgRIIb) dysfunction polymorphism and SLE has been reported. FcgRIIb polymorphism is common in patients with SLE, particularly in Asian populations. Defects in FcgRIIb are associated with SLE both in mice and humans (54, 55). Additionally. the deficiency of FcgRIIb exaggeratly induces immune responses against several antigens, including the auto-antigens. There is an age-dependency in the development of lupus characteristics in FcgRIIb deficiency (FcgRIIb-/-) mice, as anti-dsDNA, a major lupus auto-antibody, spontaneously develop lupus after 16-24 weeks old (8-10). FcgRIIb-/- mice that younger than 24-week-old are asymptomatic lupus because of the undetectable anti-dsDNA (14, 15). Meanwhile, FcgRIIb-/- mice that older than 40 weeks old develop anti-dsDNA, proteinuria and increased serum creatinine (lupus nephritis) (14, 15). Therefore, FcgRIIb-/- mice are the representative model of lupus in either asymptomatic or symptomatic stages of the diseases that become a very useful tool for the research in the topic.

Gut leakage

Gut is the common term referring abdominal tract or gastrointestinal tract (GI tract), which belongs to digestive system and help body to digest and absorb nutrients from daily food. Indeed, digestive system also includes other parts, ranging from mouth, esophagus to stomach and bowels. However, most of nutrients and minerals were absorbed in intestinal. Strikingly, it is area facing to a lot of exogenous substances and microbes, which put a critical demand for intestine to establish properly functioning barrier. Otherwise, exogenous substances like microbes or toxins are likely to penetrate epithelial cell and spread out into blood circulation. Hence, it is noticeable that plenty of protective mechanism is found on gastrointestinal tract, including physical barrier, biochemical protection and immune response (56) (Fig.6).

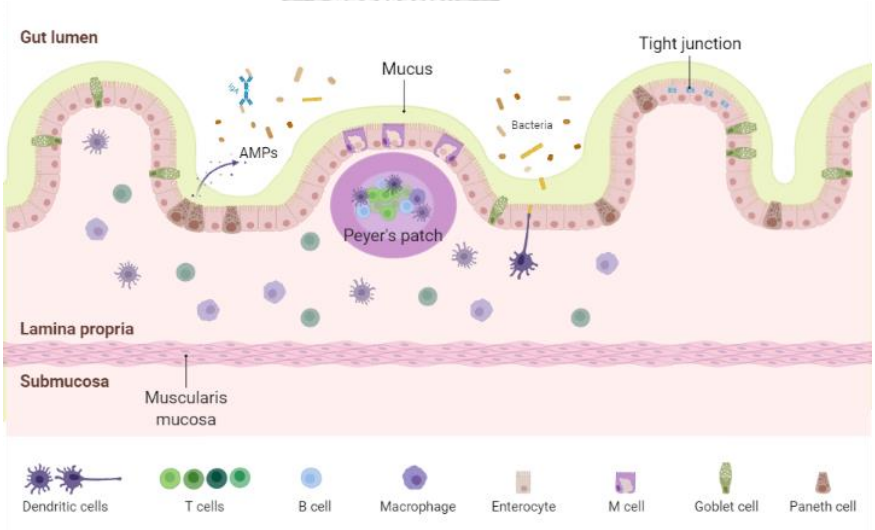


Figure 6. Components of the intestinal epithelial cells of the small intestine, including physical barrier (epithelium cells, mucus, tight junctions, commensal bacteria), biomedical barrier (antimicrobial proteins that secreted by Paneth cell), and immunological barrier (lymphocytes and IgA).

(Image, using BioRender.com, is modified from reference number 56; Mu, Kirby, 2017)

"Leaky gut" implies to the defective conditions of permeability in gut as the conjunction of intestinal epithelial cell are impaired, which leading to a decrease in protective ability of gut against the penetration of exogenous toxins. As such, when gut is leaked, exogenous substances penetrate into the body that triggers the immune responses with the recruitment of innate immune cells (57). Strikingly, in case of dysregulated immune function, such exogenous substances can be an initiation of immune activation that worsen the severity of several diseases such as rheumatoid arthritis and irritable bowel syndrome (58) There are numerous factors that leading to the defect of gut permeability, including chronic exposure to harmful food, chronic infection, or excessive uptake of alcohol (59, 60) (Fig.7).

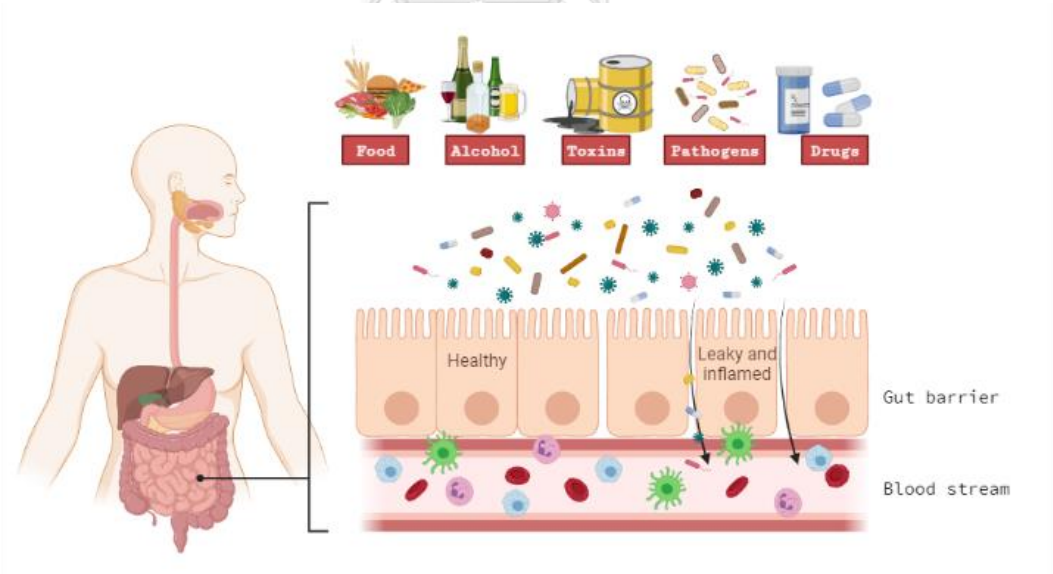


Figure 7. Comparison between a healthy and leaky gut. And Factors contributing to the development of leaky gut.

(Image, using BioRender.com, is modified from reference number 57; Sarah Ballantyne, 2019)

Accumulation of evidences show that gut microbiota and epithelial barrier can be profoundly altered by exposure to some specific nutrients or some kinds of ingredients in the ordinary diet (61). It is remarked by Qinghui Mu et al that alteration of commensal bacteria in gut is mainly caused by diet, which leads to decreasing function of gut barrier. As such, it is sought that vitamin D plays a protective role in intestine barrier by augmenting claudin-1 and TJ proteins ZO-1 expression. Hence, deficiency of vitamin D can lead to decrease in gut barrier function. Moreover, the work of Juan Kong also revealed the role of vitamin D by performing colitis model using dextran sulfate sodium (DSS) and they explored that genetic-engineered mouse without receptor of vitamin D showed more severe symptoms in colitis, which proves the role of vitamin D in gut protection (62).

Emerging publication has made an effort to verify the impact of alcohol in intestinal permeability against foreign substances such as endotoxin. The results illustrate that chronic exposure of rat to alcohol is capable of increasing permeability of mucosal intestine that allows gut translocation of high molecular weight molecules (such as horseradish peroxidase) (63, 64). The permeability of gut was also found in rat with oral administration of ethanol (65). Furthermore, the enhance in intestinal permeability in human via ethanol consumption was also demonstrated by increasing detection of polyethylene glycol (PEG) 400 after alcohol consumption in healthy persons (66). Intriguingly, alcohol-administrative rat shows a significant gut leakage with endotoxin as demonstrated by increased concentration of LPS in rat serum after

alcohol oral administration (65). Taken together, alcohol act as an initiator to induce gut leakage and endotoxemia.

Another risk factor inducing gut leakage is medical drugs encompassing anti-inflammatory drugs such as steroids or non-steroid anti-inflammatory drugs, which is considered as culprit of damaging intestinal mucus layers (60). Additionally, several publications illustrate that the increased utilization of NSAIDs lead to the exacerbation of gut permeability defect by triggering cycle of inflammation (60), partly through the mitochondrial damage-induced program cell death (67). Subsequently, gut leakage increases PAMPs (from organisms) and DAMPs (from damaged host cells) in blood circulation, which is able to activate immune cells lead to chronic inflammation (68). Perhaps, gut leakage might cause some un-healthy symptoms that triggering the increased doses of NSAIDs and worsens inflammation as a continuous cycle of pathogenesis.

Lipopolysaccharide (LPS) and SLE

In addition to, the loss of inhibitory signaling in FcgRIIb-/- mice not only causes lupus, but also results in the hyper-responsiveness against several molecules (from either host or pathogens), including lipopolysaccharide (LPS), a potent inflammatory activator from the Gram-negative bacteria. The human body contains millions of tiny living organisms in the normal condition, referred to as “human microbiota”, especially in the gut. Gram-negative bacteria are the most abundance organisms in gut that are characterized by an envelope that contains two membranes: an inner membrane and an outer membrane. These two membranes surround the aqueous cellular compartment termed the periplasm, which contains peptidoglycan cell wall. LPS, also known as endotoxins, are a large molecule composed of three structural domains: lipid A, core oligosaccharide and O-antigen (Fig.8) (69). Lipid A, the hydrophobic portion of the molecule, is an acylated β-1‘-6-linked glucosamine disaccharide that forms the outer leaflet of the outer membranes of most Gram-negative bacteria. The core oligosaccharide is a short chain of sugar residues within Gram-negative bacteria, usually contains an oligosaccharide component which attaches directly to lipid A and commonly contains sugars such as 3-deoxy-D-mannooctulosonic acid (KDO), heptoses and various hexoses. The O-antigen are major component of the surface lipopolysaccharide of Gram-negative bacteria and are highly variable in structure that is attached to the core oligosaccharide. It is composed of a repeating oligosaccharide

made of two to eight sugars (69, 70). These three structural domains are found in the outer membrane of Gram-negative bacteria that can be recognized by Toll-like receptor 4 (TLR4) that initiate inflammation and the inflammation that severe enough can promote SLE activity (69, 71, 72). Endotoxins that separated from death bacteria are able to cross gastro-intestinal barrier and pass into bloodstream (73).

