Neutrophil Extracellular Traps (NETs) formation of Fcgr2b deficient mice in lupus mouse model with ischemic reperfusion injury

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การศึกษาการเกิด Neutrophil Extracellular Traps (NETs) ในหนูเม้าส์ที่ขาดยีน Fcgr2b ที่เป็นโรคลูปัสที่เกิดจากภาวะไตขาดเลือด

น.ส.วิลาสินีย์ สายศร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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อาการบาดเจ็บจากไตวายเฉียบพลัน (AKI) ที่เกิดจากภาวะไตขาดเลือด (I/R) ถือเป็นสาเหตุสำคัญที่พบได้บ่อยส่งผลให้เกิดการเจ็บป่วยและเสียชีวิตในผู้ป่วย กับดักนิวโทรฟิล (Neutrophil extracellular traps; NETs) ถือว่าเป็นสาเหตุความสำคัญที่ส่งผลให้โรคไตอักเสบลูปัสรุนแรงเพิ่มมากขึ้น หลังการบาดเจ็บจากภาวะไตขาดเลือด เนื่องจากนิวโทรฟิลเป็นเซลล์แรกที่ตอบสนองต่อการบาดเจ็บของเนื้อเยื่อ องค์ประกอบของกับดักนิวโทรฟิล ได้แก่ ดีเอ็นเอที่ปราศจากเซลล์ (Cell-free DNA), ฮิสโตน, นิวคลีโซม รวมถึงโปรตีนในไซโทพลาสมา อาจเป็นแหล่งแอนติเจนของตนเอง (Auto-antigens) ที่กระตุ้นให้มีการพัฒนาไปเป็นโรคไตอักเสบลูปัส ดังนั้นโปรเจคนี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบการเกิดกับดักนิวโทรฟิลในอวัยวะภายในหลังการบาดเจ็บจากไตขาดเลือดในหนูที่ขาดยีน Fc gamma receptor 2b (Fcgr2b-/-) ซึ่งเป็นโมเดลจำลองของหนูลูปัส และหนูปกติ (Wildtype; WT) ผลการทดลองพบว่าที่ 24 ชั่วโมงหลังการบาดเจ็บจากภาวะไตขาดเลือด หนู Fcgr2b-/- มีการบ่งชี้กับดักนิวโทรฟิลในระดับที่สูง โดยวัดได้จากการแสดงออกของยีน *PAD4*, IL-*1β*, dsDNA และการย้อมด้วยสีอิมมูโนฟลูออเรสเซนต์ ชนิดนิวโทรฟิลอิลาสเตส (Neutrophil elastase; NE) ร่วมกับ ไมอีโลเพอร์ออกซิเดส (Myeloperoxidase; MPO) ภายในไตและในเลือด นอกจากนี้โปรตีนที่เกี่ยวกับการตายแบบอะพอพโทสิส (Activated caspase-3) ภายในไตของหนู Fcgr2b-/- นั้นก็มีการเพิ่มขึ้นส่งผลให้มีการสร้างแอนติบอดีต่อ dsDNA ในซีรัมและมีการสะสมของอิมมูโนโกลบูลิน (Immunoglobulin; IgG) ในโกลเมอรูลัสภายในไต ที่ 120 ชั่วโมงหลังการบาดเจ็บจากภาวะไตขาดเลือด การศึกษาใน *in vitro* Fcgr2b-/- นิวโทรฟิลที่ถูกกระตุ้นด้วย phorbol myristate acetate (PMA) หรือ lipopolysaccharide (LPS) เกิดกับดักนิวโทรฟิลและการตายของเซลล์นิวโทรฟิลเด่นชัดกว่านิวโทรฟิลของหนูปกติ ผลการศึกษาแสดงให้เห็นว่าหนู Fcgr2b-/- มีการสร้างกับดักนิวโทรฟิลที่สูงกว่า และไวต่อการตายของเซลล์ทั้งในไตและในอวัยวะอื่นๆ ซึ่งเหนี่ยวนำการสร้างแอนติบอดีต่อ dsDNA ในซีรัมมากขึ้นอันเป็นปัจจัยสำคัญทำให้เกิดโรคไตอักเสบที่รุนแรงขึ้น

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Wilasinee Saisorn : Neutrophil Extracellular Traps (NETs) formation of Fcgr2b deficient mice in lupus mouse model with ischemic reperfusion injury. Advisor: Assoc. Prof. ASADA LEELAHAVANICHKUL, M.D., Ph.D Co-advisor: Asst. Prof. DIREKRIT CHIEWCHENGCHOL, M.D., Ph.D

Acute kidney injury (AKI) caused by renal ischemic reperfusion (I/R) is the most prevalent cause of morbidity and mortality in patients. Neutrophil extracellular traps (NETs) are important for the progression of lupus nephritis after renal I/R injury. Because neutrophils are the first immune cell that respond to tissue damage, neutrophils might be an important cell that determines renal I/R injury. Additionally, NET components, including cell-free DNA, histone, nucleosome, and cytoplasmic protein compartment release the auto-antigens that accelerate lupus disease activity. Therefore, this project aimed to compare NETs formation in several internal organs following renal I/R injury in Fcgr2b deficient (Fcgr2b-/-) mice, a lupus mouse model, and wildtype (WT) mice. At 24 h after I/R injury, Fcgr2b-/- mice had a higher level of NET biomarkers; *PAD4*, *IL-1β* expression, serum dsDNA, and the co-staining of neutrophil elastase (NE) with myeloperoxidase (MPO), in kidney and peripheral blood when compared with WT mice. Furthermore, the cell apoptosis activated caspase-3 in the kidney, serum anti-dsDNA, and immunoglobulin (IgG) deposition in glomeruli in Fcgr2b-/- mice at 120 h after I/R injury was also higher than the WT mice. Likewise, NETs formation and apoptosis Fcgr2b-/- neutrophils after stimulation with phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) were more prominent than WT neutrophils. These findings support that Fcgr2b-/- mice with renal I/R had a higher NETs formation, cell apoptosis in the kidney and in the remote organs that induced serum anti-dsDNA and accelerated lupus disease aggression.

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CHAPTER I

INTRODUCTION

Acute kidney injury (AKI) is a common health care problem worldwide caused by ischemia and/or toxic substances (1), which are frequently represented by the renal ischemia reperfusion injury (I/R) model (2, 3). AKI is an immediate or rapid decrease in kidney function, which causes multi-organ injury and increases risk of the disease progression into the chronic phase of injury, referred to as “chronic kidney disease (CKD) or end-stage renal disease (ESRD)”(4, 5). In addition, elderly, high blood pressure, proteinuria, and diabetes are the risk factors that enhance the severity of AKI (6, 7). The pathogenesis and the consequences of AKI, including renal cell necrosis, cytokine production, and leukocyte activation (8, 9), are also the well-known exacerbating factor of systemic lupus erythematosus (SLE), a common auto-immune disease. As such, SLE is caused by auto-antibodies against several systems, due to the defect of self-tolerance, that can be facilitated by several AKI-associated factors, especially cell deaths and pro-inflammatory responses (10, 11).

The loss of the functional Fc gamma receptor 2b (Fcgr2b), which is the only inhibitory receptors in the FcgR family (12-14), lead to SLE, and the prevalence of Fcgr2b dysfunction polymorphism is high in the Asian population (15). The Fcgr2b deficient mice (Fcgr2b-/-) are used as a representative lupus model that develops lupus spontaneously in an age-dependent characteristic, ranging from “asymptomatic lupus prone” to “symptomatic lupus” at the younger and older than 16-24 weeks,

respectively (16). In Fcgr2b-/- mice, the lack of inhibitory signaling triggers immune hyper-responsiveness to self-antigens or pathogen molecules, such as pneumococcal antigens and lipopolysaccharide (LPS) (13, 16, 17). Because dsDNA is an important self-antigen in lupus, anti-dsDNA is an important lupus autoantibody. Normally, dsDNAs are retained within the nuclei, and circulating free DNA is detectable only after a certain amount of cell death that causing free DNA in blood circulation as a part of "damage associated molecular patterns (DAMPs)" for innate immunity activation (18). Likewise, dsDNA, a part of free DNA from damaged cells, could be processed as an autoantigen in lupus. Because innate immunity triggers adaptive immune responses including the production of anti-dsDNA (19, 20), innate immunity and inflammation plays an important role in lupus exacerbation (21, 22). Due to a lack of immune self-tolerance, lupus-prone persons highly expressed anti-dsDNA when exposed dsDNA (23), in contrast to the normal people. Several processes of cell breakdown (with or without apoptosis) are the primary causes of dsDNA exposure in lupus, as shown by UV-induced apoptosis in skin (24) and neutrophil extracellular traps (NETs) in several situations (25-27). Although neutrophil extracellular DNA networks in NETs are specifically designed to bind pathogens for extracellular killing activity, NETs are also detectable in severe organ injury from non-infectious causes (28).

Because neutrophils are the most abundant immune cells that migrate throughout blood circulation and to the injury site with a short lifespan and they are capable of producing reactive oxygen species (ROS) for microbicidal activity, NETs might be found in AKI and become an important exacerbating factor of lupus (29). Similarly, NETs formation associated-inflammation during the lupus exacerbation

might also become a significant source of dsDNA (30). Surprisingly, there is still a scarcity of information on the impact of AKI on lupus. Therefore, this research investigates NETs formation of the kidney after an ischemic reperfusion (I/R) injury in wild type and Fcgr2b deficient mice, acted as a lupus model, as well as explores the effects of AKI of lupus in several organs.

CHAPTER II

OBJECTIVES

1. To determine *in vitro* NET formation on Fcgr2b deficient or wild type neutrophils after several NETs inducers.

2. To investigate *in vivo* NET formation on various internal organs after renal ischemic reperfusion in wild type and Fcgr2b deficient mice.

CHAPTER III

LITERATURE REVIEW

1. Acute kidney injury (AKI)

Acute kidney injury (AKI) or acute renal failure (ARF) is an intractable problem that affects to one quarter of all hospitalized patients worldwide (6). AKI is the term of renal impaired development from mild alteration to complete organ failure, called renal injury, whereas the kidney structure has not a differentiation. This disease is characterized by acute onset and severe condition that cause renal failure in a short time, which is the leading cause of morbidity and mortality in the patients (31). The categories of risk, injury, failure, loss, and end-stage renal disease are the standardized evidence of incidence and disease progression from AKI (32). The Kidney Disease Improving Global Outcomes (KDIGO) classified the AKI severity into three stages using either a rapid increase in serum creatinine or a critical decrease in urine creatinine excretion as shown in Table 1

Table 1 The standard characteristics of incidence and result of AKI (33).

AKI stage	Serum creatinine	Urine output
Stage I	• Increase ≥0.3 mg/dL (26.5 μmol/L); or • Increase to 1.5-1.9 times from baseline	• Urine volume <0.5 mL/kg/h for 6-12 h
Stage II	• Increase to 2.0-2.9 times from baseline	• Urine volume <0.5 mL/kg/h for ≥12 h
Stage III	• Increase to ≥0.3 times from baseline; or • Serum creatinine ≥4.0 mg/dL (≥354 μmol/L); or • Initiation of renal replacement therapy; or • Decrease in glomerular filtration rate to <35 mL/min/1.73 m^2 in patients <18 years	• Urine volume <0.3 mL/kg/h for ≥24 h; or • Anuria for ≥12 h

2. AKI etiology

The filtration and excretion of nitrogenous waste products from the blood is one of the most vital functions of the kidney, which measured using the elevation of blood urea nitrogen and creatinine as indicators, indicating that the declined renal function, especially glomerular filtration rate (GFR) (34). Although, the elderly, high blood pressure, proteinuria, and diabetes are also risk factors for AKI, sepsis is the major causes of acute kidney injury in critically ill patients followed by cardiogenic

shock, complications with medications, hypovolemia, hepatorenal syndrome, and obstructive uropathy (35). The most causes of AKI are clinically classified into three primary etiologies e.g., i) prerenal (decreased renal function without pathological abnormality), ii) intrinsic renal (kidney damage-glomerular or tubular), and iii) postrenal (blockage of the urinary tract such as kidney stones, blood clot in the urinary tract or problems with the nervous system that affect the bladder and urination) (36) (Figure 1). Four structures of kidney including the tubules, the glomeruli, the interstitial, and the intrarenal blood vessels are the major area when considering etiologies of intrinsic renal failure.

2.1 Acute glomerulonephritis (AGN)

This damage occurs in an idiopathic rapidly progressive glomerulonephritis or a systemic disease i.e., bacterial endocarditis, Wegener's granulomatosis, or lupus erythematosus (34).

2.2 Acute tubular necrosis (ATN)

Two major causes are i) ischemic is caused by severe or protracted decrease in renal perfusion and ii) nephrotoxic resulting from several exogenous and endogenous compounds are potentially toxic to kidney (34).

2.3 Acute interstitial nephritis (AIN)

The injury of intrarenal vessels affects to the reduction of renal perfusion and glomerular filtration rate, which result from various causes such as malignant hypertension, atheroembolic disease, and preeclampsia or eclampsia (34).

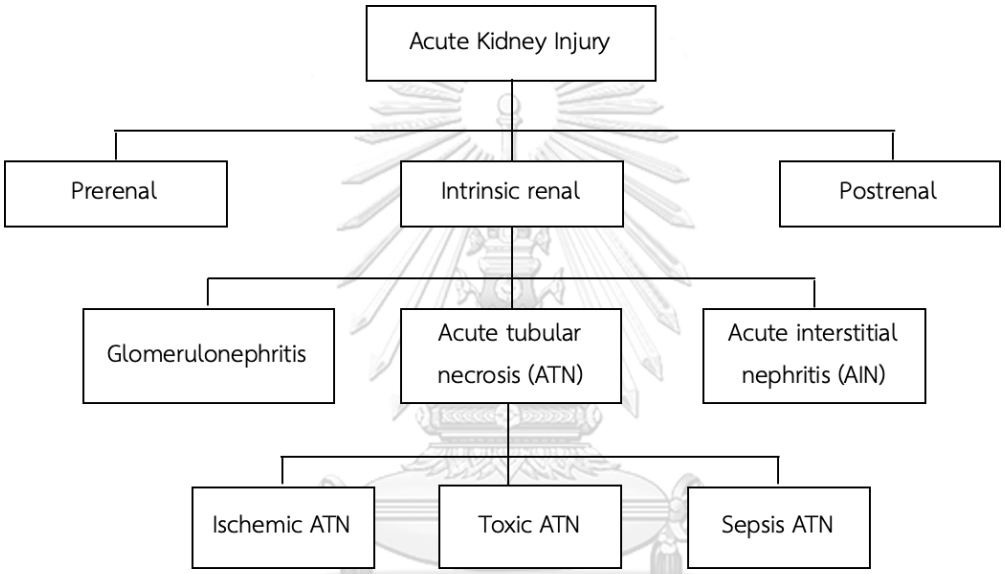


Figure 1 The most important causes of acute kidney injury (AKI): prerenal, renal, and postrenal.

3. Epidemiology of AKI

AKI causes demonstrated that approximately 25 to 60% were prerenal etiologies and 35 to 70% were renal etiologies, depending on AKI occurs in the hospital (hospital-acquired) or prior to hospitalization (community-acquired). More than 80% of renal etiologies was ischemic injury or nephrotoxins. In addition, community-acquired cases were higher than hospital-acquired cases in postrenal etiologies. AKI has been increasing an incidence and rates of hospitalized AKI patients in intensive unit (ICU) were as high as 67%, which are associated with considerable mortality (37). Importantly, patients with AKI were able to survive requiring continue dialytic therapy and recover renal function. Consequently, AKI has serious acute and chronic sequelae (Figure 2).

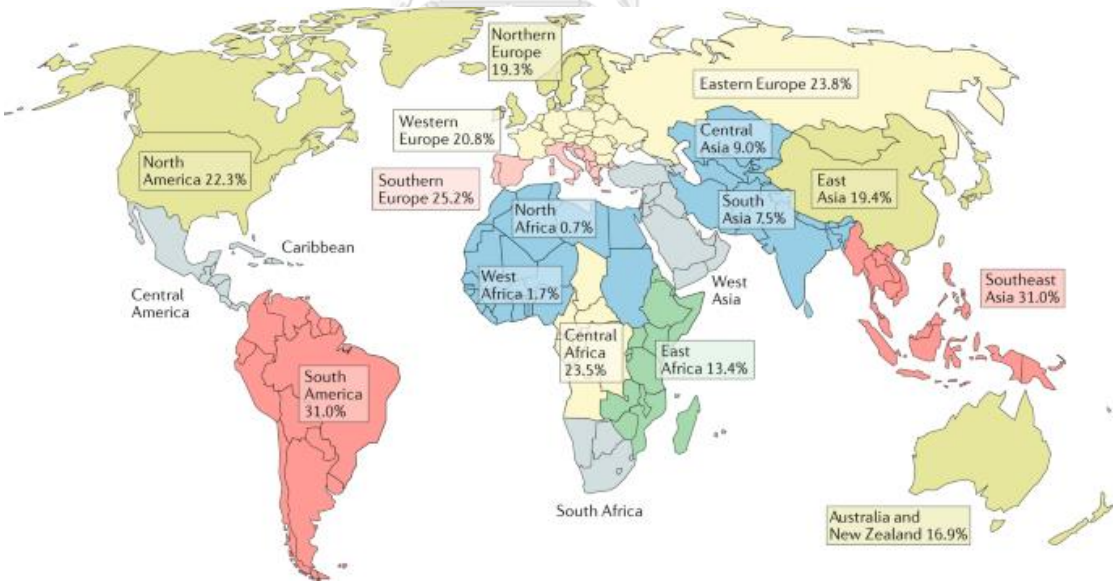


Figure 2 The variation in the incidence of AKI as shown in the percentages (38).

4. AKI pathophysiology

As over 80% of renal etiologies was ischemic injury, associations of pathophysiology, hemodynamics, and inflammation are extensively studied as the hallmark feature of AKI.

4.1 AKI-induced acute tubular necrosis

Acute tubular necrosis (ATN) correctly associated with the site of injury, related to renal epithelial cells through necrosis (39), and the decreased filtration rate of glomeruli (40, 41). The injury of renal tubular epithelial cells play a key feature of the initiation phase of AKI (42), resulting in severe cellular ATP depletion that leads to acute cell injury and dysfunction (43). Renal ischemia rapidly stimulates the differentiation of architecture and responsibility in renal proximal tubular epithelial cells through destroy the framework of filamentous actin, depending on the severity and duration of ischemic injury. The continued hypoxia following the initial ischemia and systemic inflammatory response are the major event of the extension phase (44). This phase occurs cell injury and death with necrosis and apoptosis (8). In addition, cell repairment, migration, apoptosis, and proliferation affect to slowly improving cellular and organ functions known as maintenance and recovery phases (35).

4.2 Hemodynamic-associated renal injury

The prominent infiltration of leukocytes, especially neutrophils (45), following ischemic reperfusion injury has two main events containing leukocyte adhesion on activated endothelial cells, and then red blood cell congestion, respectively, affecting to a compromise renal function through influencing renal hemodynamics. The effects of attached leukocytes lead to impair renal function in AKI including i) leukocytes may hinder the capillary flow, especially renal medulla and worsen hypoxia, ii) leukocytes may produce molecules exacerbating vasoconstriction i.e., ROS, and iii) leukocytes may be to increase tubular lumen pressure and reduce filtration rate of glomeruli when they contribute to parenchymal cell injury. These events may progress to the extension phase of AKI (46) .

4.3 Inflammatory signals in AKI

Due to leukocyte adhesiveness on activated endothelial cells, the inflammatory cytokines promoting further kidney injury such as TNF-α, IL-1β, IL-6, IL-8, and TGF-β of tubular epithelial cells and IL-1β, IL-8, and ROS of leukocytes. The elevation of IL-1β in mice with ischemic reperfusion injury indicating that the increased neutrophil infiltration, whereas IL-8 produce and act as the initial biomarker of acute injury, as well as IL-6 may also support the AKI inflammation. Toll-like receptors (TLRs) interact with the conditions of autoimmunity and inflammation, especially TLR4 (47), which expressed within 24 h of injury and may enhance the proinflammatory state of I/R injury. TLRs are transmembrane pattern recognition

receptors expressed in leukocytes and renal tubular epithelial cells (48). When exogenous microbial ligands (pathogen-associated molecular patterns (PAMPs)) are recognized, TLRs play a key role in triggering innate immune responses. Specific PAMPs are recognized by different kinds of TLRs. In mice, there are 12 TLR receptors while 10 TLR receptors in humans (49-51). Whereas TLRs are not only recognize pathogens, but they also detect endogenous ligand DAMPs, such as histones, HMGB1, and mitochondrial DNA (52). TLR9 is also suggested as a potential mediator for I/R injury because of the TLR9 activation by mitochondrial DNA release from the cell injury which plays role in several organ injury, including hepatic (53), cardiac (54) and kidney. The mitochondrial DNA is an important endogenous ligand for TLR9 during cell injury (55). When TLR9 activation leads to NFκB- mediated induction and production of pro-inflammatory chemokines, and cytokines and caspase 3/8-mediated renal tubular apoptosis (56, 57) (Figure 3).

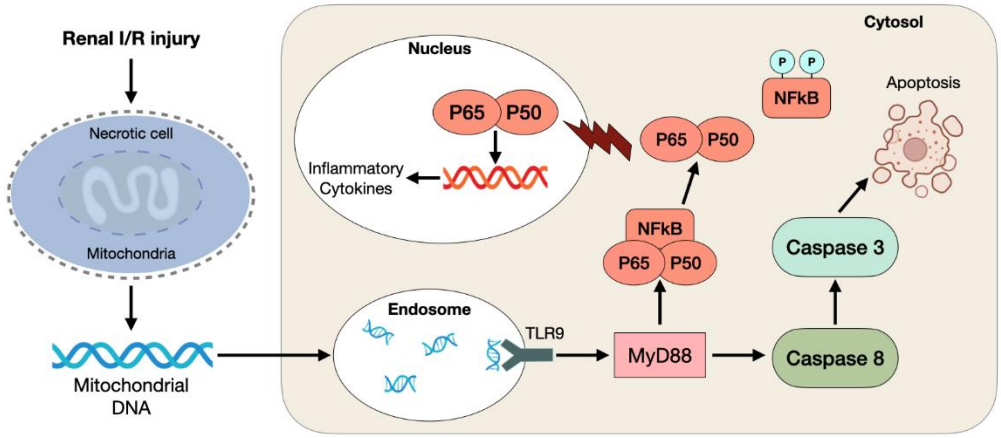


Figure 3 Proposed pathways for Toll-like receptor 9 (TLR9)-mediated exacerbation of ischemia acute kidney damage in the renal proximal tubule (Adapted from Han SJ., and Lee HT., 2019 (56))

5. Animal model of AKI

AKI is associated with varied underlying etiologies, including sepsis (58), rhabdomyolysis (59), and drug toxicity (60). Patient outcomes depend on the disease severity. The higher mortality of AKI is demonstrated in critically ill patients (61). Importantly, patients who survive an episode of AKI have a higher risk of severe adverse cardiovascular events, as well as the progression into chronic kidney disease (CKD) and end-stage renal disease (ESRD) (62, 63). The renal physiology has been studies using animal models, especially rats and mice; however, there are significant differences between the animal models and human conditions. Despite the differenced, rodents are still the frequently preferred models for the experiments because of their potential for fast colony development and the availability of genetic modification for functional investigations. As a result, animal models of AKI are important for identifying the causes of renal dysfunction and development of therapeutic and diagnostic techniques. Due to the smaller size, mice have been the major experimental species used to study AKI in order to achieve this aim. Here, some of the commonly use AKI model are mentioned.

5.1 Sepsis and sepsis-associated AKI

One of the outcomes of sepsis, which is characterized by a severe inflammation response to infection, is acute kidney injury. Sepsis is a complex illness characterized by dysregulated host response to infection and associated with a high

mortality rate (64) as well as an increased risk of chronic comorbidities. This has limited our understanding of pathophysiologic mechanisms and precluded the development of effective therapies. Systemic cytokine storm, hypoxia, mitochondrial dysfunction, tubular epithelial cell damage, and endothelial dysfunction are all factors in the pathophysiology of sepsis-associated acute kidney injury (65). The most important pathogenesis of sepsis-AKI is renal hypoxia which could be induced by several procedures.

5.2 Cecal-ligation and puncture (CLP) induced sepsis

Cecal ligation and puncture (CLP) is one of the most often utilized techniques in the laboratory to generate experimental sepsis (66). CLP causes sepsis in the form of stercoral peritonitis after a minor surgery (67). Bacteremia then follows, with an initial inflammatory phase followed by an anti-inflammatory reaction (66). Both Gram positive and Gram-negative bacteria infiltrate the bloodstream over time, causing a progressive systemic inflammatory response syndrome, septic shock, and multiorgan damage, including sepsis-associated acute kidney injury (68). The cytokine profile of CLP-induced sepsis is similar to that of human sepsis (69, 70). In addition, CLP-induced sepsis increases lymphocyte apoptosis, which mimics immunosuppression in human sepsis at the late stage (69) Furthermore, as measured by increases in BUN and creatinine, AKI is not usually detected in this model (71). As a result, CLP model contains common clinical characteristics of sepsis, it overlooks important elements such as acute lung injury (71, 72).

5.3 Folic acid-induced nephropathy

Due to renal tubular epithelial damage caused by both folic acid crystal deposition and consequent tubular blockage, as well as direct nephrotoxicity from high-dose folic acid (73). Folic acid injection could be used as an AKI model; however, it rapidly develops renal fibrosis (a key finding of chronic kidney disease; CKD) within develops 7–14 days after injection (74). The CKD continues to progress weeks thereafter and can be exacerbated with repeated administration of folic acid (75). Hence, this model is more frequently used as a rapid CKD model, despite it could be also used as an AKI model.

5.4 Ischemia reperfusion injury (I/R) model

Mouse model of kidney ischemia reperfusion (I/R) injury: warm ischemia and reperfusion injury models is the most commonly used model of AKI. In these models, renal blood flow is temporarily interrupted for some periods ranging from 20 to 45 minutes followed by the reperfusion. The atraumatic clamps are released, and the color of the kidneys on both sides is checked to ensure reperfusion (changes from dark purple to pink). After a period of ischemia or lack of oxygen (anoxia or hypoxia), I/R injury damage occurs when blood flow returns to tissue (8). The restoration of blood circulation results in tissue inflammation by reactive oxygen species (ROS) production, release of cytokines and chemokines, and the activation of leukocytes. The severity of damage depends on duration of tissue ischemia (9, 48).

Changes in plasma creatinine (76) and blood urea nitrogen (BUN) can be used for the quick evaluation on kidney function.

6. Innate immune responses in AKI

During the injury, the immune system plays a critical role in regulating wound healing response through the initiation of inflammation. Although hyper-inflammatory responses in kidney might lead to renal fibrosis, the too less inflammation also prolong the healing process. Then, an appropriate innate immune response is necessary for the complete renal healing process with the more complete tubular regeneration. The sequential activation of innate immune responses, following by cytokine release and the interaction between renal cells and immune cells, will be mentioned. Also, the adaptive immune responses in tissue damage and healing are also included.

6.1 Neutrophils

Polymorphonuclear leukocytes (PMNs or neutrophils), the most common leukocyte in human blood, are an important component of the innate immune system (77). They have multilobed nucleus (3 to 5 lobes), a short life span in the circulation, and are the first respond to injury or infections. In human, there are approximately 40-65% of neutrophils in all white blood cells while in the mice contains about 20-30% neutrophils (78, 79). Reactive oxygen species (ROS) are produced by neutrophils and can be utilized to kill pathogens. Other important functions of neutrophils including

chemotaxis, phagocytosis and neutralizing pathogens by releasing neutrophil extracellular traps (NETs) (80). The tissue damage leads to neutrophil activation and ROS production due to various factors, including hypoxia and reperfusion injury (81).