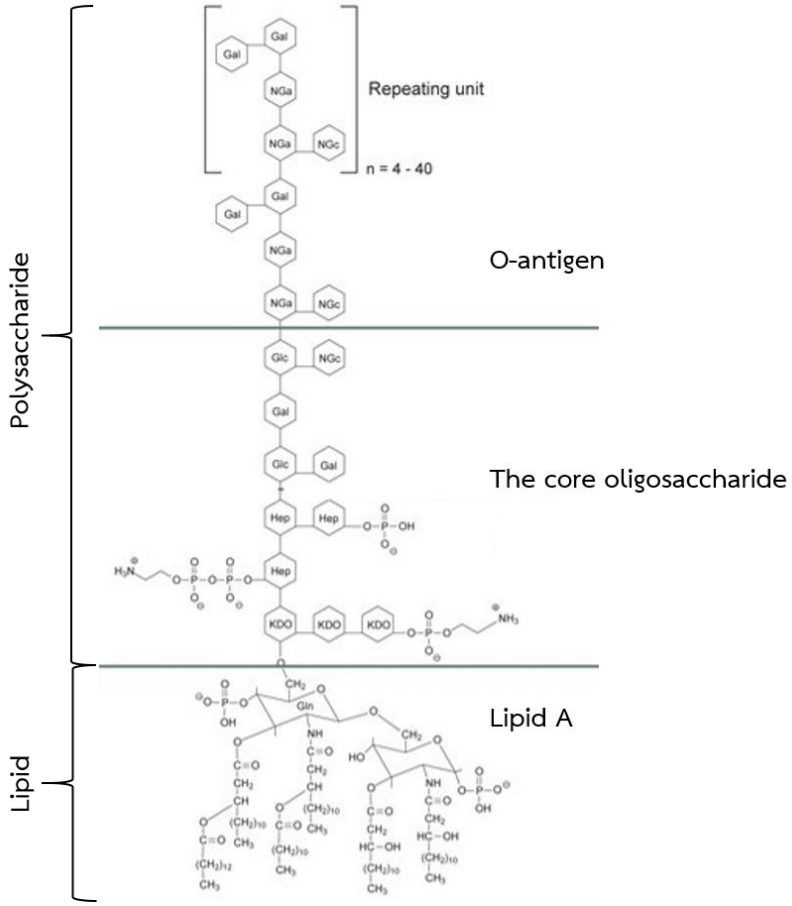


Figure 8. The structure of lipopolysaccharides.
(Image is modified from reference number 74; Sigma life science, 2021)

In SLE patients, the higher level of soluble CD14 (sCD14), which is released by monocytes when the cells are exposed to LPS, is increased in blood (75). Activation

of TLR4 also exacerbates lupus disease activity in several transgenic lupus mouse models (76, 77). The hyperresponsiveness of TLR4 responses against gut flora (which contains LPS) induce SLE activity. Moreover, the immunization of wild-type mice (C57BL/6 or BALB/c) with phospholipid-binding proteins induced lupus-like disease, that can be facilitated by LPS administration (78, 79). Taken together, these data suggest that LPS stimulation and TLR4 activation are lupus exacerbating factor.

Non-steroidal anti-inflammatory drugs (NSAIDs)

Inflammation is a process by which the immune response defends the body from harmful stimulants, such as pathogens and the damaged cells. General inflammatory symptoms are swelling, pain and fever (80). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to reduce pain and inflammation (in joints and other parts) which are the most commonly used medications in the world. Appropriate doses of NSAIDs is effective for fever, pain and other inflammatory signs (81). The anti-inflammatory property of NSAIDs is to inhibit the function of the cyclooxygenase (COX) enzyme and thereby reduce the production of prostaglandins (PGs) that cause pain and swelling in inflammation. When the tissue is damaged, polyunsaturated phospholipids in the cell membrane are initially converted to arachidonic acid by phospholipase A_2 and the arachidonic acid is either converted to prostaglandins (PGs) by enzyme cyclooxygenase (COX) or converted to leukotrienes by enzyme lipoxygenase (LOX) (Fig.9) (25, 82, 83). There are two types of COX enzymes, constitutively expressed COX-1 and inducible COX-2. COX-1 is a housekeeping enzyme for protecting the gastric mucosa lining and maintaining several functions including lung (airway smooth muscle), blood flow (kidney and gut), platelet aggregation, and intestinal mucosa (25). Meanwhile, COX-2 is an inducible enzyme for the synthesis of several proinflammatory-PGs including in macrophages/ monocytes (26, 27).

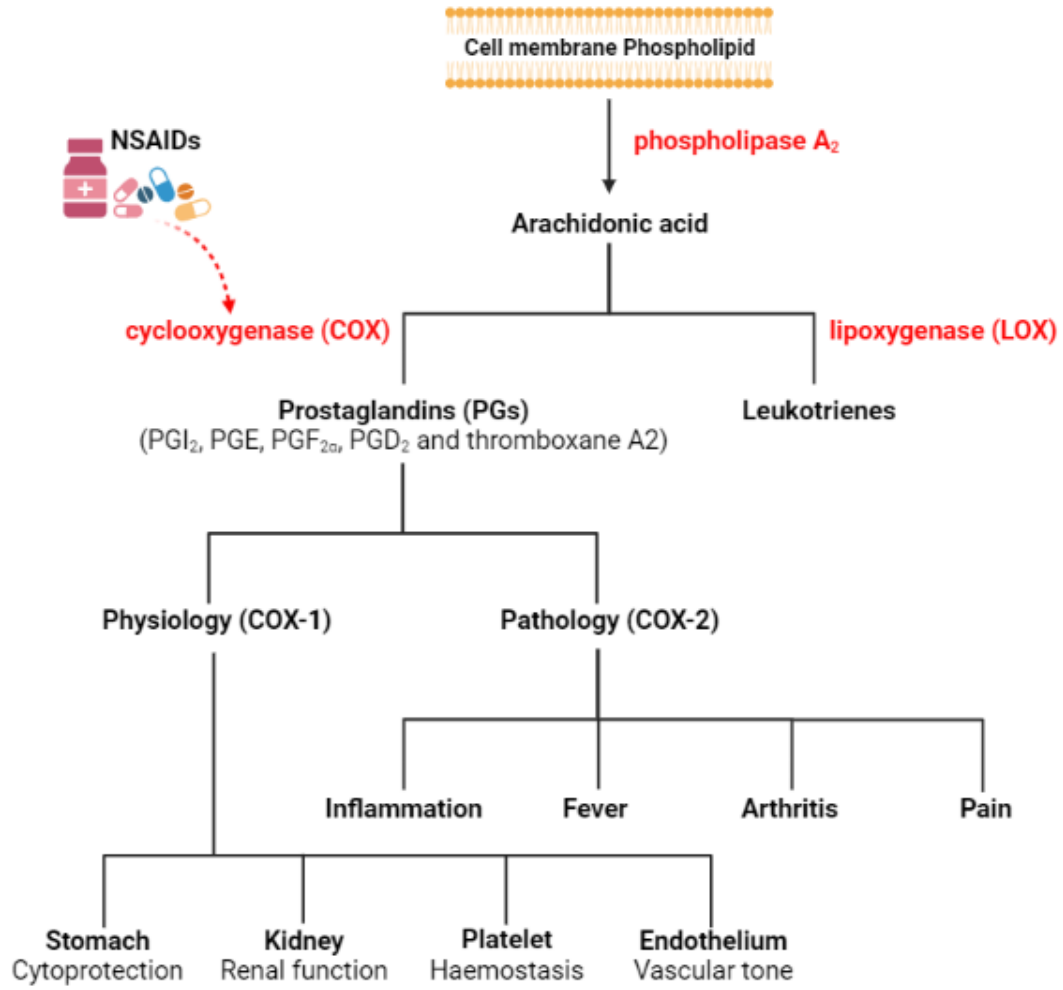


Figure 9. Arachidonic acid pathway showing production of prostaglandins from membrane phospholipids.

(Image, using BioRender.com, is modified from reference number 80; Thongchai Korsuntirat, 2010)

NSAIDs are drugs that reduce the Inflammation with a chemical formula related to aspirin. Classification of NSAIDs can be classified into 3 main groups of drugs, including (25, 80):

1) Classification of NSAIDs based on structure

   i. Salicylates such as sulfasalazine, acetylsalicylic acid, sodium salicylate and diflunisal

   ii. Aryl and heteroaryl acetic acid derivatives such as ibuprofen, fenoprofen, naproxen and oxaprozin

   iii. Indole/indene acetic acid derivatives such as indomethacin, sulindac and etodolac

   iv. Anthranilates such as diclofenac, mefenamic acid and meclofenamic acid

   v. Oxicams (enol acids) such as piroxicam and meloxicam

2) Classification of NSAIDs based on the basis of plasma half-life ($t_{1/2}$)

   i. Short duration of action (half-life less than 8 h.) such as ibuprofen, diclofenac, indomethacin

   ii. Moderate duration of action (half-life approximately 8-24 h.) such as naproxen, nimesulide, celeccoxib

   iii. Long duration of action (half-life more than 24 h.) such as piroxicam, meloxicam and etoricoxib

3) Classification of NSAIDs based on activity of inhibiting cyclooxygenase enzyme

   i. Traditional NSAIDs (IC_{50} ratio of COX-2/COX-1 more than 1) such as ibuprofen, diclofenac and indomethacin

ii. Selective COX-2 inhibitors (IC_{50} ratio of COX-2/COX-1 between 0.01-1) such as meloxicam and nimesulide

iii. Specific COX-2 inhibitors (IC_{50} ratio of COX-2/COX-1 less than 0.01) such as celecoxib, etoricoxib, rofecoxib, valdecoxib and parecoxib

NSAIDs are weak organic acids, pH between 3-5, high lipid solubility, absorbed in the stomach and small intestine. The half-life of NSAIDs are not equal. Most drugs are metabolized in the liver and excreted through the kidneys (80). Benefits of NSAIDs are demonstrated by a wide range of effect, ranging from anti-inflammation, analgesia, antiplatelet, and antipyretic. NSAIDs provide enormous benefits for many patients, but they need to be carefully used so that their risks can be minimized and their benefits are maximized. With their anti-inflammation, anticancer and antinociception, NSAIDs often become over-use and over-dose, leading to multiple adverse effects, especially in the gastrointestinal (GI) tract (25, 81). Most of the patients using NSAIDs for chronic inflammatory conditions (eg. rheumatoid arthritis or pain) were observed to have a high prevalence of gastric mucosal lesions (84, 85). In a study performed in India, NSAIDs gastrointestinal complications are reported as high as 30% of the regular NSAIDs user (86). In Pakistan, 820 patients were analyzed by upper gastrointestinal (GI) endoscopy (1998-2000), 15% of patients with gastric ulcers are associated with NSAIDs use. Interestingly, percentage of duodenal ulcer (65.3%) is higher than gastric ulcer (42.3%), although stomach symptoms are more frequently found (85). Taking into

account, the length of administration and dosage of NSAIDs are the main culprit inducing several complications.

NSAIDs-induced enteropathy

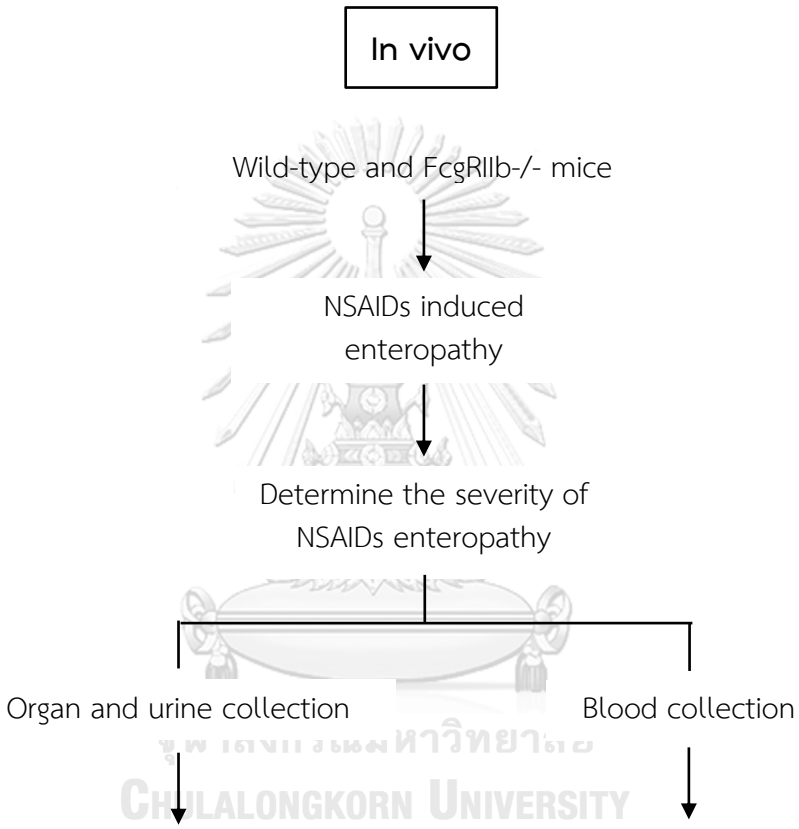
It is well known that NSAIDs can damage the gastrointestinal tract, especially in elderly (87, 88). Around 80% of NSAIDs users have acute hemorrhages, mucosal erosions, gastric erosions and small intestinal lesions as detected by endoscopic examination which, sometimes, are lethal (33, 89, 90). Chronic NSAIDs administration decreases absorptive capacity and increases intestinal permeability (33). NSAIDs, such as indomethacin, damage small intestine within 24 h, mainly in the jejunum and ileum (91, 92). There are several factors involved in the pathogenesis of NSAIDs-induced intestinal damage, including a deficiency in prostaglandins (PGs), bile acid, bacterial flora, and nitric oxide (NO) (93-97). The deficiency of prostaglandins has been identified as the most important factor for the occurrence of these lesions. A critical function of the intestinal mucosa is to form a barrier between the body and gut environment. The dysfunction of intestinal mucosa barriers can lead to activated immune signaling and intestinal inflammation which causes gastrointestinal leakage (90, 98). Up to 80% of patients with SLE need NSAIDs to reduce pain and inflammation (in joints and other parts) (99). The association between gut-microbiota composition and disease progression of lupus have been reported (100), but the information of gut-leakage against lupus is still limited. Normally, gut-barrier is a natural protection that protects the translocation of pathogen associated molecular patterns (PAMPs) and viable organisms from gut into blood circulation (101). The defect of gut-permeability causes

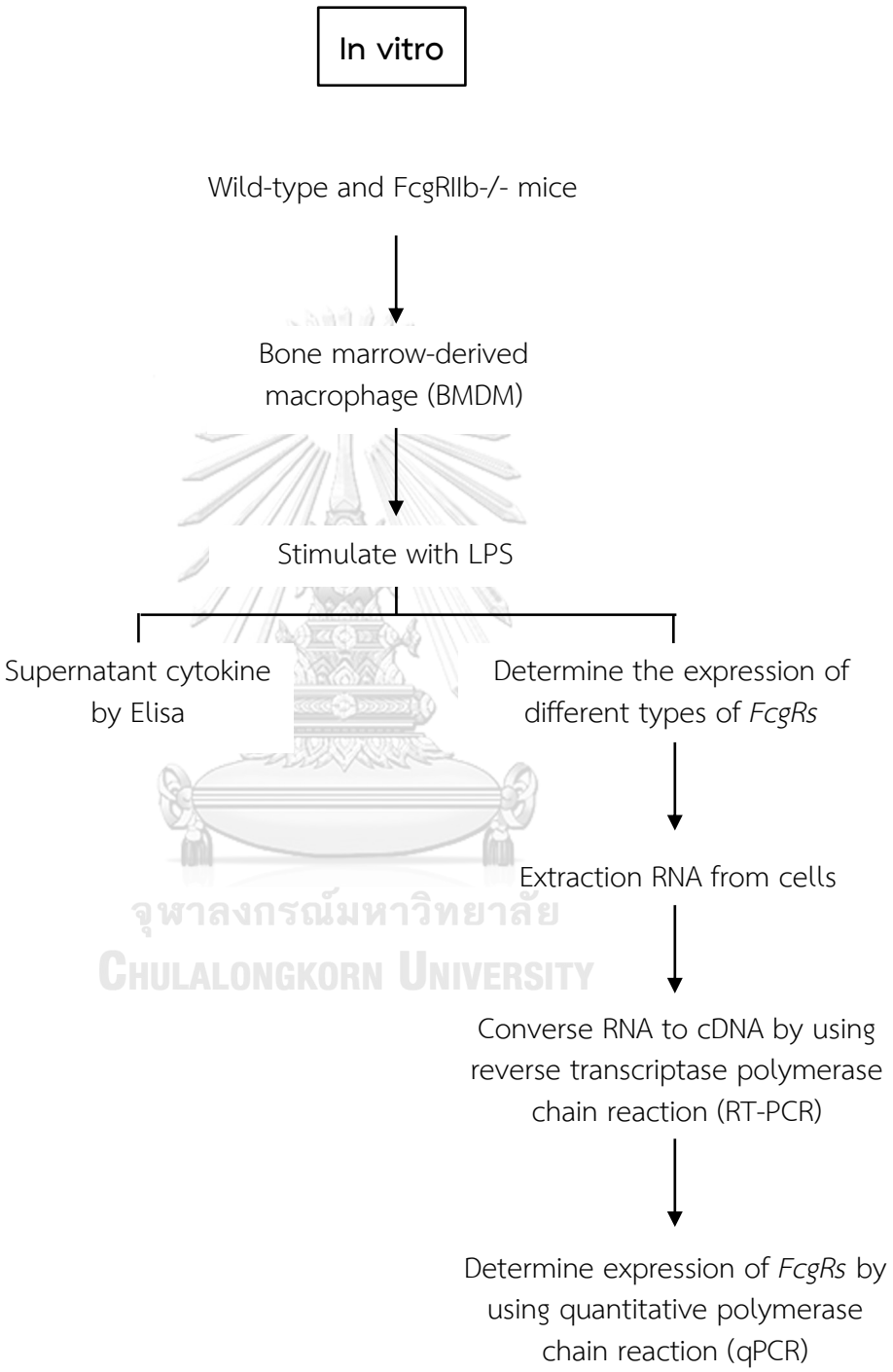
the translocation of PAMPs (102). Indeed, spontaneous gut leakage in active lupus mice and in patients with active lupus due to the deposition of circulating immune complexes (CIC) in gut are reported (9, 19). Because GI tract is the endogenous source of endotoxin which is a major molecular component of Gram-negative bacteria, gut leakage causes endotoxemia (11).

Hence, NSAIDs are one of the common drugs with obvious gastrointestinal complications that are commonly prescribed in patients with lupus. Unfortunately, the gastrointestinal complications from NSIADs, due to prolong use and/ or over doses, in patients with lupus is possible. To make the matter worst, inflammatory responses in patients with lupus could be more severe than the normal host, at least with some genetic defects. Then, the inflammatory responses against NSAIDs-induced mucositis in patients with lupus might be more prominent that the normal population that possibly lead to gut leakage. Subsequently, we performed the experiments to test this topic.

CHAPTER IV

METHODOLOGY





CHAPTER V

MATERIALS AND METHODS

Animals and Animal model

FcgRIIb deficient mice on a C57BL/6 background (FcgRIIb-/-) weight 25–30 g (n=5) and C57BL/6 mice (wild-type) weight 20–25 g (n=5) were used. The mice were received standard chow and water during the whole experiment. Only female mice were used in experiments and maintained in the facility until 24-week-old before use. FcgRIIb-/- mice develop anti-dsDNA autoantibodies as early as 16–24 weeks without kidney injury and have lupus nephritis at 40-week-old (8-10, 20, 21), FcgRIIb-/- mice at 24-week-old were used as a representative model of asymptomatic lupus. All experimental methods were approved by The Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and followed the protocols of the National Institutes of Health (NIH), USA.

For Non-steroidal anti-inflammatory drugs (NSAIDs) induced enteropathy model, the mice were induced by daily oral administration of indomethacin (Sigma-Aldrich, St. Louis, MO, USA) at 25 mg/kg diluted in 0.2 mL of phosphate buffer solution (PBS) for 7 days before sample collection. Mice were sacrificed with cardiac puncture and followed an established protocol under isoflurane anesthesia before blood and organs collection. Spot urine collection was performed at 6 h before sacrifice by

placing mice in the metabolic cage (Hatteras Instruments, NC, USA). Proteinuria was calculated by spot urine protein creatinine index (UPCI) with an equation; UPCI = urine protein (μg/mg) urine creatinine (mg/dL).

Gut permeability test

Gut permeability was investigated by measurement of serum Fluorescein isothiocyanate-dextran (FITC-dextran) assay, a non-absorbable molecule in the gut. The mice were gavaged with 0.5 mL of FITC-dextran (molecular weight, 4.4 kDa; FD4; Sigma-Aldrich) at a concentration of 25 mg/mL diluted in sterile PBS. Blood was collected by tail-vein at 3 hours after FITC-dextran administration, and serum FITC-dextran was measured by fluorospectrometry (Microplate reader Varioscan Flash LemiSens option; Thermo Fisher Scientific, Wilmington, DE) with the excitation and emission wavelengths at 485 and 528 nm, respectively, using a standard curve of serially diluted FITC-dextran.

Blood collection and serum analysis

Blood was collected by using cardiac puncture. During blood sample collection, the mouse was deeply anesthetized and lay it on back. Then, the mouse was inserted with a 21-gauge needle slightly left of and under the sternum, directed toward the animal's head. The syringe was pulled on the plunger to fill the syringe. When completion of blood collection, the mouse was immediately euthanatized. Blood was collected in the sterile tube and be centrifuged at 5,000 rpm for 5 minutes at 4°C.

then, serum was stored in the sterile tube at -80ºC for the additional analyses. Before used in experiment, serum was thawed on ice. Lupus characteristics were determined by proteinuria, serum creatinine, and serum anti-dsDNA. Assays for serum creatinine used the QuantiChrom Creatinine-Assay (DICT-500). serum anti-dsDNA was analyzed following a protocol using coated Calf-DNA (Invitrogen, Carlsbad, CA, USA). Assays for serum endotoxin (lipopolysaccharide; LPS) was analyzed by HEK-Blue LPS Detection (InvivoGen, San Diego, CA, USA) and serum cytokines (TNF-α, IL-6, and IL-10) was measured by ELISA kit (Invitrogen, Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's instruction.

Organ collection for Histology analysis and Immunofluorescent imaging

At sacrifice, renal and different intestinal parts, including duodenum, jejunum, ileum and colon were collected. The semi-quantitative evaluation of renal histology on paraffin-embedded slides was performed with Periodic acid-Schiff (PAS) color at 200× magnification in 10 randomly-selected fields for each animal. Renal injury was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation. The intestines were rinsed several times in cold PBS, and further divided into three parts. One part was frozen and stored for cytokine analysis. In brief, around 20 mg of different intestinal parts were placed in PBS, sonicated with Ultrasonic Disruption (Sonics & Materials, VCX 750, USA) and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected in the

sterile 1.5 ml microcentrifuge tube for the presence of cytokines in tissue. Assays for cytokines in tissue (TNF-α, IL-6, and IL-10) was measured by ELISA (Invitrogen, Thermo Fisher Scientific, Wilmington, DE, USA). Second part were fixed in 10% formalin, embedded in paraffin and sectioned into slide before staining with hematoxylin and eosin (H&E) for representative figures of intestinal histology. For the third part, the intestines were prepared in Cryogel (Leica Biosystems, Richmond, IL, USA) and store at -80 °C until ready for sectioning. Then, tissue was cut into 5-20 μm thick sections, tissue sections was put onto slides and stained with goat anti-mouse IgG (Alexa Fluor 488, Abcam, Cambridge, MA, USA) and DAPI (4′,6-diamidino-2-phenylindole), a blue-fluorescent DNA stain, then photographed and analyzed the fluorescent intensity by ZEISS LSM 800 (Carl Zeiss, Germany).