Following I/R injury, neutrophils are the first leukocytes to accumulate in the kidney, and their production of ROS, proteinases, elastase, and myeloperoxidase contributes to further renal injury after reperfusion (82). Stimulated neutrophils are the most intense physiological producers of superoxide anions through the activation of the NADPH oxidase upon adhesion or by pro-inflammatory cytokines (56). Furthermore, renal neutrophil recruitment, which includes transmigration, peaks at approximately 24 h after damage (25, 82).

6.1.1 Neutrophil extracellular traps (NETosis)

Neutrophil extracellular traps (NETs) were discovered in 2004 (83), also known as NETosis, are web-like structures that eliminate extracellular microorganisms by trapping invasive pathogens (bacteria, viruses, fungi, and protozoa) (84). NETs structure consists of nuclear components such as de-condensed DNA and histones, as well as neutrophil granules such as myeloperoxidase (MPO), citrullinase histone H3 (CitH3), and neutrophil elastase (NE) (83). Excessive NETosis causes physical abnormalities and organ damage in several diseases, including SLE, atherosclerosis, and rheumatoid arthritis (9, 85). In addition, Peptidyl arginine deiminase-4 (PAD4) is involved in the NET formation. Meanwhile, PADs 1-3 and PAD6 are cytoplasmic enzymes (86). PAD4 is found in both the cytoplasm and the

nucleus (87, 88), with granulocytes being the most abundant (86). The function of PAD4 is to eliminate residual arginine in histone structure, resulting in a reduction in positive charge and de-condensation of DNA strands (89).

PAD4 in neutrophils are important for renal I/R-induced AKI. I/R injury increases PAD4 in renal tubular cell, and PAD4-deficient mice are protected against I/R-induced kidney injury (90). As a result, PAD4 might be implicated in signal transduction, resulting in inflammatory responses following I/R injury. In the *in vitro* study, Cl-amidine (PAD inhibitor) prevents citrullination of histone 3 and significantly decreased NETs formation (91) in lupus prone mice (92). Therefore, PAD inhibitors are expected to reduce the production of the pathogenic autoantibody in the SLE model (93). Furthermore, platelets and DNA-released necrotic cells promote the development of NETs in granulocytes after I/R damage (94) (Figure 4). Likewise, cell-free DNA (cfDNA) in SLE serum is significantly higher than in healthy control and cfDNA in patients with active LN is higher than patients with non-active LN. The cfDNA quantification in LN is positively associated with lupus characteristics and neutrophil counts (95, 96). Then cfDNA counted by the PicoGreen assay for the determination of lupus activity is interesting.

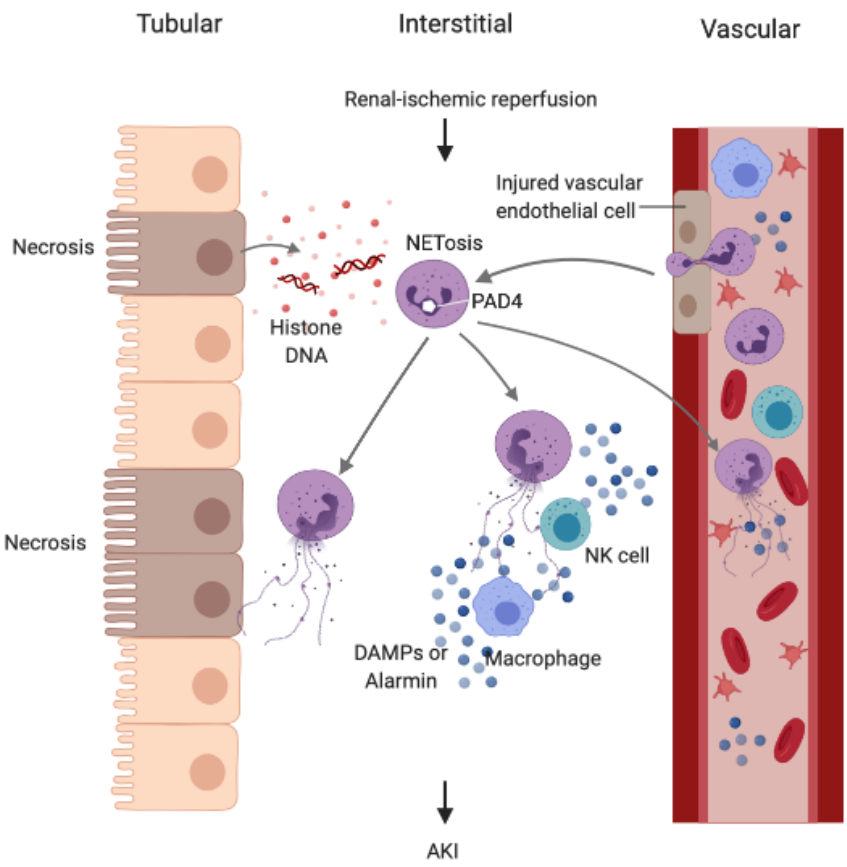


Figure 4 Neutrophil extracellular traps in ischemic acute kidney injury. (Adapt from Bolisetty S., and Agarwal A., 2009 (97))

6.1.2 NETosis pathway

After neutrophils detect the stimulators, they can generate the NADPH oxidase complex, which activates ROS production (98). ROS is not only has microbicidal properties, but it also enhances the activity of PAD4, a protein that citrullinates histones. After that, neutrophil elastase (NE) and myeloperoxidase MPO translocate to the nucleus (99), where they process and cleave core histones resulting

in chromatin de-condensation and mobilization (Figure 5) (100). Hence, NETs consist of decondensed chromatin (DNA, nucleoproteins, and histones) and cytoplasmic proteins which include NE, MPO, protease 3, cathepsin G and neutrophil serine protease 4 (101). Moreover, calcium flux and zinc signals are linked to the production of ROS and histone citrullination by PAD4, two main important processes of NETosis (102).

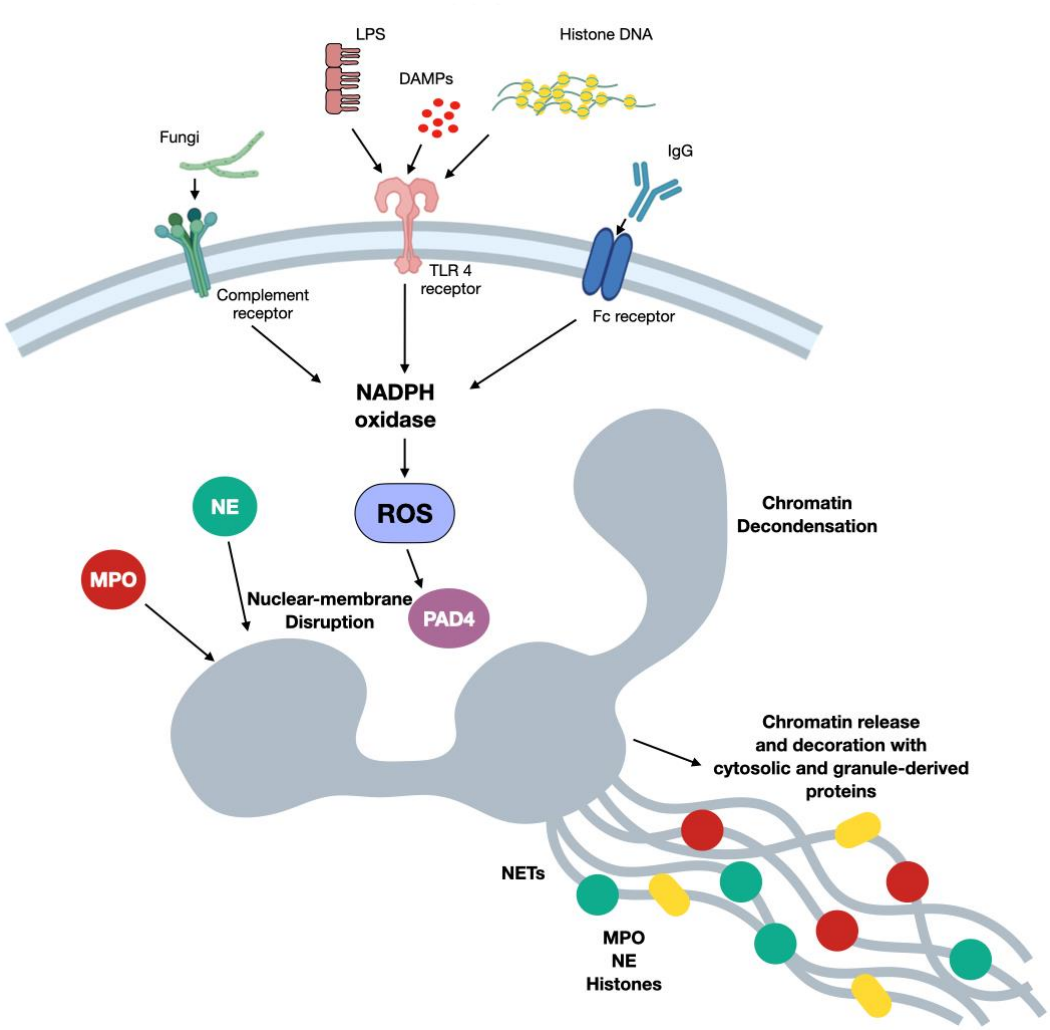


Figure 5 Sequential steps of suicidal NETosis (Adapt from Van Avondt K.,and Hartl D., 2018 (103)).

6.2 Monocytes/Macrophages

In the early phases of renal I/R injury, macrophages play a role in the innate immune response, but they also promote tubular healing and long-term kidney fibrosis. After recruitment into injury site, monocytes differentiate into functionally different macrophage subsets (M1 and M2 macrophage) (104). M1 macrophage release chemokines, pro-inflammatory cytokines (105) whereas M2 macrophages are involved in wound healing and immunoregulation. These immuno-regulatory macrophages release anti-inflammatory mediators (106). In the ischemic kidney of mice, macrophage infiltration begins to increase at 1 hour, peaks at 24 hours, and continues for 7 days after reperfusion.

6.3 Dendritic cells (DCs)

Dendritic cells that express CD11c and MHC class II are abundant in the interstitial of normal kidneys and play an important role in the link between innate and adaptive immunity (107). Renal DCs have two main functions: they act as antigen-presenting cells to T cells, and they release TNF-α, a major pro-inflammatory mediator early after injury.

7. Fc gamma receptors

People of Southeast Asian and African ancestry, who live in malaria-endemic areas (108), reduced Fcgr2b function may provide a survival advantage against parasite infection (13). Reduced inhibitory function produced by Fcgr2b- I232T (threonine substitution for isoleucine at the 232 position) causes increased B cell and myeloid cell activation. Even though it predisposes to SLE, a highly active immune system may be beneficial in response to infection (109). As such, Fcgr2b-deficient mice are resistant to severe clinical symptoms following infection with *Plasmodium chabaudi chabaudi*, a rodent malaria parasite with some parallels to *Plasmodium falciparum* infection in humans (13)

Consequently, Fc gamma receptors (FcgRs) are immune receptors that have an important role in response to antigens and clearance of immune complexes (110). FcgRs are a family of proteins that are produced by a variety of immune cells and perform biological functions by binding to the Fc region of IgG to effectively regulate inflammation and infection. Fcgr2b is most well-known function to regulate serum antibody levels via a negative feedback loop, suppressing B cells when sufficient antibodies are produced (11, 111). Fcgr2b deficiency interacts with a number of C57BL/6-specific genes to induce a spontaneous SLE-like disease, characterized by the presence of autoantibodies against chromatin and the development of lethal glomerulonephritis (10, 112). Fcgr2b signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) to suppress downstream processed such as cellular proliferation, phagocytosis, and inflammatory cytokine release when immune

complexes bind to activating Fc receptors on myeloid-lineage cells or the B cell receptor on B cells (113).

FcgRs are divided into two types; activating and inhibitory receptors, which are mostly found on the surface of immune cells. In human, the activating receptors are the high-affinity FcgRI (CD64), low-affinity FcgRII (2a and 2c; CD32), FcgRIII (3a and 3b; CD16), and FcgRIV (114). In mice, FcgRs consist of FcgrI, FcgrIII, and FcgrIV receptors and Fcgr2b is the only inhibitory receptor in humans and mice (115). Furthermore, activating signaling occurs through immunoreceptor tyrosine-based activation motifs (ITAMs), and inhibitory signaling occurs through immunoreceptor tyrosine-based inhibitory motifs (ITIMs), both of which are important negative feedback mechanisms for controlling the antibody production (116). In previous study, the induction of autoimmune disease in mice using resveratrol (polyphenol compound) in Fcgr2b deficient mice develop autoantibody production and IC-mediated glomerulonephritis (117). The meta-analysis illustrates that the abnormality of FcgRs contributes to SLE susceptibility and progression (118). Therefore, the balance between FcgR activation and inhibition signals is important for immune regulation of antibody production, and the loss of Fcgr2b activity accelerates lupus progression.

8. Disease-stimulated AKI

The systemic inflammation and tissue injury play a pivotal characteristic in the pathophysiology of AKI, which associated between autoantibody and antibody-driven diseases e.g., systemic lupus erythematosus (SLE), lupus Nephritis (LN), and neutrophil-related lupus nephritis.

8.1 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease that effects several organs and has a wide range of symptoms, ranging from minor cutaneous involvement to severe major organ damage. SLE is more prevalent in woman than in males with all ethnic group, with a ratio of female: male at 8:1 (119). The pathogenesis of SLE is a complex interaction between the immune system, hormones, and environmental factors, as well as genetic predisposition and epigenetic modifications, which can make the patient more susceptible to various pathogens (120, 121). SLE is caused by a loss of self-tolerance accompanied by excessive activation of autoreactive T cells, which increase autoantibody production by activated B cells, as well as high releases of pro-inflammatory cytokines that further exacerbate the immune responses (122). SLE patients may experience a variety of symptoms, including fatigue, skin rashes, fevers, and arthritis (123). Furthermore, lupus nephritis (LN), a kidney disease caused by SLE, is a major cause of morbidity and mortality of lupus and a leading cause of CKD and ESRD (124).