Bone Marrow-derived Macrophage

The femur bone marrow cells from wild-type (WT) and FcgRIIb-/- mice were obtained and centrifuged at 6,000 rpm in 4°C for 10 min. Then, the cells were incubated for 7 days in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, 1.3% penicillin/streptomycin, 1% HEPES, 1% sodium pyruvate, 5% Hi-Horse serum and macrophage colony-stimulating factor (M-CSF) in a humidified 5% CO_2 incubator at 37°C. After that, the cells (2×10^6 cells/well) were cultured in 6-well plates with DMEM supplemented with 10% fetal bovine serum, 1.3% penicillin/streptomycin, 1% HEPES and 1% sodium pyruvate under 5% CO_2 incubator

at 37°C for 24 h, then washed and stimulated with LPS (*Escherichia coli* 026: B6; Sigma-Aldrich) at 150 ng/mL for 3, 6 and 24 h under 5% CO_2 incubator at 37°C before supernatant and cell collection. The supernatant and cells were collected in the sterile 1.5 ml microcentrifuge tube for the presence of cytokines and *FcgRs* expression, respectively and store at -80 °C until ready for use. Supernatant cytokines were measured by ELISA kit (Invitrogen, Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's instruction and *FcgRs* expression (both activating and inhibitory receptor) in macrophages were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

RNA Isolation and real-time PCR Analysis

After cells stimulation with LPS, total RNA from cells were extracted using FavorPrep™ Tissue Total RNA Mini Kit (Favorgen, Biotech Corp, Taiwan) according to the manufacturer's instruction. And its concentration was measured on NanoDrop spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA USA). total RNA was converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA USA) in RT-PCR machine (ProFlex™ PCR System, Applied Biosystems, Waltham, MA USA). For the conditions of RT-PCR is shown in Table 1.

The cDNA templates were used for determine expression of FcgRs by quantitative real-time PCR (qRT-PCR). Real-time PCR was performed using QuantStudio6 Flex Real-Time PCR Systems (Thermo Scientific, Waltham, MA USA) with

PowerUp™ SYBR™ Green Master Mix (Thermo Scientific, Waltham, MA USA) in a 10 µl final reaction volume. The thermal cycling conditions were 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Each sample was tested in triplicate. Templates were omitted for the negative controls. The housekeeping gene *Beta-2-Microglobulin* (*β2M*) was used as an internal standard. A list of primers for PCR is shown in Table 2. The relative expression of each gene was calculated using the comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta Ct}$).

Table 1. The conditions of RT-PCR in reverse-transcribe to cDNA are demonstrated.

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	58	30 s	
Extension	72	45 s	

Table 2. List of Primers in the study are demonstrated.

Primers	Forward	Reverse
Beta-2-Microglobulin (*β2M*)	5'CCACTGAAAAAGATGAGTATGCCT-3'	5'-CCAATCCAAATGCGGCATCTTCA-3'
Fc gamma receptor I (*FcgRI*)	5'-CACAAATGCCCTTAGACCAC-3'	5'-ACCCTAGAGTTCCAGGGATG-3'
Fc gamma receptor IIb (*FcgRIIb*)	5'-TTCTCAAGCATCCCGAAGCC-3'	5'-TTCCCAATGCCAAGGGAGAC-3'
Fc gamma receptor III (*FcgRIII*)	5'-AGGGCCTCCATCTGGACTG-3'	5'-GTGGTTCTGGTAATCATGCTCTG-3'
Fc gamma receptor IV (*FcgRIV*)	5'-AACGGCAAAGGCAAGAAGTA-3'	5'-CCGCACAGAGAAATACAGCA-3'

Statistical analysis

All data will be analyzed by the Statistical Package for Social Sciences software (SPSS 22.0, SPSS Inc., IL, USA) and Graph Pad Prism version 7.0 software (La Jolla, CA, USA). The results were presented as mean ± standard deviation (S.D). The Mann-Whitney unpaired t-test were carried out to determine the differences in the expression between groups and control. $P < 0.05$ was considered as statistically significant.

CHAPTER VI

RESULT

High dose of indomethacin induced intestinal ulcers and enhanced gastro-intestinal permeability (gut leakage) more prominently in 24-week-old FcgRIIb-/- mice than wild-type (WT) mice suggesting a prominent adverse effect of NSAIDs in lupus.

The characteristics of lupus after indomethacin administration in FcgRIIb-/- mice compared with wild-type mice

Female FcgRIIb-/- mice at 24-week-old were used as representative lupus mice and age-matched wild-type mice were used as a control group, both FcgRIIb-/- mice and WT mice were administered by 25 mg/kg indomethacin once daily for 7 days which caused a 40% mortality rate only in FcgRIIb-/- mice but zero mortality in WT mice without difference in body weight between strains of mice (Fig.10A,B). However, administration of indomethacin enhanced the levels of anti-dsDNA, serum creatinine, and urine protein creatinine index in FcgRIIb-/- mice but not in WT mice (Fig.11A-C). Renal injury at NSAIDs-administered condition was more prominent in FcgRIIb-/- mice compared with the WT as indicated by renal histological score (Fig.12 and Fig.13) and glomerular immune complex (IC) deposition (Fig.14 and Fig.15). The common observed abnormality in lupus nephritis (11) including proteinaceous casts, red blood cell casts

(Fig.13; arrow heads and dotted line arrow) and glomerular IC deposition (Fig.15) at 7 days were prominently presented in NSAIDs-administrated FcgRIIb-/- mice, but neither WT mice nor PBS-control FcgRIIb-/- mice, suggesting an exacerbation of lupus activity by NSAIDs.

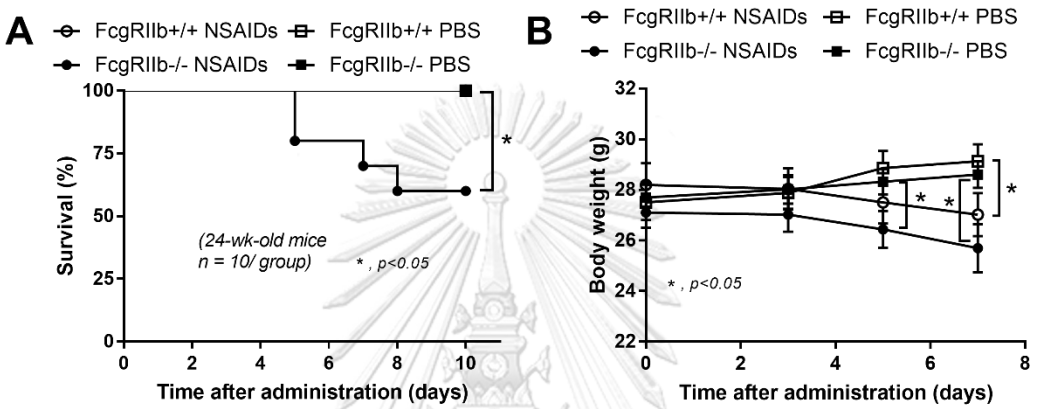


Figure 10. Characteristics of mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control in FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice as indicated by survival analysis (A) and body weight alteration (B) are demonstrated.

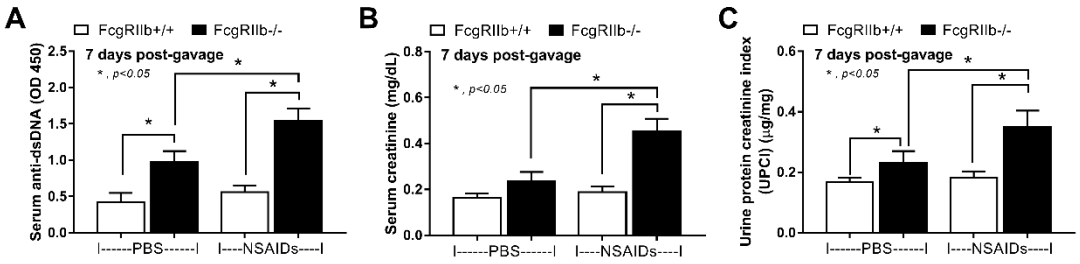


Figure 11. Characteristics of mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control in FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice as indicated by anti-dsDNA (A), serum creatinine (B), and urine protein creatinine index (C) are demonstrated.

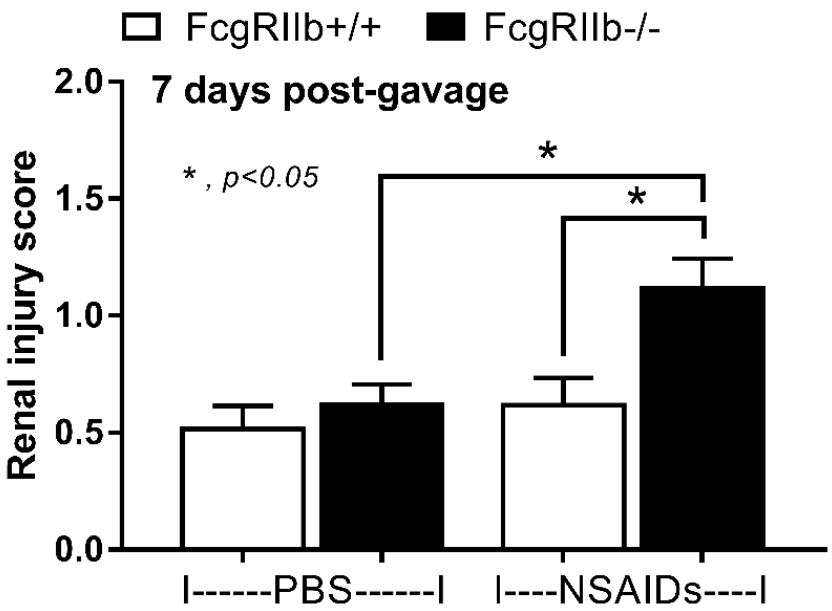


Figure 12. Representative figures of renal injury score of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control at 7 days of the administration were demonstrated.

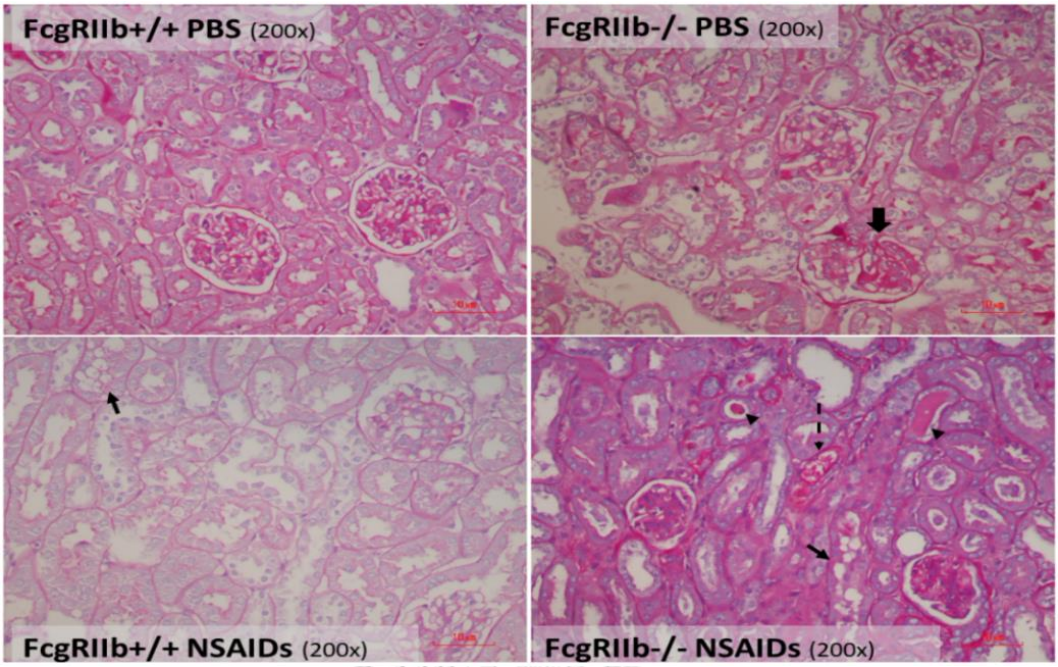


Figure 13. Representative of Periodic acid-Schiff staining (PAS) histological pictures (original magnification 200x) of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control were demonstrated. Thick arrow, prominent mesangial staining in FcgRIIb-/- PBS; Thin arrows, NSAIDs-induced tubular vacuolization in both mouse strains; arrow heads, proteinaceous cast formation in renal tubule of NSAIDs administered FcgRIIb-/- mice; Dotted-line arrow, red blood cell casts in renal tubule of NSAIDs administered FcgRIIb-/- mice.

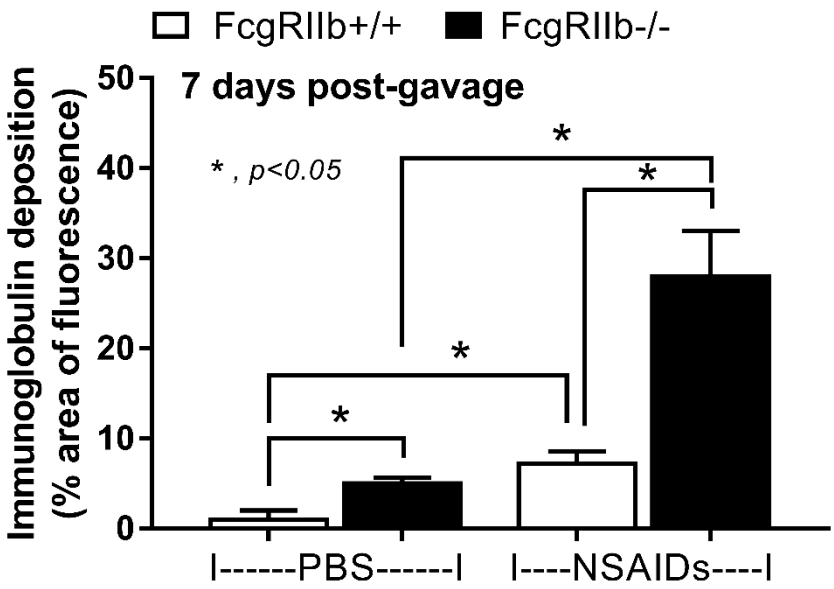


Figure 14. Representative figures of immunofluorescent score of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control at 7 days of the administration were demonstrated.

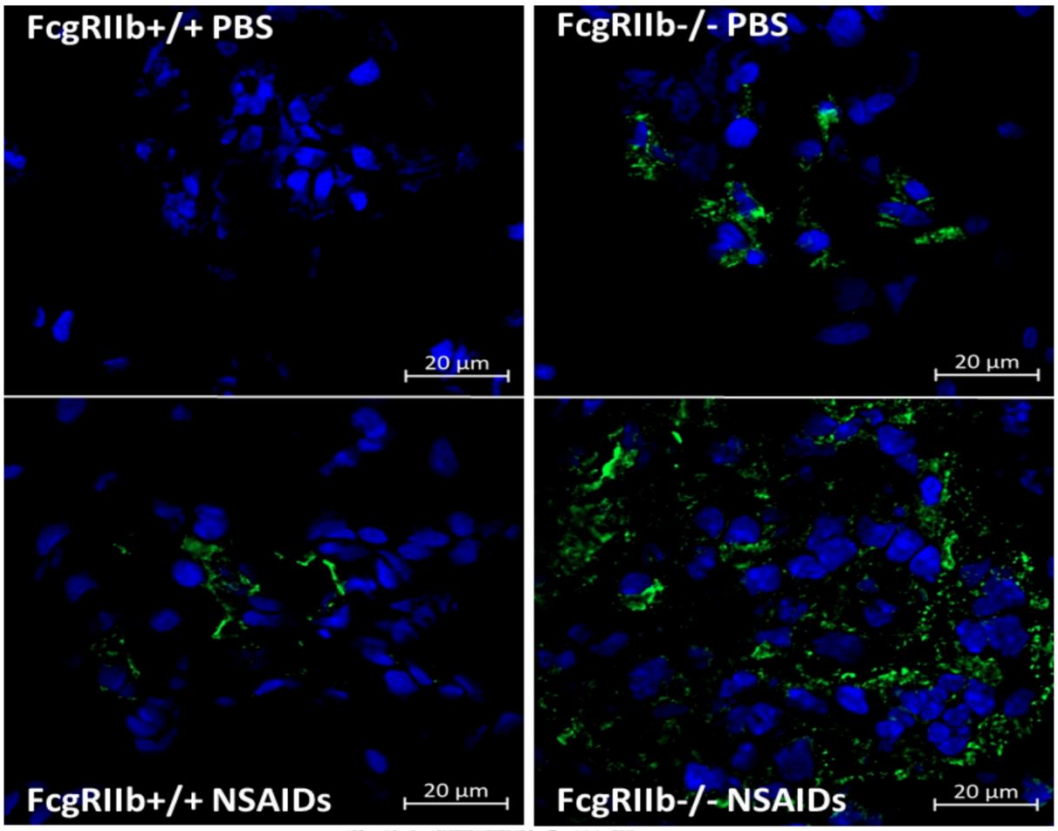


Figure 15. Representative figures of glomerular immune complex deposition of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control (original magnification 200x) at 7 days of the administration were demonstrated. Green and blue colors demonstrated immunoglobulin and cell nuclei, respectively.

Indomethacin-induced enterocolitis in FcgRIIb-/- mice compared with wild-type mice

The severity of enterocolitis in FcgRIIb-/- mice was more prominent throughout the intestines from duodenum to colon (Fig.16A-D and Fig.17) compared to the WT mice. The ulceration wounds were detectable in the duodenum, jejunum, ileum and colon of indomethacin-administered FcgRIIb-/- mice (Fig.17, arrows), while only mononuclear cells infiltration was found in WT mice (Fig.17). In parallel, the immune deposition in the intestine of the control FcgRIIb-/- mice (non-NSAIDs administration) was detectable in FcgRIIb-/- mice, but not in WT (Fig.18A-D and Fig.19). After NSAIDs administration, immunoglobulin (Ig) was also detectable in the intestines of WT mice indicating the Ig of wound repairing processes (29). However, the immunoglobulin intensity in NSAIDs-administered WT mice was less than in NSAIDs-administered FcgRIIb-/- mice (Fig.18A-D and Fig.19), possibly due to the immune deposition before NSAIDs administration in asymptomatic lupus mice. In addition, cytokines from the intestinal tissue of indomethacin-administered FcgRIIb-/- were higher than WT mice with indomethacin, while the cytokine levels exhibited no difference between mouse strains in the control groups (Fig.20A-D).

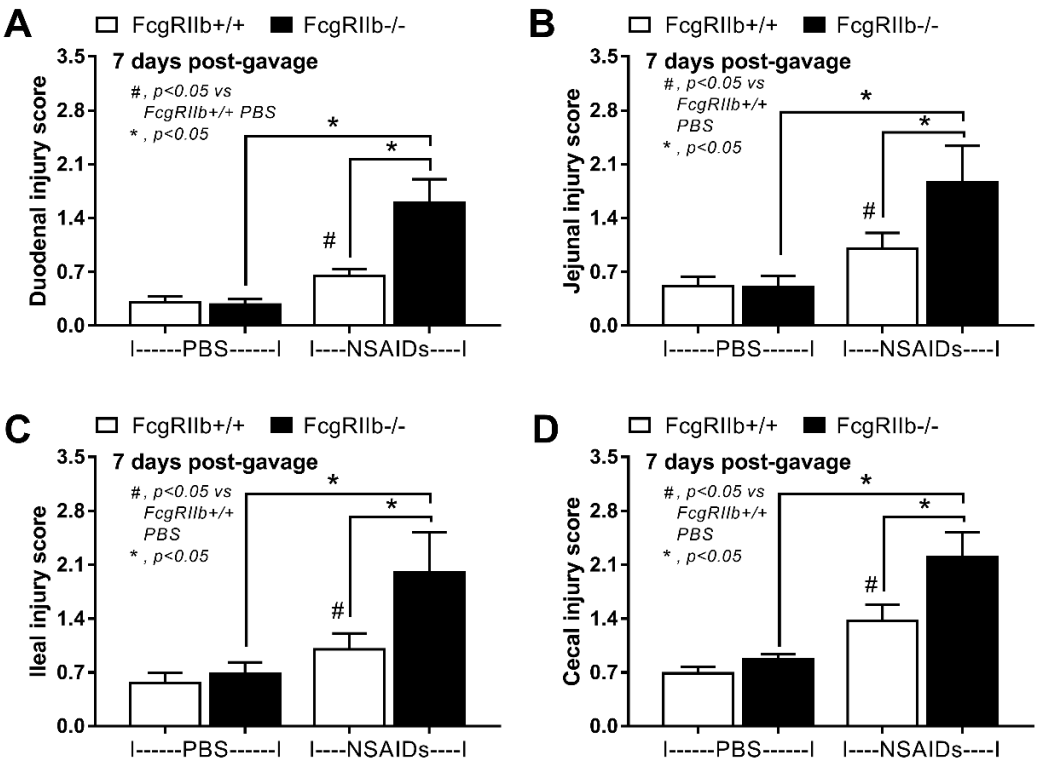


Figure 16. Characteristics of intestinal injury in FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control as determined by intestinal histopathological scores (A-D).