8.2 Lupus Nephritis (LN)

Lupus nephritis (LN) in Asians and Africans are more severe than the Caucasians leading to CKD in these ethnics (125). LN is characterized by renal deposition of immune complexes (IC). IgG antinuclear autoantibodies against several self-components (dsDNA, nucleosome, and histones) (126, 127) correlate with clinical symptoms and contribute to renal pathology in LN patients (128-130). Clinically, LN is characterized by periods of remission interspersed with episodes of disease activity (flare-ups). If these inflammatory processes are not effectively and rapidly treated, glomerulosclerosis, interstitial fibrosis, tubular atrophy, and progressive kidney failure will develop, leading to ESRD and the requirement for renal replacement therapy (125). Furthermore, anti-dsDNA can promote inflammation and contribute to disease development by the formation of IC with chromatin exposed on apoptotic cells and subsequent recruitment of Fc gamma receptor myeloid cells, as well as the fixation of complement (131). According to previous studies, during inflammation, an increasing number of cells undergo apoptosis (and secondary necrosis), resulting in the release of intracellular components such as nucleosome. Antibodies against dsDNA may bind to these nucleosomes, causing the kidney deposition (132, 133). Moreover, the Fc gamma receptor (FcgR) polymorphism in human is one of the susceptible factors of SLE and LN (134).

8.3 Neutrophil-related lupus nephritis

Lupus nephritis is caused by the deposition of circulating immune complex (CIC) in kidney. Hence, an increase in production of CIC increases lupus activity and severity. The source of autoantigens in lupus is derived from several processes, especially cell apoptosis and NETosis. As such, NETs release several immunogenic structures that are recognized by several autoantibodies, such as antibodies against myeloperoxidase or proteinase-3, which produce anti-double-stranded DNA, anti-histones, and anti-nucleosomes (78). Moreover, antimicrobial peptides (AMPs) could bind with HMGB1, which is recognized by toll-like receptors that might induce several mediators including IFN-α and promote NET formation.

The pro-inflammatory cytokines, including IL-6, enhance the differentiation of autoantibody-secreting autoreactive B cells leading to increased production of circulating immune complex (CIC). Another well-known pathway associated with increased antibody production in lupus is mediated by the releasing of autoantigens from apoptotic cells or secondary necrotic cells (SNECs). Finally, the autoantigens from all of these cell death pathways enhance CIC production and the recognition of CIC, in part by Fc gamma receptor, leads to the pro-inflammatory state that contributes to the more tissue injury in patients (135) (Figure 6).

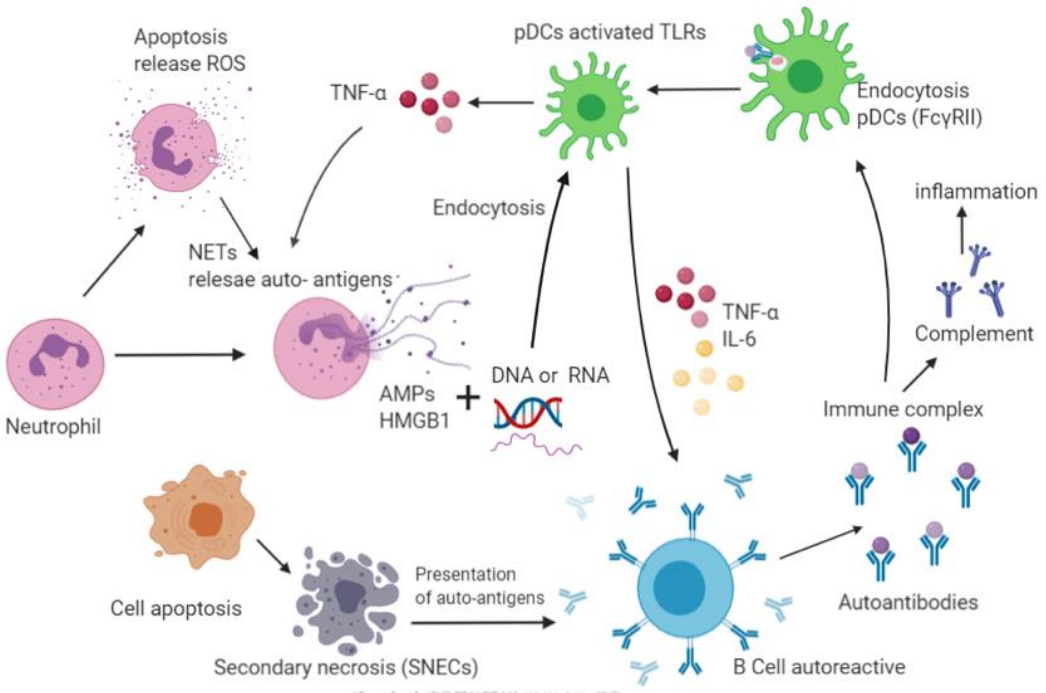


Figure 6 Pathogenesis of systemic erythematosus lupus (Adapted from Delgado-Rizo V, *et al.*, 2018 (135))

CHAPTER IV

MATERIALS AND METHODS

1. Animal studies and surgical protocol

The Institutional Animal Care and Use Committee of Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, under approval number 009/2564 and followed the protocol of the National Institutes of Health (NIH), USA, consented to all animal experimental methods. C57BL/6 wild type mice and Fcgr2b-/- deficient mice between 8 to 10 weeks old that were weight between 20 to 25 grams were used in this study. Fc gamma receptor 2b deficient on a C57BL/6 background (Fcgr2b-/-) have been provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA), and wild type (WT) mice have been purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand). Mice were maintained with standard diet and water during the experiment.

1.1 Renal ischemia reperfusion injury model

Renal ischemic reperfusion injury (I/R) was performed by clamping both renal arteries according to a previous study (136). In brief, bilateral renal arteries were clamped for 35 min through the abdominal incision under isoflurane anesthesia on a 37°C heated operation table before 2 layers abdominal suture. Both renal arteries were identified through abdominal incision before the abdominal wall for the sham surgery.

The final concentration of 10 mg/kg/dose of tramadol was administered subcutaneously after surgery and mice were sacrificed at 24, 48, 72, and 120 h under isoflurane anesthesia for sample collection. Serum was kept at -80°C until analysis and various internal organs were processed in 10% formalin or Tissue-Tek O.C.T compound (Sakura Finetek, CA, USA) for histological analysis or snap frozen and stored separately at -80°C.

2. The AKI characterization of blood serum

Serum creatinine and serum urea nitrogen acted as the AKI indicator, whereas the elevation of serum anti-dsDNA was used for determination of the lupus characteristics. In addition, serum pro-inflammatory cytokine, liver injury, and the quantification of NETs were also evaluated the inflammatory after renal ischemia reperfusion injury. The amount of serum anti-dsDNA and NETs were assessed using blood serum of the WT mice as the baseline. Parameters of AKI characteristics containing serum cytokine, liver injury, and NETosis (cell death from NETs formation) were evaluated from blood serum as follows.

2.1 Serum creatinine assay

Serum creatinine was measured using QuantiChrom Creatinine-Assay kit (BioAssay, Hayward, CA, USA) following the manufacturer instructions. Briefly, 200 µL of mixed working reagent A and B were immediately added and mixed into wells

containing 30 µL of blood serum in a clear bottom 96-well plate. The optical density at a wavelength of 510 nm was quickly determined and then at 5 min by a Varioskan Flash spectrophotometer (Thermo) using the standard creatinine as the baseline. The amount of creatinine in blood should be more than 0.3 mg/dL.

2.2 Serum urea nitrogen (BUN) assay

The standard assay of urea nitrogen (BUN) colorimetric detection kit (Invitrogen) was used for quantified the level of urea in serum as the following. The serum samples were diluted with distilled water and transferred 50 µL of diluted sample to 96-well plate, and then 75 µL of the color reagent A and B was added in to each well, respectively. The absorbance at a wavelength of 450 nm was measured after a prolonged 30 min incubation at room temperature using the urea nitrogen standard as the baseline. The normal range of urea nitrogen in serum is 5 to 20 mg/dL.

2.3 Serum anti-dsDNA assay

The anti-dsDNA was evaluated using a protocol with coated-calf thymus DNA (Invitrogen, Carlsbad, CA, USA) (29). Briefly, plate was incubated with calf thymus DNA at 4°C overnight, then filled with blocking solution at room temperature, and washed with 1X phosphate buffered saline (PBS; pH 7.4) with 0.05% Tween 20, respectively. Subsequently, mouse serum samples were added into the plate and incubation was continue overnight at 4°C. Then, HRP-conjugated goat anti-mouse

antibody (BioLegend, USA) and TMB peroxidase substrate (TMB Substrate Set; BioLegend, USA) were added to the analyzed plates and incubated at room temperature for 1 h and 15 min, respectively. The solution of 2 N H_2SO_4 was added to stop the reaction. The measurement at a wavelength of 450 nm was determined using spectrophotometer (Thermo).

2.4 Alanine transaminase assay

Liver injury was evaluated by the activity of alanine transaminase (ALT) using EnzyChrom ALT assay (EALT-100, BioAssay). Briefly, 20 µL of each sample was added with the working reagent was prepared by mixing 200 µL of assay buffer, 5 µL of cosubstrate, 1 µL of lactate dehydrogenase (LDH), and 4 µL of reconstituted NADH per well reaction. The analyzed plate was immediately tapped to mix and incubated at room temperature. The kinetics of alanine transaminase were recorded with the rate of NADH consumption by the absorbance at a wavelength of 340 nm after incubation at 5 and 10 min, respectively. The average normal range of ALT kinetics is 24 U/L.

2.5 Determination of serum pro-inflammatory and NETs associated cytokines

Serum pro-inflammatory cytokines (TNF-α and IL-6) and NETs associated cytokine (IL-1β) were quantified using enzyme-linked immunosorbent assay (ELISA; Invitrogen) according to the manufacturer instructions. First, 100 µL of capture

antibody diluted with 1X PBS was added into each ELISA-plate well, and plates were then incubated at 4°C overnight. The coated ELISA plate was washed 3 times with wash buffer (1X PBS with 0.05% Tween 20), blocked with 1X ELISA/ELISPOT diluent, and incubated at room temperature for 1 h, respectively. Serum samples were diluted using 1X ELISA/ELISPOT diluent, transferred 100 µL of diluted sample into each well of ELISA plate, and incubated at 4°C overnight. After a prolonged overnight incubation, ELISA plate was washed thrice with wash buffer before adding 100 µL of diluted detection antibody to all wells and incubating at room temperature for 1 h. Subsequently, ELISA plate was washed thrice with wash buffer, added 100 µL of diluted Streptavidin-HRP or Biotin-HRP, and then incubated for 30 min at room temperature. The ELISA plate was washed 5 times with wash buffer, added 100 µL of 1X tetramethylbenzidine (TMB) substrate solution, incubated at room temperature for 15 min, and then added 100 µL of 2 N H_2SO_4 to stop the reaction, respectively. A Varioskan Flash spectrophotometer (Thermo) was used to determine the measurement at a wavelength of 450 nm.

2.6 Quantification of NETs

A Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, USA) was used to quantify NETs following the manufacturer instructions. Briefly, 100 µL of serum sample diluted with 1X TE buffer was added with 100 µL of working reagent and incubated for 5 min a room temperature in the dark. After incubation, the sample was quantified the fluorescent intensity by a spectrophotometer (Thermo) using the

standard fluorescein wavelengths of excitation at 480 nm and those of emission at 520 nm.

3. The AKI characterization on polymorphonuclear leukocytes (PMN)

3.1 AKI-associated PMN characterization

3.1.1 Neutrophil isolation

To evaluate the differentiation of PMN after renal ischemia reperfusion injury in Fcgr2b-/- and wild type mice, peripheral blood was collected into heparin tubes by cardiac puncture under isoflurane anesthesia. Whole blood was separated using Polymorphprep™, containing sodium diatrizoate ($C_{11}H_8I_3N_2NaO_4$) and a polysaccharide solution (8% (w/v); Alere Technologies AS, Norway), at a ratio of 1:1, and by centrifugation at 1800 rpm for 30 min at room temperature with no deceleration force, respectively. Then, the layer of polymorphonuclear cells (PMN) was collected and washed with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) by centrifugation at 1800 rpm for 5 min. Subsequently, the contaminated red blood cells (RBCs) were removed using ammonium chloride lysis buffer, polymorphonuclear cells were pelleted at 1800 rpm for 5 min, and rested in fresh FBS-contained RPMI 1640, respectively. Neutrophils was >95% of purity and variability as visualized using the trypan blue exclusion method. The concentration of 2×10^5 cells of neutrophils was used for the functional assays.

3.1.2 Determination of AKI-associated PMN characterization

The concentration of 2×10^5 cells of neutrophils was laid on the poly L-lysine coated cover slip in 24-well plates and incubated in a 5% CO_2 incubator at 37ºC for 2 h. Neutrophil-attached glass cover was fixed with 4% paraformaldehyde in 1X PBS for 10 min, carefully removed the cover glass, and put down the side of fixed sample on a drop of PBS (parafilm sheet covered 24-well plate). Then, the fixed sample was thrice washed for 5 min in PBS, incubated for 1 min in 1X PBS with 0.05% Tween 20 to permeabilize the cells, and blocked using 1X PBS with 2% bovine serum albumin (BSA) for 30 min at room temperature, respectively. To visualize the AKI-induced NETs, NETs markers were detected with antibodies against neutrophil elastase (NE; ab68672), myeloperoxidase (MPO; ab25989), and citrullinated histone H3 (citrulline R2 + R8 + R17; ab5103) (Abcam, Cambridge, MA, USA) were used to identify the cells, which were incubated at 4ºC overnight. Subsequently, the cover glass was rinsed 3 times with 1X PBS and incubated for 1 h at room temperature with various secondary antibody including i) Alexa Fluor 488 goat anti-rabbit IgG (green color; ab150077), ii) Alexa Flour 647 goat anti-mouse IgG (red color; ab150115), and iii) donkey anti-rabbit IgG (red color; ab150077) (Abcam). Then, the cover glass was washed 3 times and stained with DAPI (4′,6-diamidino-2-phenylindole), a blue-fluorescent DNA stain (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature, and was increased fluorophore photostability by prolong antifade mountant (Prolong, Life Technologies), respectively. The fluorescent images were visualized with ZEISS LSM 800 Airyscan confocal laser scanning microscope at 630× magnification (Carl Zeiss, Jena, Germany).