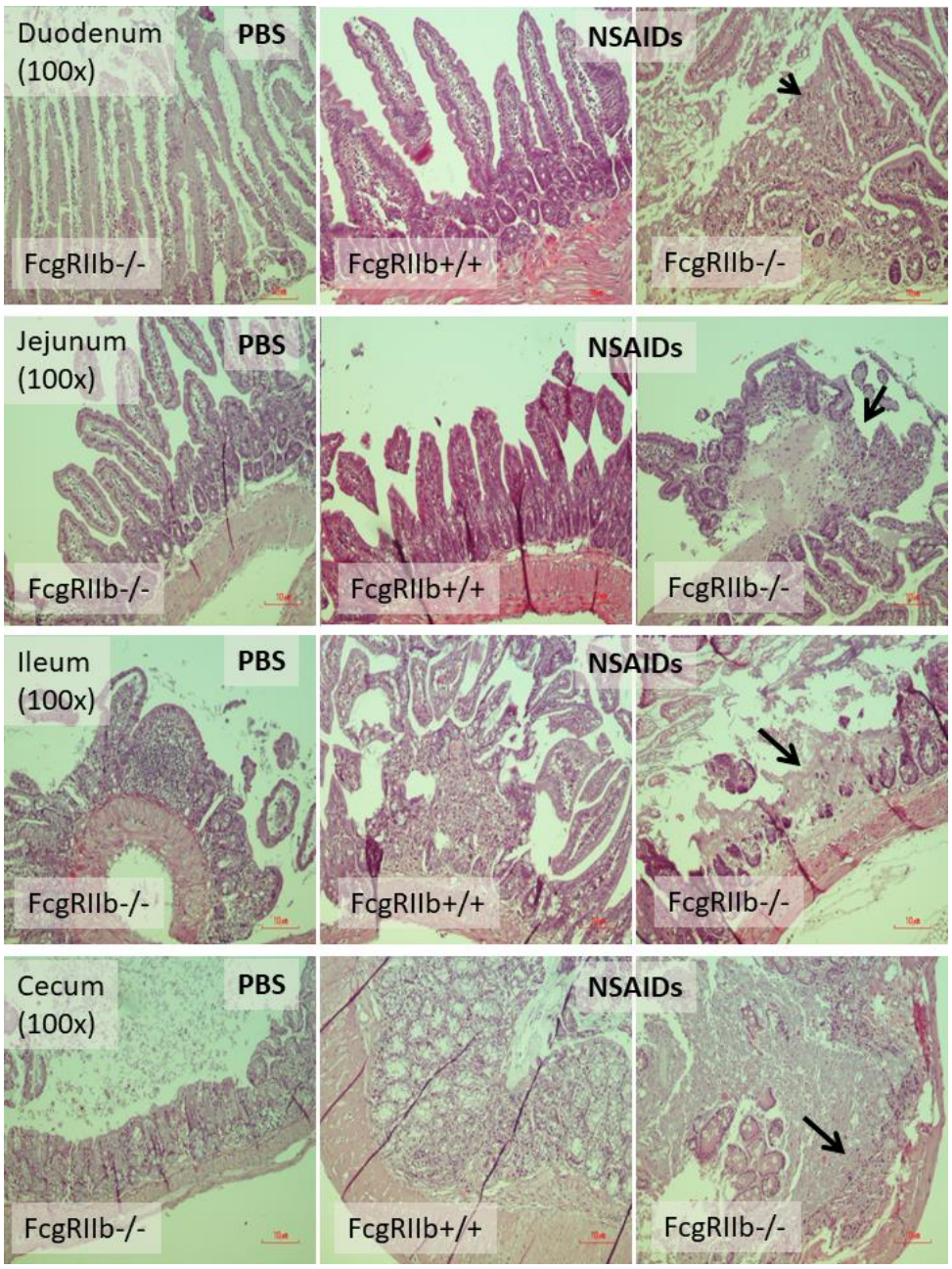


Figure 17. Representative figures of intestinal histology (hematoxylin and eosin staining) of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control (original magnification 200x) were demonstrated. Figures of PBS-administered wild-type control mice (FcgRIIb+/+) was not demonstrated due to the similarity to FcgRIIb-/- PBS control mice. Arrow, raw surface of the intestinal mucosa indicating the intestinal ulcers.

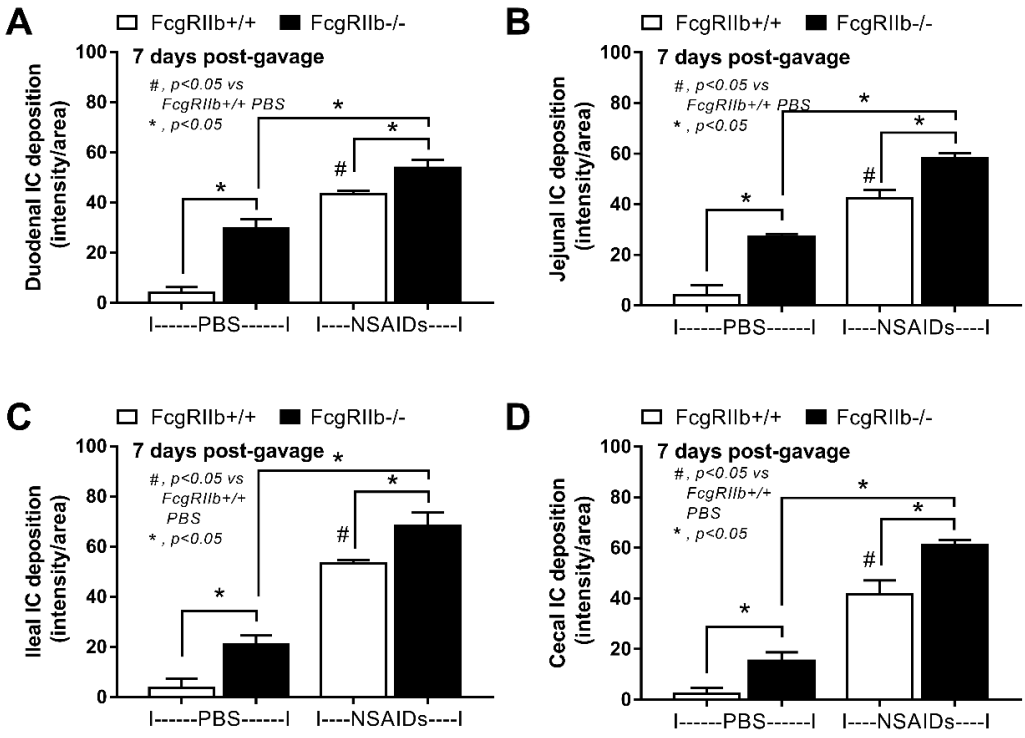


Figure 18. Characteristics of intestinal injury in FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control as determined by immune complex deposition (A-D).

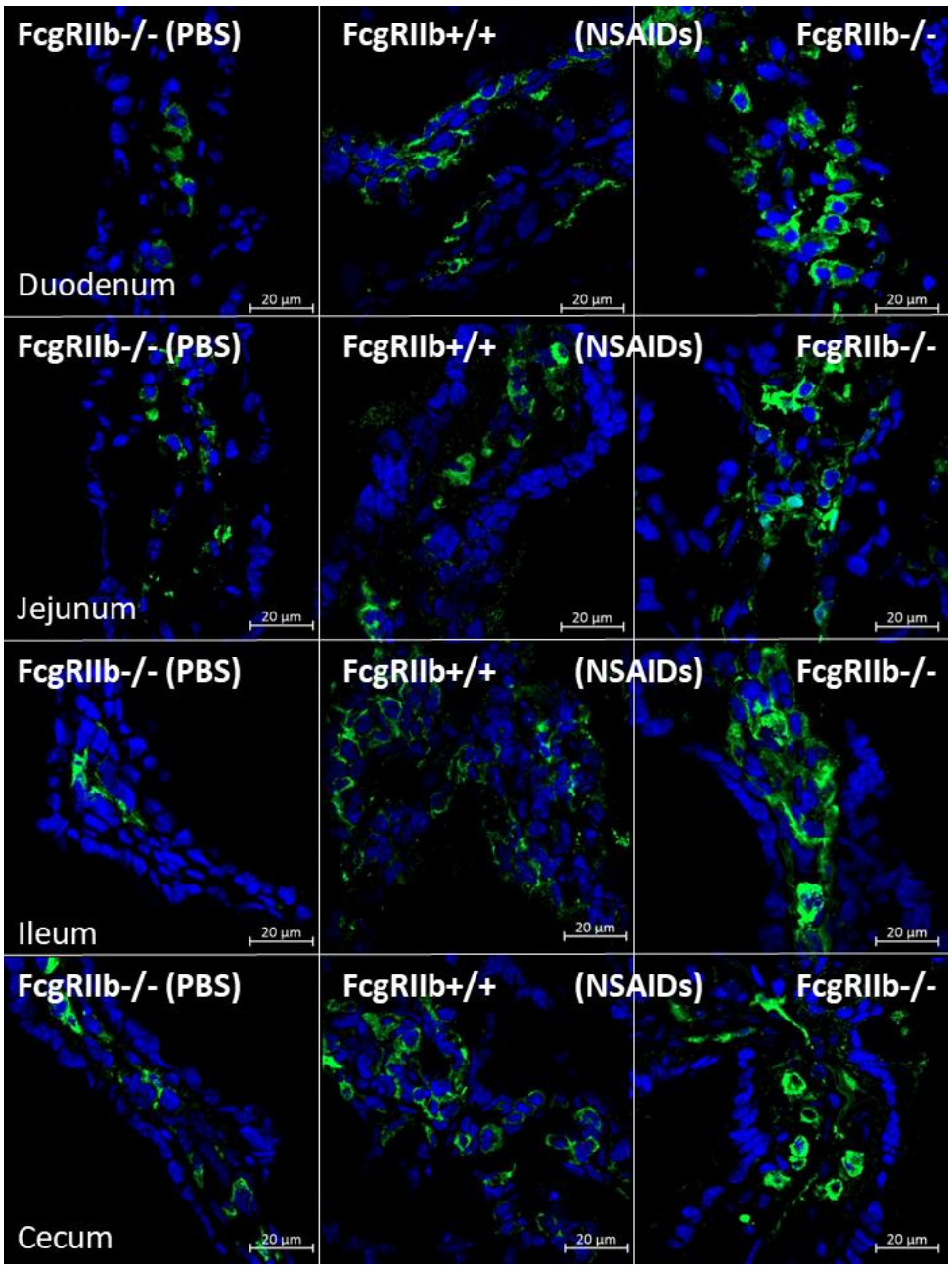


Figure 19. Representative figures of immune complex deposition in the intestines of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control (original magnification 200x) were demonstrated. Figures of PBS-administered wild-type control mice (FcgRIIb+/+) was not demonstrated due to the non-detectable of immune complex deposition. Green and blue colors demonstrated mouse IgG and intestinal nuclei, respectively.

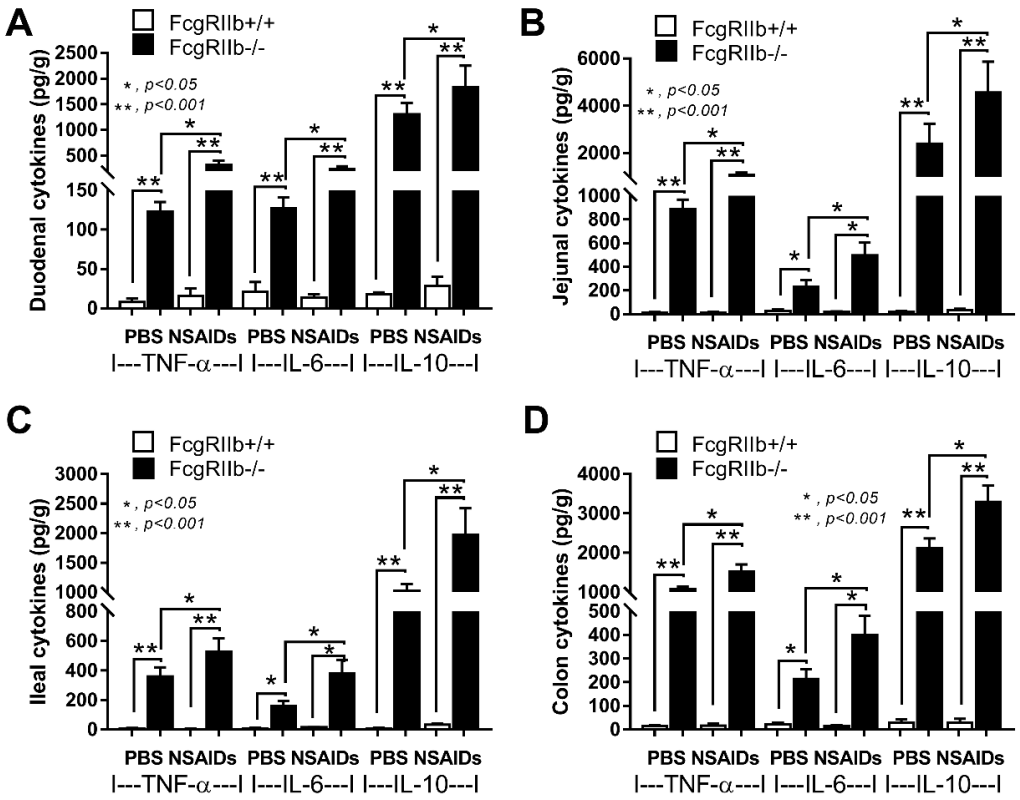


Figure 20. Characteristics of intestinal injury in FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control as determined by intestinal cytokines (A-D).

Indomethacin-induced endotoxemia in FcgRIIb-/- mice compared with wild-type mice

After indomethacin administration, gut permeability defect (gut leakage) as determined by FITC-dextran assay and endotoxemia was higher in FcgRIIb-/- mice than WT mice (Fig.21A, B). Gut leakage was not detectable in FcgRIIb-/- control mice (Fig.21A), despite the detectable immune deposition (Fig.18A-D), supporting asymptomatic immune deposition in gut of these lupus mice. Unsurprisingly, the endotoxemia-induced systemic inflammation in indomethacin-administered FcgRIIb-/- mice was more severe than in WT mice (Fig.22).

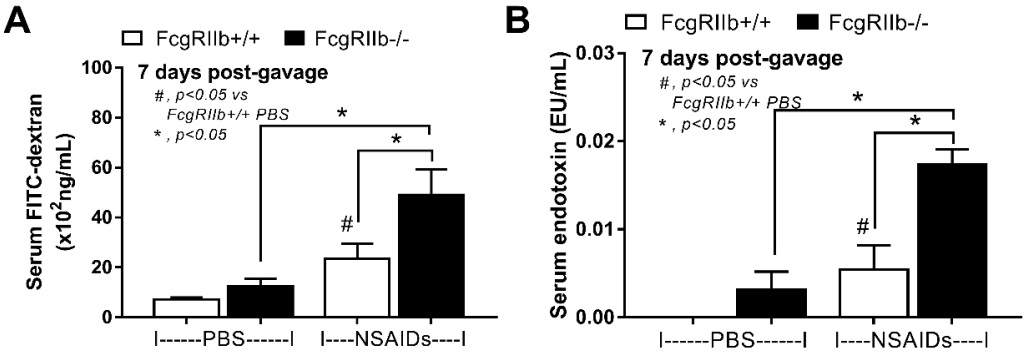


Figure 21. Representative serum FITC-dextran (A) and endotoxemia (B) from the serum of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control (n=5/group).

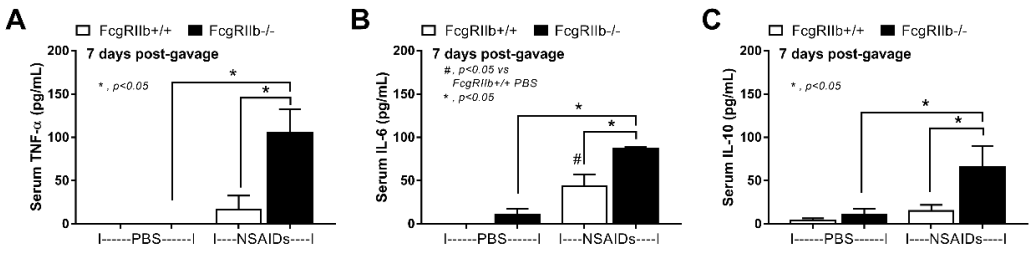


Figure 22. Representative serum cytokines of FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after the administration of indomethacin (NSAIDs) or phosphate buffer solution (PBS) control (A-C) (n=5/group).

Responses against endotoxin in FcgRIIb-/- macrophages compared to wild-type macrophages

As LPS from gut translocation might activate macrophages in either acute or chronic exposure manners, the *in vitro* was performed with a single LPS stimulation. As such, the hyper-inflammatory response of FcgRIIb-/- macrophages was demonstrated by the higher TNF-α and IL-6 in supernatant after LPS stimulation (Fig.23A, B) supporting a previous publication (13). The gene expression of *FcgRIIb*, an inhibitory receptor, was determined along with other activating *FcgRs*. Accordingly, expression of all *FcgRs*, except *FcgRI*, rapidly increased from the baseline as early as 3 h after LPS activation (Fig.24A-D). In addition, A higher expression of *FcgRIIb* with the similar *FcgRIII* and *FcgRIV* expression were demonstrated in most of time-points after LPS stimulation in WT macrophages when compared to FcgRIIb-/- cells (Fig.24A-D). Notably, a higher *FcgRIII* expression at 6 h (Fig.24C) and a lower *FcgRIV* expression (Fig.24D) at 3 h of LPS activation in WT macrophages in comparison to FcgRIIb-/- cells were demonstrated.

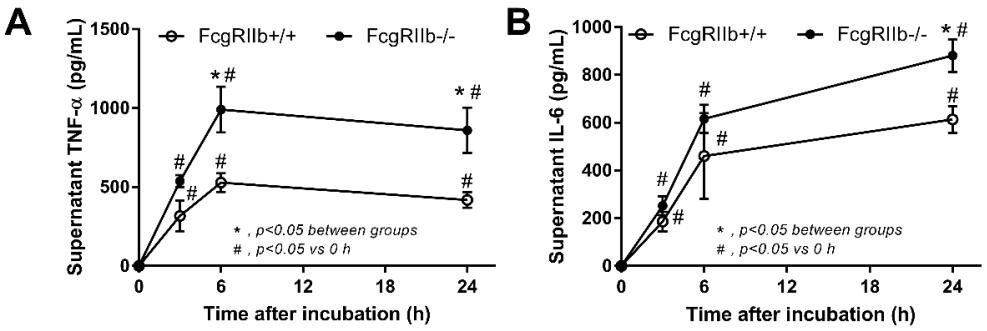


Figure 23. Representative supernatant cytokines secreted by macrophages from FcgRIIb-/- lupus mice and wild-type (FcgRIIb+/+) mice after 6 h incubation with LPS stimulation (A, B). Independent triplicate experiments were performed.

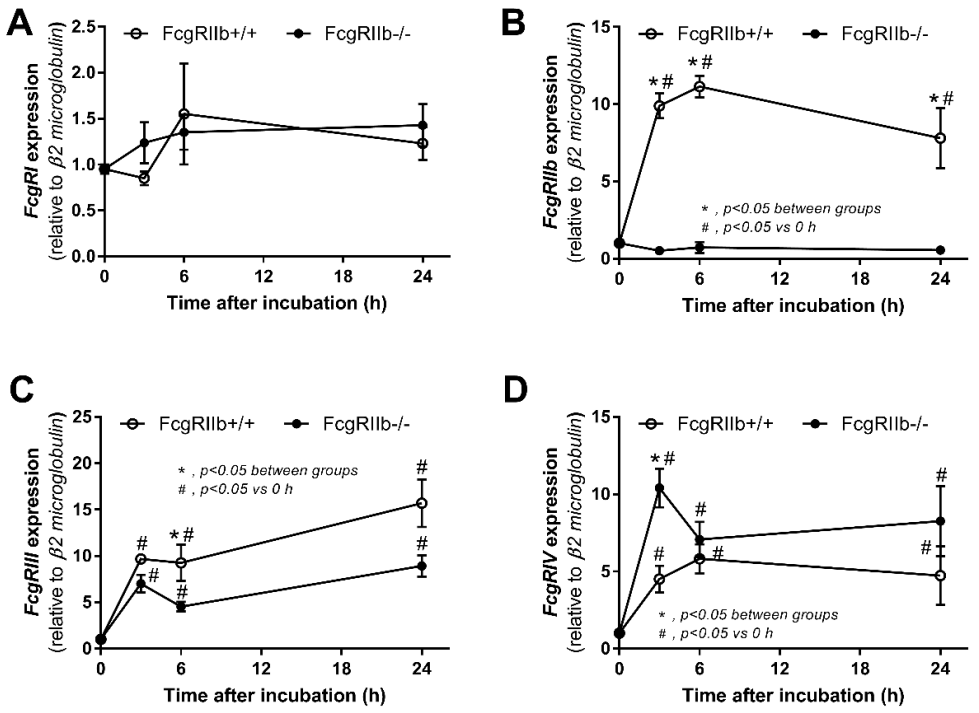


Figure 24. Representative the gene expression of *FcgRs* by quantitative polymerase chain reaction in macrophages from wild-type (FcgRIIb+/+) mice and FcgRIIb -/- mice after 6h incubation with LPS stimulation (A-D) are demonstrated. Independent triplicate experiments were performed.

CHAPTER VII

DISCUSSION

The elevation of anti-dsDNA in FcgRIIb-/- mice at 24-week-old resulted in asymptomatic immune complex deposition on intestines that enhanced the susceptibility to indomethacin-induced enteropathy. Despite the anti-inflammatory property of NSAIDs, the high dose of indomethacin caused systemic inflammation through gut-leakage induced endotoxemia, and possibly exacerbated lupus activity.

Prominent indomethacin-induced enteropathy in FcgRIIb-/- mice over wild-type mice

Due to the fact that cyclooxygenase (COX) enzyme is necessary in the homeostasis of several biological systems, NSAIDs adverse effects are demonstrated in multi-organs especially nephropathy and enteropathy (103). Among the different manifestations of NSAIDs nephropathy (renal ischemia, cortical necrosis, proteinuria and interstitial nephritis) (34, 35), renal ischemic tubular injury is the most common with the subtle histological findings including renal tubular vacuolation and loss of brush borders (104-106). Here, indomethacin in WT mice caused proteinuria with only tubular vacuolization indicating NSAIDs-induced minimal change of disease (107) possibly because of podocyte injury from leukotrienes-activated T cells (increased leukotrienes conversion from arachidonic acid due to the blockage of prostaglandin

synthesis) (108) In parallel, NSAIDs administration in 24-week-old FcgRIIb-/- mice exacerbated lupus nephritis as indicated by red blood cell casts, proteinaceous casts and glomerular immune complex deposition (11). Perhaps, NSAIDs induced uremia and uremia-induced inflammation could exacerbate lupus activity (37, 38). However, NSAIDs nephropathy might not be a main lupus exacerbating factor in NSAIDs-mouse model because i) the uremia in NSAIDs model (no obvious renal histological damage) is less severe than other direct renal damage models (ischemia and bilateral nephrectomy) (109, 110) and ii) endotoxemia from uremia (an indirect mucosal damage) is less severe than endotoxemia from direct gut mucosal injury from NSAIDs (111, 112).