3.2 PMA or LPS induces apoptosis and purification

3.2.1 Neutrophil proliferation and purification

To assess the apoptosis and NETosis susceptibility between Fcgr2b-/- and WT neutrophils using the *in vitro* experiment, neutrophils were derived from peritoneum using a published protocol (56). Briefly, 1 mL of 3% thioglycolate was intraperitoneal administered in 8-week-old mice. At 3 h administration, mice were sacrificed under isoflurane anesthesia, and peritoneal cavity was thoroughly collected and washed with ice-cold phosphate buffer solution (PBS) before centrifugation at 1800 rpm, 4°C for 5 min to separate the PMN cells, which was evaluated by Wright's-stains (54). Only the preparation with more than 90% neutrophils was further used for the experiments. Neutrophils at a concentration of 2×10^5 cells/mL were cultured in RPMI 1640 supplemented with 10% FBS. Only the preparation with more than 90% of neutrophils was further used for the experiments.

3.2.2 PMA or LPS-stimulated apoptosis and NETosis

Each condition of 2×10^5 neutrophils was added with phorbol myristate acetate (PMA; Sigma-Aldrich), a NETs stimulator or lipopolysaccharide (LPS) (*Escherichia coli* 026: B6; Sigma-Aldrich) at a final concentration of 25 and 100 ng/mL, respectively, in the 24-well plate containing cover glass-coated poly- L-lysine, and incubated at the same condition for 2 h. Supernatants were stored at -80ºC until used for cytokine measurement by ELISA (Invitrogen) and were quantified for dsDNA by Picogreen assay kit (Invitrogen) according to the manufacturer's protocol

as described above. NETosis or apoptosis-induced neutrophils were detected the cell morphology using fluorescent imaging as described above, and those by flow cytometry.

3.2.3 Flow cytometry

Neutrophils were suspended in 1X PBS at a concentration of 5×10^5 cells/mL, stained for apoptosis/ necrosis by 5 µL per reaction of annexin V-FITC and propidium iodide (PI) (BD Biosciences), respectively. Then, the samples were washed with FACS flow buffer, 1X PBS supplemented with 1% (v/v) FBS, and 0.05% NaN_3, and processed by the BD LSR II Flow Cytometry (BD Biosciences) using the FlowJo software (Tree Star Inc., Ashland, OR, USA). Reactive oxygen species (ROS) was determined by DHE (Dihydroethidium) assay (ab236206; Abcam, Cambridge, MA, USA), according to the manufacturer's instructions.

4. The AKI characterization on internal mouse organs

4.1 Histological analysis

Several tissues after renal ischemic reperfusion injury were fixed overnight in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Tissue was sectioned at 4 µm and stained with hematoxylin and eosin (H&E) according to the standard protocols. Each individual was visualized using an inverted microscope (Olympus, Inc., Japan) at 200× magnification in 10 randomly selected fields (32-34).

Renal injury was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation using the following score in Table 2, whereas lung injury was determined by alveolar hemorrhage, alveolar congestion, neutrophil infiltration, and alveolar wall thickness with the following score in Table 2.

Table 2 Injury score using histological analysis

Organ	Score				
	0	1	2	3	4
Kidney	area of damage < 5%	area of damage 5% –10%	area of damage 10% – 25%	area of damage 25% – 50%	area of damage > 50%
Lung	no injury in the observed field	injury up to 25%	injury up to 50%	injury up to 75%	injury in the entire field

4.2 Immunofluorescent analysis

Internal organs containing kidney, liver, lung, and heart were prepared in Tissue-Tek O.C.T compound (Sakura Finetek, CA, USA), sliced at 5 μm, and stained with several fluorescent-tagged mouse antibodies to elucidate the characteristics of AKI using immunofluorescence as the following.

NET detection

Antibody (Abcam, Cambridge, MA, USA) against neutrophil elastase (NE; ab68672), myeloperoxidase (MPO; ab25989) with secondary antibodies; goat anti-rabbit IgG (ab150077, green color), and goat anti-mouse IgG (ab150115, red color) and DAPI (Sigma Aldrich) were used for NETs visualization.

Cell apoptosis

The anti-Cleaved Caspase 3 (Asp175, 9661S; Cell Signaling Technology, Boston, MA, USA) with secondary antibody against goat anti-rabbit IgG (ab150077; Abcam) and DAPI (Sigma Aldrich) were used to identify cell apoptosis.

Immune complex deposition

The immunoglobulin deposition was detected using goat anti-mouse IgG (ab150113, green color; Abcam). After immunofluorescent staining, the stained samples were mounted by prolong antifade mountant (Prolong, Life Technologies) to increase the fluorophore photostability. The fluorescent images were analyzed with ZEISS LSM 800 Airyscan CLSM (Carl Zeiss, Jena, Germany) at 630× magnification as described above.

5. Expression of inflammatory cytokines

The various interesting genes in mouse tissues and neutrophils after renal ischemic reperfusion injury, and apoptosis or NETosis-induced neutrophils containing injury-associated inflammatory cytokines (*TNF-a, IL-6, and IL-10*) and NETs including peptidyl arginine deiminase 4 *(PAD4)* and *IL-1β* were quantified the overexpression using real time-polymerase chain reaction (qRT-PCR).

5.1 Sample preparation

Each internal organs including kidney, lung, liver, and heart were weighted at 20 mg and homogenized by a rotor-stator homogenizer in 350 µL of FARB buffer (Favorgen, Taiwan) supplemented with 3.5 µL of β-mercaptoethanol. For cultivated neutrophils at the concentration of 1×10^6 cells/mL were collected by centrifugation at 1800 rpm for 5 min at 4ºC. After that, 350 µL of FARB buffer (Favorgen) and 3.5 µL of β-mercaptoethanol were added into each condition and vortexed to resuspend the cells completely. All samples were incubated at room temperature for 5 min and then transferred the sample mixture to the filter column and centrifuged at 13,000 rpm for 2 min. Subsequently, 1 volume of 70% RNase-free ethanol was added into the clarified supernatant in a new microcentrifuge tube and mixed well by vortexing.

5.2 RNA extraction

Total RNA was extracted using the FavorPrep™ Tissue Total RNA Mini kit (Favorgen, Taiwan) following the manufacturer instructions. After the sample preparation, the ethanol-added sample mixture was put into the FARB Mini column, centrifuged for 1 min at 13,000 rpm at 4ºC, and discarded the flow through, respectively. The FARB Mini column were cleaned with 500 µL of wash buffer 1, centrifuged at 13,000 rpm for 1 min at 4ºC, and discarded the flow through, respectively. Then, 750 µL of wash buffer 2 was put into the FARB Mini column, centrifuged at 13,000 rpm for 1 min at 4ºC, and discarded the flow through, respectively. To completely remove the residual wash buffer, the FARB Mini column was centrifuged at 13,000 rpm for 3 min at 4ºC. The FARB Mini column was placed onto an elution tube, added 30 µL of RNase-free ddH_2O to the membrane, and incubated for 1 min at room temperature before centrifuging at 13,000 rpm for 1 min to elute RNA. The purity of extracted RNA was measured at a wavelength of 260 and 280 nm using a Nanodrop 100 spectrophotometer (Thermo Scientific). The RNA was kept at -80°C until analysis.

5.3 cDNA synthesis

The contamination of genomic DNA was conducted in 20 µL of reaction mixture containing 10 µL of total RNA, 2 µL of 10X Reaction Buffer with $MgCl_2$, 2 µL of 1 U of DNase I with RNase-free, and 6 µL of RNase-free water. After that, the reaction mixture was incubated at 37°C for 45 min. Then, two microliters of 50 mM

EDTA were added and incubated at 65°C for 10 min to stop the enzyme reaction. A High-Capacity cDNA Reverse Transcription kit (Thermo Scientific) was used for reverse transcription of whole RNAs as follows. The cDNA synthesis was performed in 20 µL of reaction mixture is composed of 10 µL of 100 ng of the total RNA, 3.2 µL of sterile milliq water, 2 µL of 10X RT buffer, 2 µL of RT Random primers, 1 µL of RNase inhibitor, 0.8 µL of 25X dNTP mix, and 1 µL of MultiScribe reverse transcriptase. Then, the reaction mixture was incubated at 25°C for 5 min, followed by 37°C for 120 min, and 85°C for 5 min, respectively. The cDNA solution was stored at -20°C.

5.4 Quantitative real-time PCR

The expressed genes were performed with quantitative real-time PCR by QuantStudio 6 Flex Real-time PCR System (Thermo Scientific) with 10 µL of mixture containing 1 µL of nuclease-free water, 5 µL of 2X SYBR® Green PCR master mix (Applied Biosystems), 0.2 µL of 10 µM of each primer, and 2 µL of 100 ng of cDNA template. The experiment was carried out in triplicate with the following cycling parameter as follows; the initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min. The results were demonstrated using the relative quantitation of the comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta Ct}$) as normalized by β-actin (an endogenous housekeeping gene). The list of primers is shown in Table 3.

Table 3 The primers were used in the investigation

Primer	**Sequence**	
Tumor necrosis factor-α (*TNF-α*)	Forward	5´-CCTCACACTCAGATCATCTTCTC-3´
	Reverse	5´-AGATCCATGCCGTTGGCCAG-3´
Interleukin-6 (*IL-6*)	Forward	5´-TACCACTTCACAAGTCGGAGGC-3´
	Reverse	5´-CTGCAAGTGCATCATCGTTGTTC-3´
Interleukin-10 (*IL-10*)	Forward	5´-GCTCTTACTGACTGGCATGAG-3´
	Reverse	5´-CGCAGCTCTAGGAGCATGTG-3´
Peptidyl arginine deiminase (*PAD4*)	Forward	5´-ACAGGTGAAAGCAGCCAGC-3´
	Reverse	5´-AGTGATGTAGATCAGGGCTTGG-3´
Interleukin-1β (*IL-1β*)	Forward	5´-GAAATGCCACCTTTTGACAGTG-3´
	Reverse	5´-TGGATGCTCTCATCAGGACAG-3´
β-actin	Forward	5´-CGGTTCCGATGCCCTGAGGCTCTT-3´
	Reverse	5´-CGTCACACTTCATGATGGAATTGA-3´

6. Statistical analysis

Statistical differences among groups were examined using the unpaired Student's t-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with two groups or more than two groups, respectively, all of which are presented as the mean ± standard error (SE). Statistical comparisons of data were conducted by paired Student's t-test in the experiment condition of before and after treatment. SPSS 11.5 software (SPSS, Chicago, IL, USA) was used for all statistical analyses.

CHAPTER V

RESULTS

1. The AKI characteristics were accelerated after renal ischemia reperfusion of Fcgr2b-/- lupus prone mice

Blood serum, polymorphonuclear cells, and internal organs, as well as NETs production-associated genes have been evaluated in Fcgr2b-/- lupus prone mice after renal ischemia reperfusion injury.

1.1 Detection of AKI-represented blood serum

At the onset of renal damage, the reperfused 8-week-old Fcgr2b-/- mice with asymptomatic lupus prone state, blood urea nitrogen (BUN), serum creatinine (Scr), and anti-dsDNA have no AKI and lupus characteristics in 8-week-old Fcgr2b-/- mice (Figure 7A and B). The highest concentration of BUN and Scr of Fcgr2b-/- mice were performed at 24 h and remained elevated 120 h after I/R, but Scr had retrieved to baseline similar to WT mice. In addition, anti-dsDNA, which acted as a key lupus auto-antibody, highly enhanced at 120 h indicating that renal I/R modulates lupus activity (Figure 7C). To evaluate the inflammatory after renal injury, alanine transaminase (ALT) presented the highest activity at 24 h which is similar to serum IL-1β and serum dsDNA, whereas serum TNF-α and IL-6 continue expressed at 72 to 120 h (Figure 7D-H). These results demonstrated that the systemic inflammation in

fcgr2b -/- mice following AKI injury might be more severe than WT mice at 120 h after I/R.