On the other hand, NSAIDs induced prominent enteropathy in the upper and lower gastrointestinal tract (113) and caused endotoxemia from the direct gut mucosal injury (114). More specifically, indomethacin-induced endotoxemia due to the intestinal mucosal damage is well-known (92, 115), partly due to the enterohepatic drug recycling resulting in a prolonged and repeated exposure of the intestinal mucosa to the compound. Because the gut-leakage induced spontaneous endotoxemia in full-blown FcgRIIb-/- mice at 40-week-old and in patients with active lupus (9), FcgRIIb-/- mice at 24-week-old which demonstrated only increased anti-dsDNA, but not lupus nephritis, are selected to use for NSAIDs evaluation. Accordingly, NSAIDs-induced gut leakage was demonstrated by endotoxemia without systemic Gram-negative bacterial infection and endotoxin, a potent immune activator, together with systemic cytokines

in NSAIDs-administered mice were higher in FcgRIIb-/- mice compared with WT mice. In addition, NSAIDs induced more severe intestinal mucosal injury in FcgRIIb-/- mice as the ulcers could be demonstrated in all intestinal parts while induced only monoclonal cell infiltration in NSAIDs-administered WT mice. As such, the increased susceptibility toward NSAIDs enteropathy might be due to the pre-conditioning intestinal injury as demonstrated by the intestinal immune deposition in FcgRIIb-/- mice, but not in WT, before the NSAIDs administration. The immune deposition in gut increased with NSAIDs administration in both mouse strains but was more predominant in FcgRIIb-/- mice. While immune deposition in gut of NSAIDs-administered WT mice was for a wound healing process (116), prominent immune deposition in FcgRIIb-/- mice cause by either a wound healing process or an increase antibody production. Furthermore, NSAIDs enteropathy was severe enough to cause local intestinal inflammation only in FcgRIIb-/-, but not in WT mice, as indicated by the increase intestinal cytokines in all intestinal parts.

Overwhelming inflammatory responses against endotoxin of FcgRIIb-/- macrophages over the wild-type cells

Hyper- immune responsiveness related to a defect in negative signaling is demonstrated in FcgRIIb-/- lupus mice (4) and in macrophages (13). In addition, LPS is a pathogenic molecule foreign to the host, which potently activate innate immune responses in the host through TLR- 4 (117), resulting in systemic inflammatory

responses (9, 36, 118-120). In FcgRIIb-/- macrophages, there was a high cytokine production after LPS stimulation. However, there might be the heterogeneity of macrophages *in vivo* that LPS tolerance might be induced in some cells. Hence, we further tested LPS activation in macrophages by stimulations with LPS. Because of i) the cross-talk between TLR-4 and FcgRs (121) and ii) the balance between activating and inhibitory FcgRs, an alteration of FcgRs might be associated with the LPS responses of FcgRIIb-/- macrophages. After LPS stimulation, both of the activating FcgRs (*FcgRIII* and *FcgRIV*) and the inhibitory FcgR (*FcgRIIb*) were enhanced in WT macrophages, while only the activating FcgRs (*FcgRIII* and *FcgRIV*), but not the inhibitory *FcgRIIb*, was increased in FcgRIIb-/- cells.

While *FcgRIV* expression in FcgRIIb-/- macrophages was higher than WT cells at 3 h post LPS stimulation, *FcgRIII* in WT cells was higher than FcgRIIb-/- cells at 6 h of the activation suggesting a possible different type of activating FcgRs between WT and lupus cells. In align with receptor expression, there was an obvious difference in cytokines between FcgRIIb-/- and WT macrophages as LPS-stimulated FcgRIIb-/- macrophages showed the higher level of inflammatory cytokines (TNF-α and IL-6) at every timepoints (3h, 6h and 24h post-stimulation), suggesting that FcgRIIb depletion leads to excessive inflammatory responses in the innate immune cells. Perhaps, the prominent cytokine production in LPS-stimulated FcgRIIb-/- macrophages was associated with the enhanced *FcgRIV* without *FcgRIIb* inhibitory receptors. Indeed, mouse FcgRIV is more functionally active than FcgRIII as FcgRIV recognizes 3 out of 4

isoforms of mouse IgG (IgG1, IgG2a and IgG2b), while FcgRIII recognizes only mouse IgG1 (mouse IgG3 was non-recognizable by FcgRs) (2, 122).

Several limitations of the study are noted. First, our study tested only one model of lupus mouse focusing on a single gene as the possible cause, when a variety of lupus models from different pathophysiology exist. Lupus is a considered a clinical syndrome with multiple factors and multi-gene involvement (123). Second, there is a limitation in the mouse model due to the very high dose of indomethacin compared to a more typical lower dose in patients. Likewise, only indomethacin, a short-acting drug with a high GI side effect, was tested due to its popular utilization in animal models (42, 43) despite a variety of newer drugs in the clinical practice. Third, only the gene expression, but not the protein abundance, of FcgRs was explored. Fourth, only an association, but not the more physiologic evaluations (cause-effect), between the macrophage metabolic profiles and LPS stimulation was performed. Nevertheless, our data provide a proof of concept that NSAIDs (indomethacin) could induce the inflammatory responses, including gut leakage, that subsequently affects lupus activity. Our initial findings suggest that the additional studies in patients are warranted.

APPENDIX A

MATERIALS & EQUIPMENT

1.	96 wells flat bottom plate	USA
2.	96 wells PCR microplate	US
3.	6 well plate (cell culture plate)	USA
4.	Conical centrifuge tube 15, 50 mL	USA
5.	Ultrasonic Disruption (Sonics & Materials, VCX 750)	US
6.	Pipet tips 10, 20, 200, 1000 µL	US
7.	Micro centrifuge	Malaysia
8.	Micropipette 1, 10, 20, 200, 1000 µL	Germany
9.	NanoDrop 1000	USA
10.	Pipet controller	India
11.	Serological pipette 5, 25 mL	USA
12.	ProFlex™ PCR System	USA
13.	QuantStudio6 Flex Real-Time PCR Systems	USA
14.	Terumo syringe 1, 5, 10 mL	Japan
15.	Ultra-Low Temperature Freezer U570 premium	Germany
16.	Varioskan Flash Multimode ELISA reader	USA
17.	Vortex Genie 2	US
18.	ZEISS LSM 800 Confocal microscopes	Germany

19.	Centrifuge 5415R / 5417R	Germany
20.	CO_2 incubator NU-5500E	USA
21.	Biosafety Cabinet Class II NU-4oo-600E	USA
22.	Water bath memmert	Germany
23.	QuantiChrom Creatinine-Assay (DICT-500)	USA
24.	coated Calf-DNA	USA

APPENDIX B

CHEMICAL AND REAGENTS

1.	Indomethacin	USA
2.	Fluorescein isothiocyanate–dextran	USA
3.	Lipopolysaccharide (LPS; *Escherichia coli* 026: B6)	USA
4.	Absolute ethanol	USA
5.	Cryogel	USA
6.	DAPI (4′,6-diamidino-2-phenylindole)	USA
7.	Goat anti-mouse IgG	USA
8.	Isoflurane	PA
9.	Primer	USA
10.	Normal saline	Thailand
11.	Phosphate Buffer Saline (PBS)	Thailand
12.	Enzyme-linked immunosorbent assay (ELASA) kit	USA
13.	PowerUp™ SYBR™ Green Master Mix	USA
14.	Tissue-Tek OCT compound	UK
15.	RNase/DNase free H_2O	Germany
16.	HyClone™ 100 mM Sodium Pyruvate solution	USA
17.	FavorPrep™Tissue Total RNA Mini Kit	Taiwan
18.	HEK-Blue™ LPS Detection Kit	USA

19.	Macrophage colony-stimulating factor (M-CSF)	USA
20.	RevertAid First Strand cDNA Synthesis Kit	USA
21.	HyClone™ DMEM/HIGH GLUCOSE	USA
22.	Gibco™ Fetal Bovine Serum, qualified, Brazil	Brazil
23.	HyClone™ Penicillin-Streptomycin 100X solution	USA
24.	Cytiva HyClone™ HEPES Solution	USA

APPENDIX C

REAGENTS PREPARATION

1. Indomethacin 25 mg/Kg

   Mouse weight 25 g

Indomethacin	0.625	mg
PBS	200	µL

2. Fluorescein isothiocyanate–dextran (FitC-dextran) 25 mg/mL

Fluorescein isothiocyanate–dextran	25	mg
PBS	1	mL

3. Standard for Fluorescein isothiocyanate–dextran (FitC-dextran)

   Stock solution (1 mg/mL FitC-dextran)

FitC-dextran	1	mg
PBS	1	mL

   2 flod dilution for standard

Stock solution (1 mg/mL FitC-dextran)	500	µL
PBS	500	µL

4. 70% ethanol

100% ethanol	70	mL
Sterile water	30	mL

5. Cell culture for HEK-Blue™ LPS Detection

Growth and Selection Medium	10	mL	
Complete DMEM high glucose		10	mL
1X Normocin		20	µL
1X HEK-Blue™ Selection		40	µL
QUANTI-Blue™ Solution	100	mL	
QB reagent		1	mL
QB buffer		1	mL
Sterile water		98	mL

6. 1X DNase I Buffer

10 mM Tris-HCl	78.8	mg
2.5 mM MgCl2	11.9	mg
0.5 mM CaCl2	2.8	mg
ddH20	50	mL

7. 1X Phosphate Buffer Saline (PBS)

Stock solution (10X PBS)	100	mL
ddH_2O	900	mL

8. Lipopolysaccharide

Stock solution (10 µg/mL)

Lipopolysaccharide (1 mg/mL)	5	µL
PBS	495	µL

Working solution (150 ng/mL)

Stock solution (10 µg/mL)	150	µL
cDMEM	9,850	µL

9. Macrophage colony-stimulating factor (M-CSF)

Working solution (25 ng/mL)

Stock (50 µg)	12.5	µL
ddH20	500	µL

10. Primer

Working solution (10 mM)

Stock solution (100 mM)	10	µL
ddH20	90	µL

11. Quantitative Real-Time Polymerase Chain Reaction (qPCR) master mix

PowerUp™ SYBR™ Green Master Mix (2X)	5.0	µL
Forward primer	0.2	µL
Reverse primer	0.2	µL
Nuclease-free H_2O	2.6	µL
cDNA	2.0	µL

12. Wash Buffer

1X PBS	1000	mL
0.05% Tween 20	500	µL

13. Stop solution ELISA

2N H_2SO_4	2.805	mL
ddH_20	47.195	mL

14. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) master mix

Oligo (dT)	1	µL
RNA	10	µL
Nuclease-free H_2O	1	µL
5X Reaction Buffer	4	µL
Thermo Scientific™ RiboLock RNase Inhibitor	1	µL
RevertAid Reverse Transcriptase	1	µL
10 mM dNTP Mix	2	µL

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