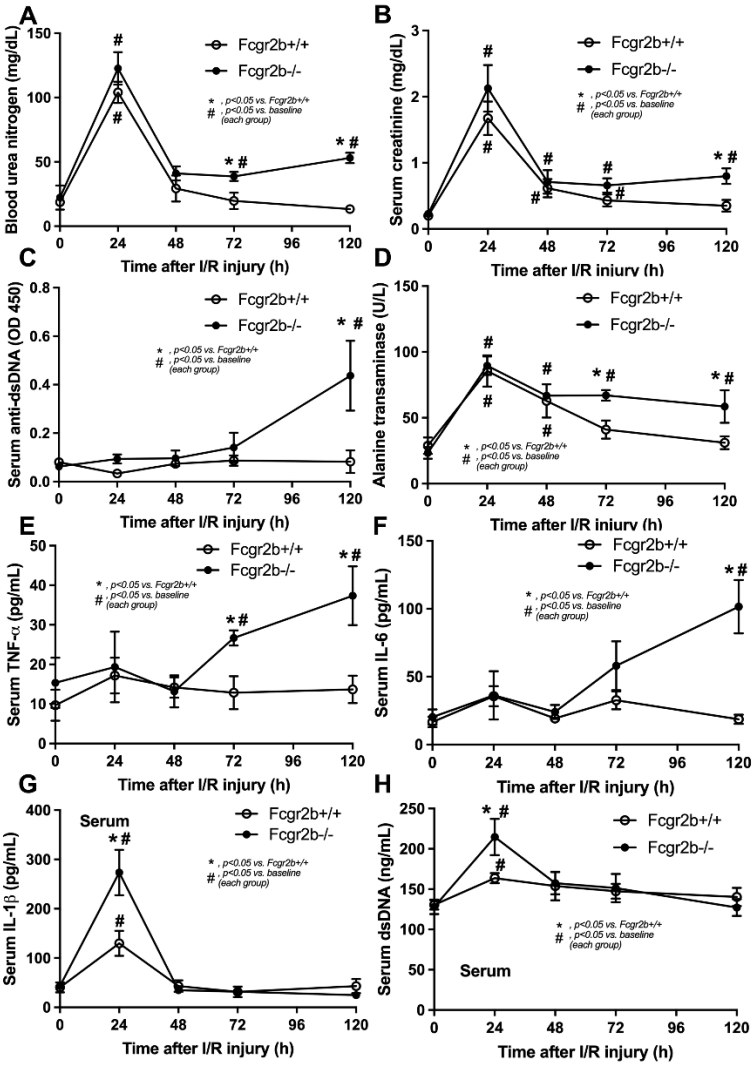
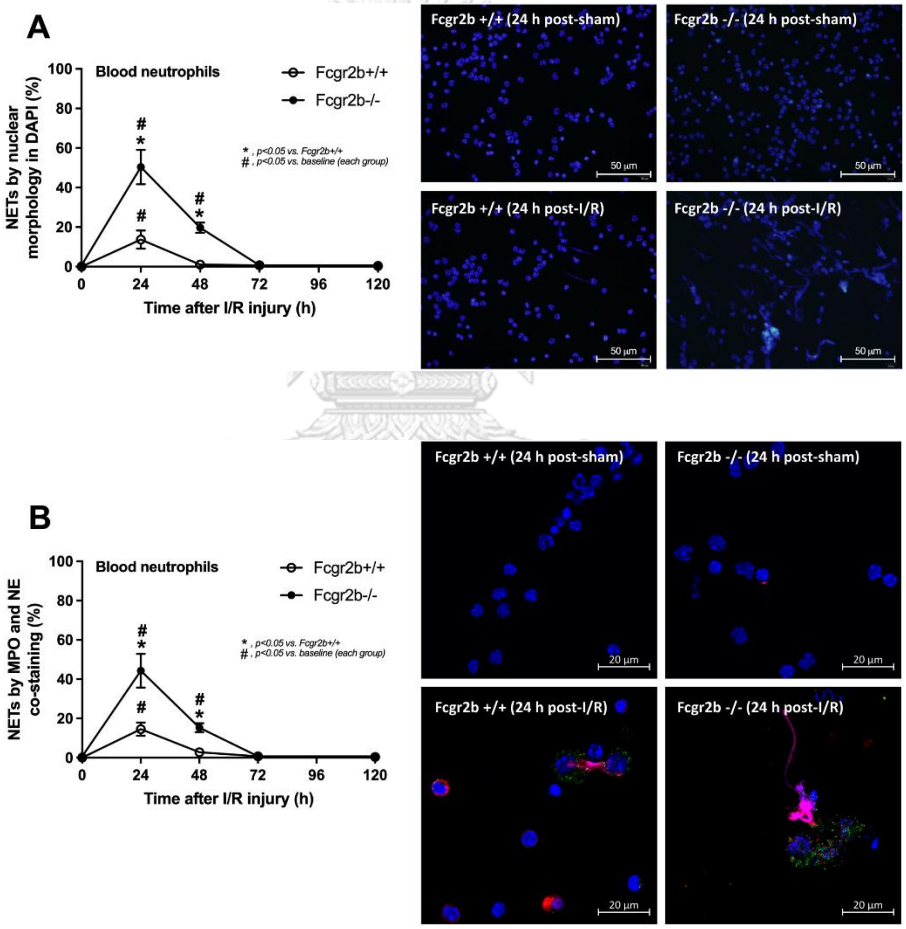


Figure 7 The AKI characterization after renal ischemia reperfusion. A; blood urea nitrogen, B; serum creatinine, C; serum anti-dsDNA, D; alanine transaminase, E; serum TNF-α, F; serum IL-6, G; serum IL-1β, and H; serum dsDNA.

1.2 Determination of NET-performing neutrophils

Prominent NETs in Fcgr2b-/- mice at 24 h after I/R compared to WT mice, as indicated by nuclear morphology (DAPI), which was co-stained with myelo-peroxidase (MPO) and neutrophil elastase (NE), as well as citrullinated histone 3 (CitH3) staining (Figure 8A-C) was demonstrated. These data indicated that renal I/R injury in Fcgr2b-/- mice induced NETs formation at 24 h.



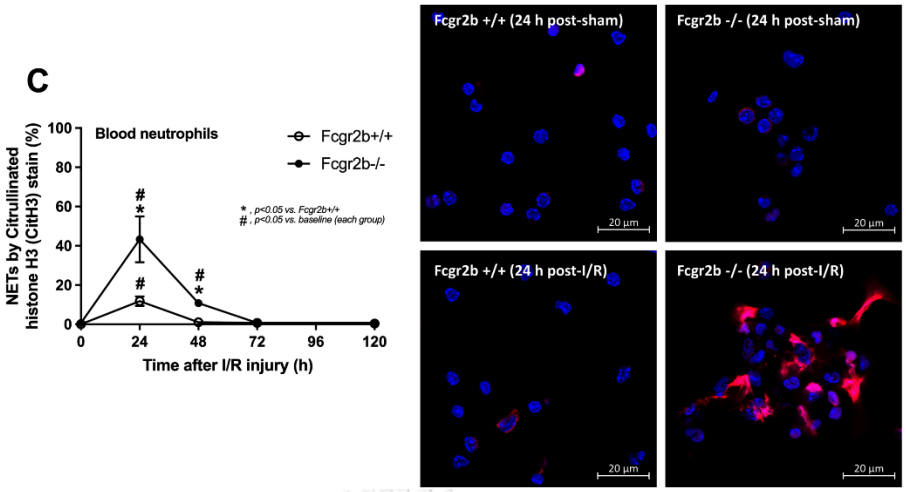


Figure 8 Neutrophils of Fcgr2b-/- and WT mice performed NET formation at 24 h after renal I/R, which are detectable by CLSM using DAPI (A), MPO and NE (B), and CitH3 (C) staining.

1.3 Analysis of the injury of internal organs

Figure 9 showed the infiltration of mononuclear cells in kidney, liver, and lung indicating that the injuries of kidney and liver occurred after 120 h I/R have significantly the differentiation between Fcgr2b-/- and WT mice, whereas lung presented the organ damage over 72 to 120 h of renal I/R injury. The damage of heart after 120 h I/R has no the differentiation (data not shown). As a result, the injuries of internal organs indicated that Fcgr2b -/- mice with AKI injury might be more severe than WT mice at 72 to 120 h after I/R depending on the organ.

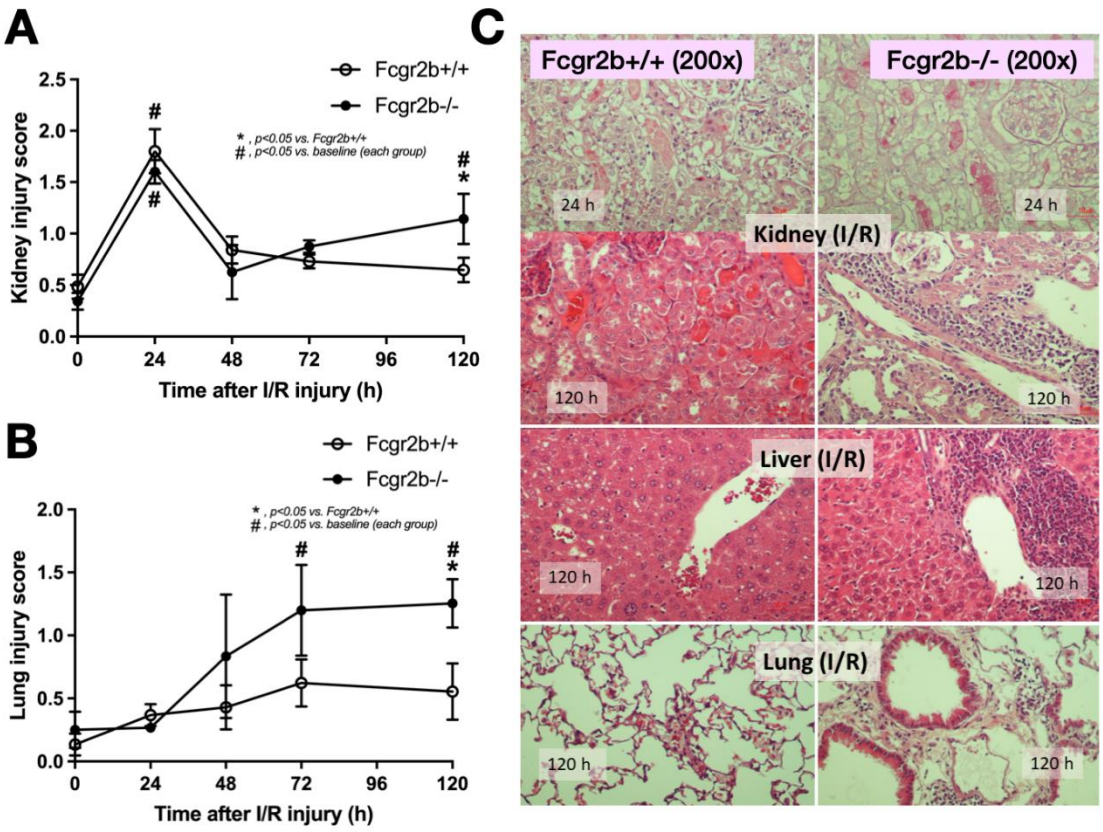


Figure 9 The injurious histological score of kidney (A) and lung (B), as well as the photographs of kidney and lung (C) with hematoxylin and eosin staining.

In parallel, the production of NETs on kidney, lung, liver, and heart were visualized by immunofluorescent staining using MPO and NE as the biomarker demonstrated that Fcgr2b-/- mice showed fluorescent score was higher than WT mice after 24 h I/R injury (Figure 10 A and B). These results implied that NETs formation in Fcgr2b-/- mice following I/R injury might be an auto-antigens, which have the effect on lupus exacerbation. Meanwhile, the absence of NETs in liver and heart after 24 h I/R injury suggested that the renal I/R induced remote organ injury via non-NETs inflammatory pathways (Figure 10 C and D).

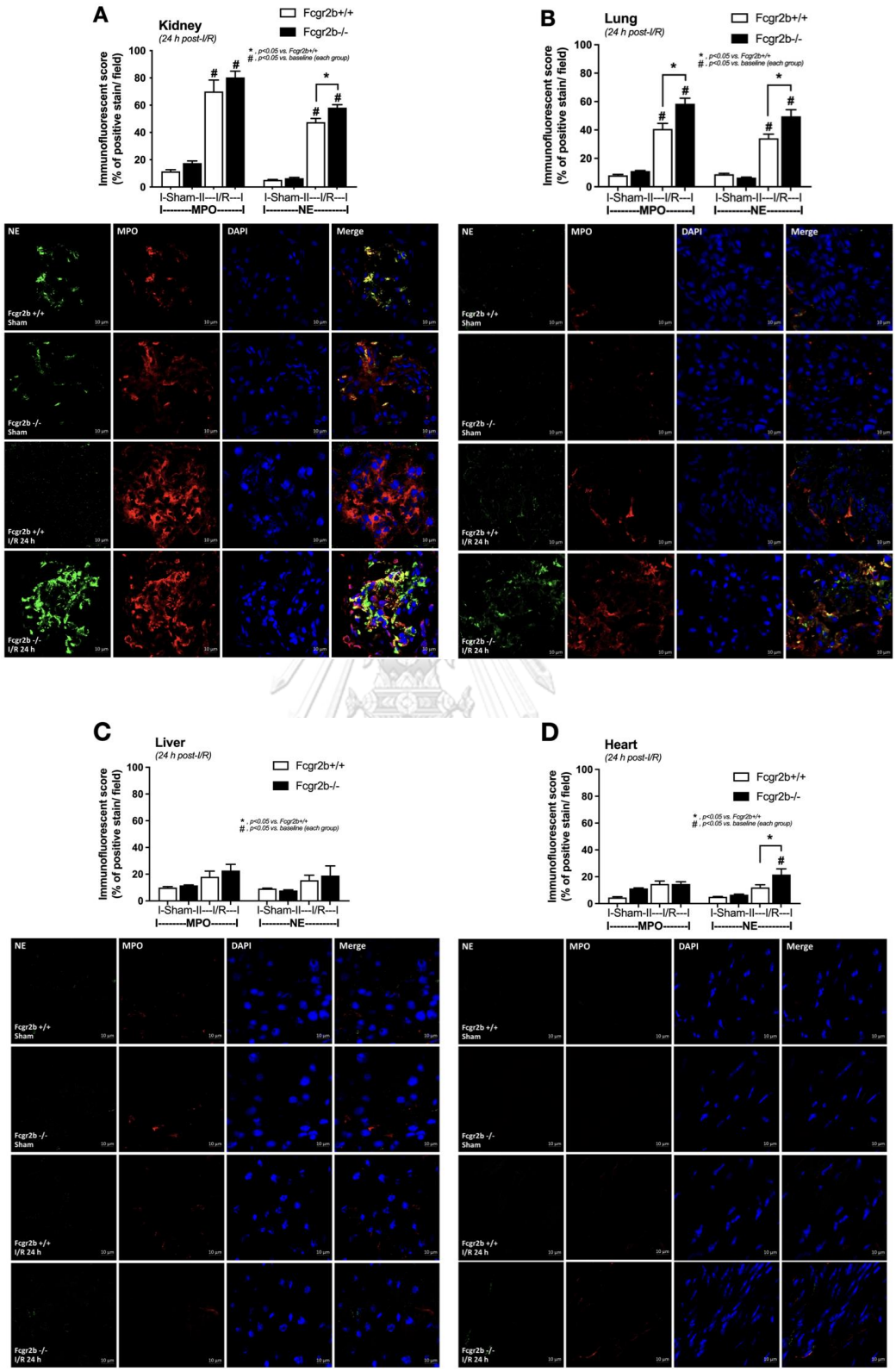


Figure 10 NET formation on the damageable kidney (A), lung (B) liver (C), and heart (D) after 24 h of renal I/R injury.

Because of the NETs that occurred in kidney and lung, these organs were evaluated for apoptosis and immunoglobulin deposition, Fcgr2b-/- mice had high apoptotic cells in the kidney and lung than those of WT mice at 24 h renal I/R injury, and the detected Caspase 3 of lung decreased at 120 h of renal I/R injury (Figure 11A and C). In addition, cell apoptosis was exclusively found only in Fcgr2b-/- mice (Figure 11A). These data suggested that Fcgr2b-/- mice showed significantly more cell damage (apoptosis and NETosis) than WT mice. Although renal IgG deposition was seen in both mouse strains at 120 h of I/R injury, the deposition in Fcgr2b-/- mice were more apparent when compared with WT mice (Figure 11B). Indeed, IgG deposition was observed in WT mice as part of the wound healing process. Therefore, IgG deposition was much higher in Fcgr2b-/- mice than WT mice, suggesting that ischemia-induced inflammation causes enhanced antibody production in lupus mice (Figure 11B and D). These data implied that renal I/R injury through the exacerbated NETs and apoptosis affected to lupus disease aggravation and autoantibody production.

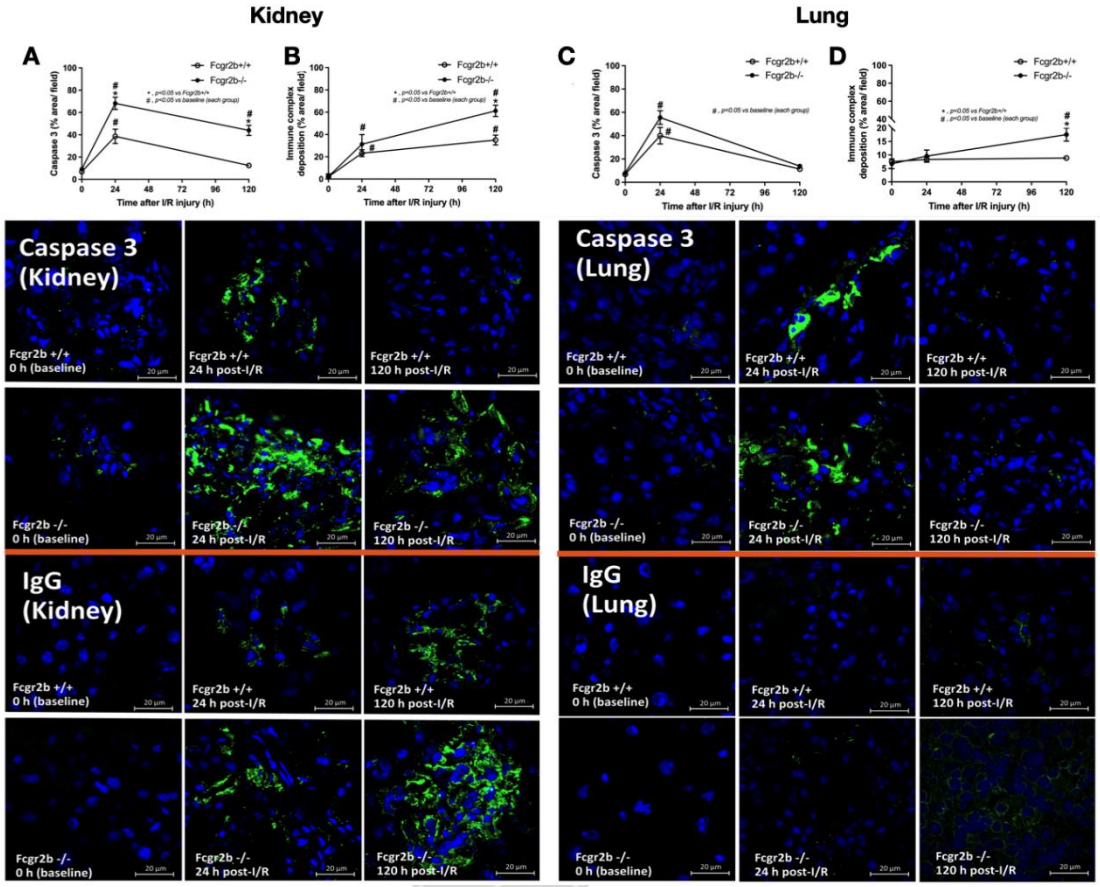
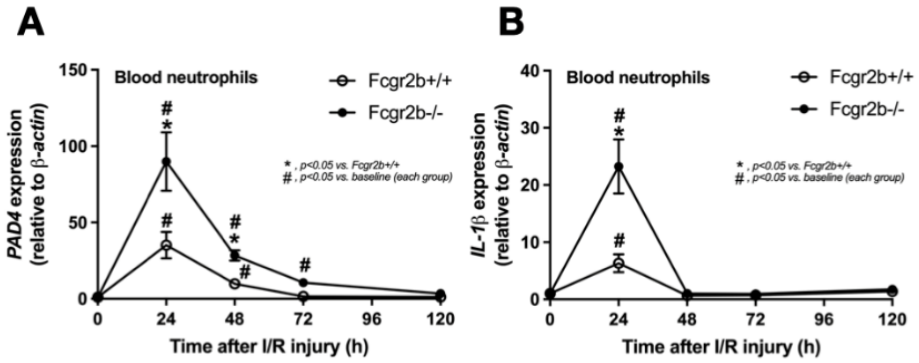


Figure 11 Renal ischemia reperfusion (I/R) induced more prominent apoptosis at 24 h and induced immunoglobulin G (IgG) deposition at 120 h in renal and lung of lupus prone mice.

1.4 Evaluation of the overexpression of apoptosis or NETosis-induced neutrophil and neutrophil extracellular trap genes on Fcgr2b-/- and WT neutrophils and internal organs

Parameters of NETs, including *peptidyl arginine deiminase 4 (PAD4*) and *IL-1β* genes, (Figure 12A and B) demonstrated the more prominent NETs in Fcgr2b-/- neutrophils at 24 h after I/R when compared to WT neutrophils, which associated with the increased serum dsDNA (Figure 7H). These data indicated that renal I/R injury in Fcgr2b-/- mice induced NETs formation at 24 h. Furthermore, NETs formation-associated genes were performed in the internal organs after I/R, which demonstrated that Fcgr2b-/- mice had the enhanced expression levels of *PAD4* and *IL-1β* in kidney and lung, and these of levels elevated again after 120 h (Figure 12C and D).



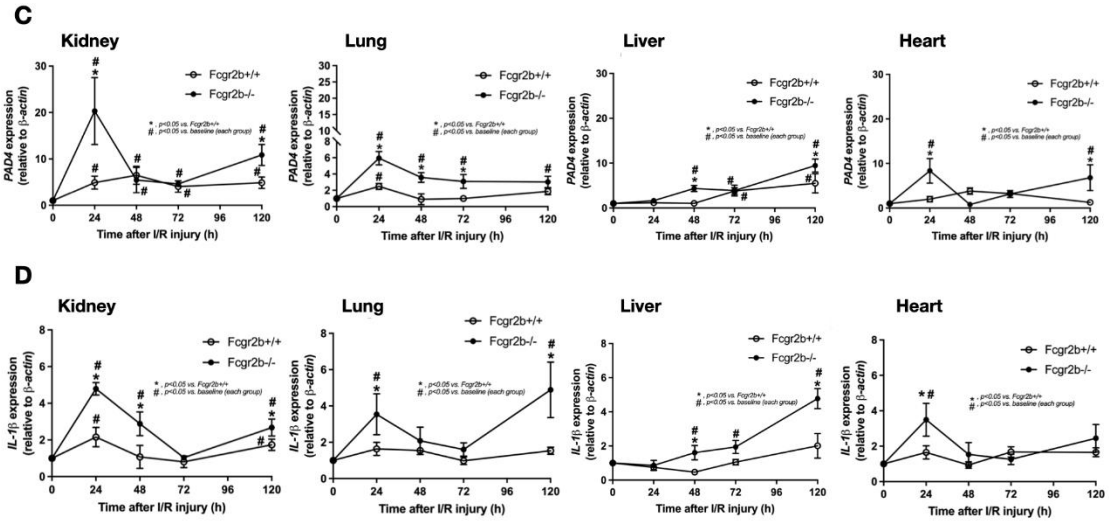


Figure 12 The gene expression of *PAD4* (A) and *IL-1β* (B) was detected in blood neutrophils and the differentiation of *PAD4* (C) and *IL-1β* (D) overexpression in each internal organ.

Because of local organ inflammation (*TNF-α, IL-6,* and *IL-10* gene expression) following I/R injury, the expression of *TNF-α, IL-6,* and *IL-10* genes were only found to be high in the kidney of Fcgr2b-/- and WT mice at 24 h after I/R, in contrast to lung, liver, and heart. On the other hand, Fcgr2b-/- mice showed higher cytokine expression than WT mice at 120 h after renal I/R (Figure 13A-L). These results implied that Fcgr2b-/- mice had the higher responses toward I/R damage than WT mice and induced more severe remote organs inflammation.

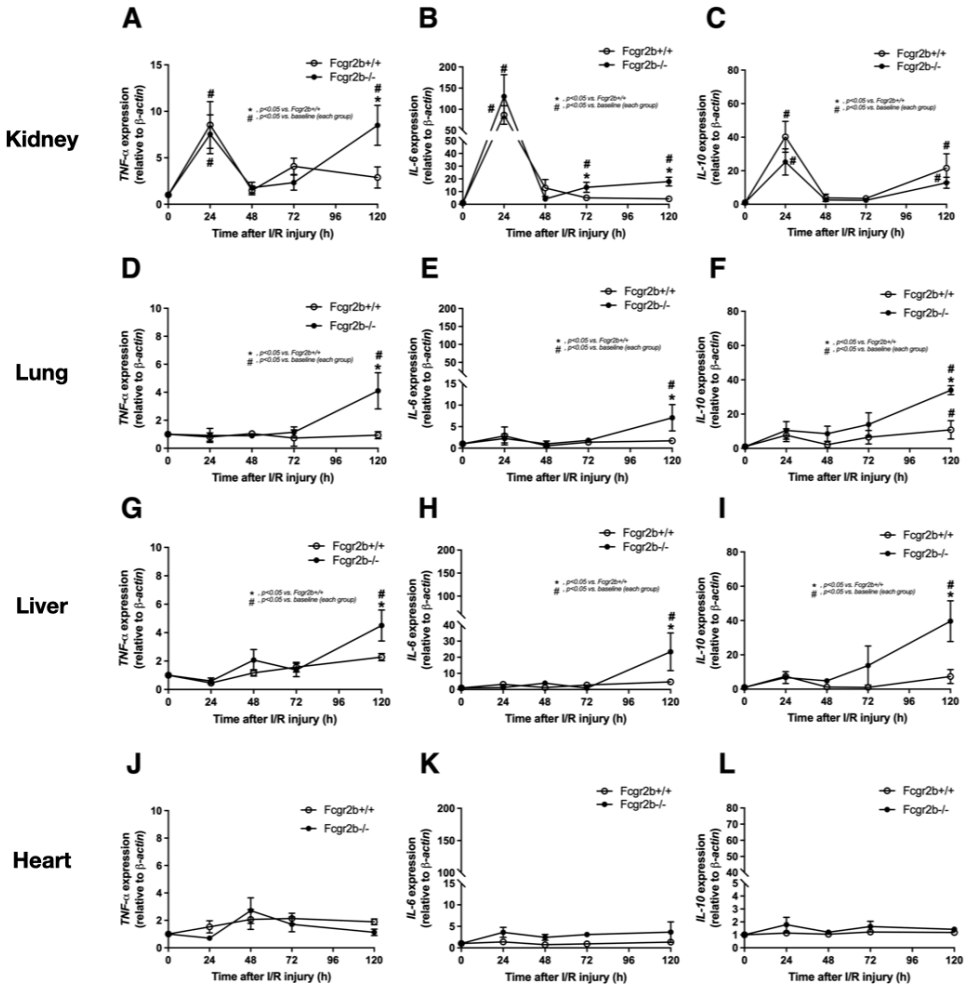


Figure 13 The expression of inflammatory cytokine-related genes (*TNF-α*, *IL-6*, and *IL-10*) of kidney (A-C), lung (D-F), liver (G-I), and heart (J-L) in Fcgr2b-/- and WT mice with ischemia reperfusion injury are demonstrated.

2. Investigation of the susceptibility of NET formation, inflammatory activation, and apoptosis on Fcgr2b-/- neutrophils

PMA and LPS are well-known for study the stimulation of immune pathway, especially cell apoptosis and NET formation. Because Fcgr2b-/- neutrophils might be more susceptible to apoptosis and NETosis than WT cells (the loss of inhibitory signaling), PMA and LPS are used as the representative stimulators. At 4 h of the stimulation with PMA or LPS, NETosis occurred in neutrophils from both mouse strains which were detectable with DAPI nucleus morphology and co-staining of MPO and NE. PMA similarly induced NETs in neutrophils of both mouse strains at 4 h post-stimulation, while LPS inducing higher NETs in Fcgr2b-/- than WT cells (Figure 14A and B), which were consistent with the amount of dsDNA and the expression of PAD4 gene as well as the production of prominent pro-inflammatory cytokine production (TNF-α and IL-6) (Figure 14C-G).

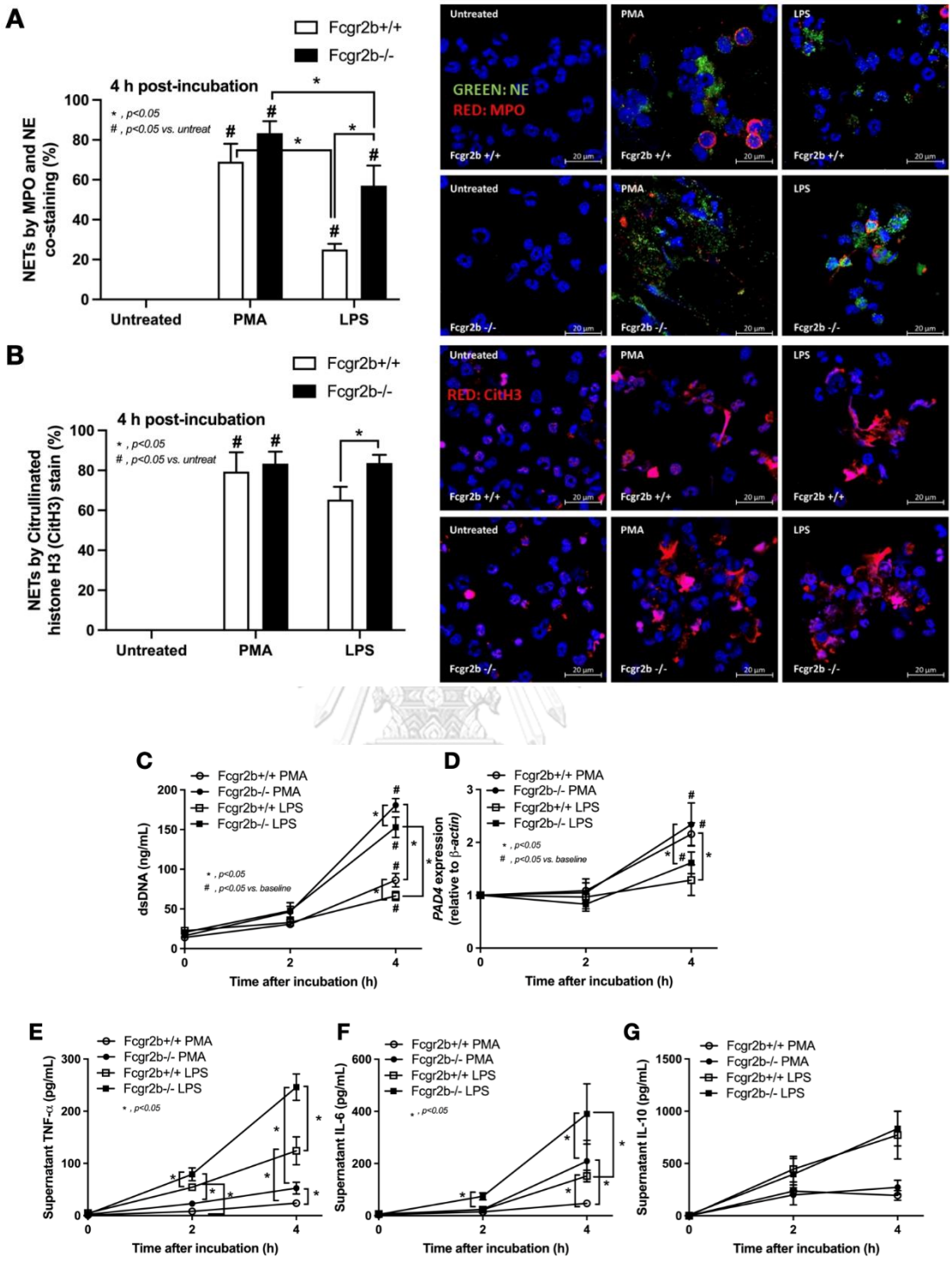


Figure 14 NET formation in Fcgr2b-/- or WT neutrophils after activation by PMA or LPS with several NET parameters are visualized by CLSM (A-B), the amount of

several NET parameters in Fcgr2b-/- or WT neutrophils after activation by PMA or LPS are demonstrated.

In addition, PMA or LPS-stimulated neutrophils were analyzed the apoptosis, necrosis, and the production of reactive oxygen species indicating that Fcgr2b-/- stimulated neutrophils demonstrated more prominent apoptosis (and necrosis) than WT cells at 2 h of the stimulation, accompanying with the increased production of ROS (Dihydroethidium; DHE) (Figure 15A-C). Furthermore, LPS induced more severe necrosis with lower ROS in Fcgr2b-/- neutrophils when compared with PMA (Figure 15B and C). These results demonstrated that the stimulated cells especially Fcgr2b-/- neutrophils were susceptible to apoptosis at 4 h of post-stimulation by PMA and LPS.

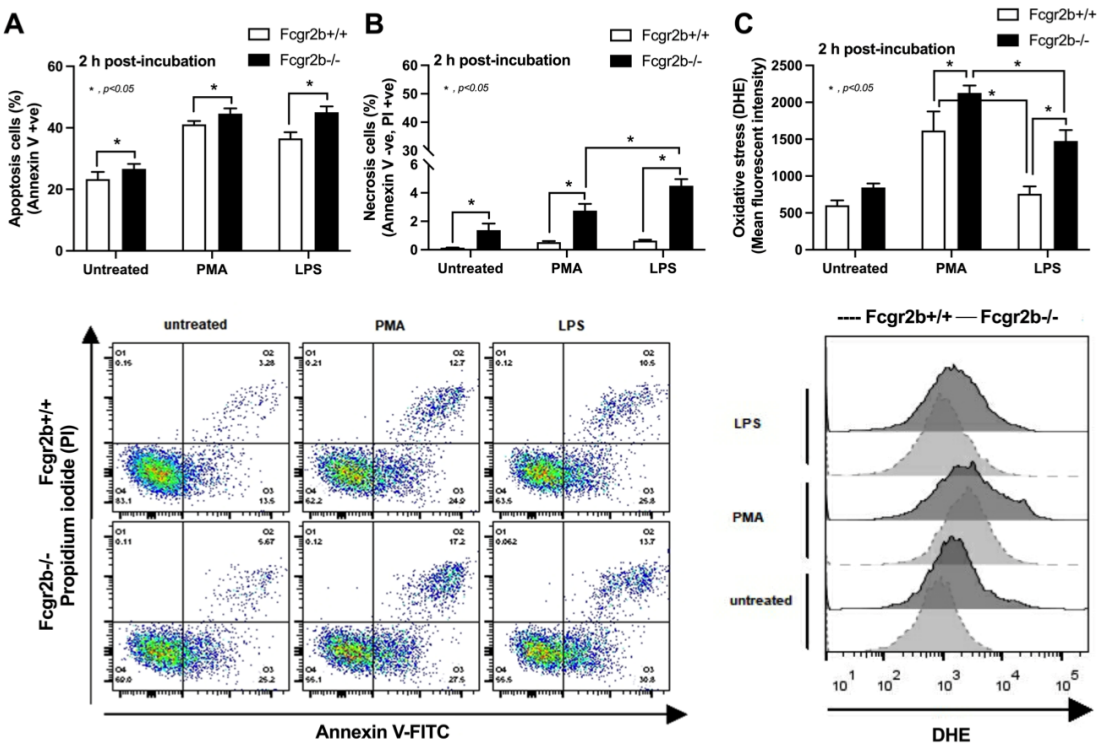


Figure 15 The validation of apoptosis (A), necrosis (B), and the production of reactive oxygen species (C) of PMA or LPS-activated neutrophils of lupus prone mice.

CHAPTER VI

DISCUSSION

Acute kidney injury or AKI is one of the important factors that decrease the function of kidney and lead to internal organ injury as well as stimulate the series of disease into chronic phase of injury. Several parameters in AKI, including the enhanced inflammatory cytokines, renal cell apoptosis or necrosis, and the increased responsibility of leukocytes facilitate chronic kidney disease (CKD). The deficient Fcgr2b (Fcgr2b-/-) mouse model was used as a representative of lupus, using the NETs stimulators (PMA and LPS). While PMA is a well-known direct NETs inducer, LPS is an indirect NETs stimulator with a potent inflammatory property.

The increase in BUN, Scr, and anti-dsDNA in 8-week-old Fcgr2b-/- mice, the asymptomatic lupus prone state without AKI and lupus characteristics, after I/R injury demonstrates that renal I/R could facilitate lupus disease activity. In addition, the systemic inflammatory including IL-1β, TNF-α, IL-6, and serum dsDNA were remained increased when compared with WT mice, indicating that the inflammatory processes after renal ischemia reperfusion in 8-week-old Fcgr2b-/- mice might be more severe than WT mice. Furthermore, Fcgr2b-/- and WT mice developed the characteristics of NETs on peripheral blood neutrophils and in various internal organs after 24 h of renal I/R injury. Despite the damage of kidneys and remote organs is similar in both mouse strains, Fcgr2b-/- mice produced more NETs in peripheral blood and internal organs, especially kidney and lung, compared to WT mice,

indicating that Fcgr2b-/- mice was more sensitive to the organ injury and led to propound NET formation within 24 h after renal I/R injury (137, 138). Consequently, these evidences demonstrated that the inflammatory hyper-responsiveness due to the loss of Fcgr2b receptor in asymptomatic lupus mice exacerbate lupus activity. Perhaps, the loss of inhibitory Fcgr2b-/- enhances activating FcgRs, which then cross-talks with TLR4 (139), an innate immune receptor that recognizes ischemia-induced damage associated molecular pattern (DAMPs), in Fcgr2b-/- mice results in the higher inflammation in Fcgr2b-/- mice than WT mice (140-142). On the contrary, the hepatic and cardiac damage caused by renal injury after 24 h I/R might not be linked to NETs formation because very less NETs were detected in these organs at 24 h after I/R.

The elevated anti-dsDNA induces immune complexes (ICs) that deposit in several organ, including glomeruli. On the other hand, the renal cell apoptosis at 120 h after I/R injury of Fcgr2b-/- mice (but not in WT), indicating that an impact of the loss of tolerance in lupus (23). Hence, the loss of inhibitory Fcgr2b with renal I/R facilitated NET formation (143) and induced cell apoptosis (144) through the possible enhanced presentation of self-antigens, auto-antibody production and IC deposition(145). The renal damage in lupus prone mice induced NETs at 24 h after I/R that causes accumulation of renal IC at 120 h after I/R injury without NETs (undetectable renal MPO and NE co-staining). The influences of acute kidney injury as a lupus aggravation factor were explained through I/R induced NETs formation at 24 h post-I/R and NETs enhanced self-antigen presentation, facilitated anti-dsDNA production and caused lupus at 120 h post-I/R. To support the increased anti-dsDNA production at 5 days after I/R injury, flow cytometry analysis of spleen were further analyzed. Accordingly, the results indicated that the inducible anti-dsDNA after 5

days of renal I/R injury was associated with the non-specific activation of autoreactive B cells post renal I/R that was prominent in Fcgr2b-/- mice. An immunoreceptor tyrosine-based activation motif (ITAM) and Fcgr2b receptors on B cells play a dominant role for negative feedback mechanism of activation, proliferation, antigen internalization, and antibody production, as well as induce cell apoptosis in the previous publication (10). Therefore, the depletion of Fcgr2b function extensively control antibody secretion and apoptosis, as well as led to antibody-driven diseases e.g., lupus-like diseases with more autoreactive germinal center B-cell clones (146). The possible processes that profound I/R-induced NETs and apoptosis in Fcgr2b-/- mice lead to an aggravation of lupus activity at 120 h are i) renal necrosis causes NETs (138), ii) prominent NETs promoting apoptosis, a well-known lupus b exacerbating factor (144), iii) both NETs and apoptosis increase an auto-antibody production by enhancing self-antigen presentation (147), and iv) inflammation exacerbates lupus activity (145). Nonetheless, increased self-antigen exposure via any cell death mechanisms may aggravate lupus disease activity.

PMA and LPS are generally associated to NET formation and release of ROS, which have been verified in Fcgr2b-/- neutrophil cells, and NETosis after PMA activation was more prominent that LPS which induced NETs through the pro-inflammatory cytokines. Similarly, proteomic analysis of NETs demonstrated that the different inducers stimulated NET formation with different mechanisms (148). In addition, ROS activity was correlated with apoptotic assay because ROS are generated via the NADPH oxidase, which is necessary for phosphatidylserine exposure during PMA and LPS-induced cell death (149, 150). As a result, ROS has several important roles in NETs, including cell signaling as well as regulation of cell

survival and death pathway. The disruption of these pathways was expected to have a significant clinical implication for oxidative stress-associated diseases such as cancer, ischemia-reperfusion injury, and diabetes (151).

This study presents the impact of AKI characteristics following I/R damage, the NET formation, and apoptosis in several organs between Fcgr2b deficient and wild type mice, as well as compares characteristics of Fcgr2b-/- and wild type neutrophils after stimulation with PMA and LPS. In summary, AKI increased lupus activity aggravation through renal I/R-induced significant NETs in kidney and led to inflammatory activation in several organs of Fcgr2b-/- mice. Due to the loss of inhibitory signaling of Fcgr2b receptor on neutrophils, NETs significantly enhanced dsDNA in Fcgr2b-/- mice with I/R injury and also induced several systemic inflammations, anti-dsDNA, as well as immune complex between dsDNA with anti-dsDNA in several organs. These occurrences suggesting that inhibition of NET formation might be beneficial and should be investigated in lupus patients with AKI, as well as exploration of a new strategy for treatment in lupus patients. In addition**,** this thesis is part of journal acute kidney injury induced lupus exacerbation through the enhanced neutrophil extracellular traps (and apoptosis) in Fcgr2b deficient lupus mice with renal ischemia reperfusion injury (152).

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