

รายงานการวิจัย

การศึกษาเชิงระบบของการส่งสัญญาณผิดปกติในเซลล์ของระบบภูมิคุ้มกันในหนู
โรคตุปัส

Systems-investigation of aberrant signaling in immune cells of SLE mouse
model

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Abstract

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โรคแพ้ภูมิตนเองเกิดขึ้นเมื่อเซลล์ภูมิคุ้มกันตอบสนองต่อแอนติเจนของตัวเองผิดปกติและนำไปสู่การอักเสบในเนื้อเยื่อ ปฏิสัมพันธ์ระหว่างพันธุกรรมและสิ่งแวดล้อมเป็นส่วนส่งเสริมให้อาการของโรคเป็นมากขึ้น สารไซโตไคน์ที่ทำให้เกิดโรคเอสแอลอีและโรครุมตอยด์ที่สำคัญคือ อินเตอร์เฟอรอนแอลฟา โรคเอสแอลอีเกิดจากภูมิคุ้มกันผิดปกติเรื้อรังซึ่งจะทำให้เกิดการสร้างแอนติบอดีต่อสารเนื้อเยื่อตนเองและทำให้เกิดไตอักเสบ การส่งสัญญาณผ่านเซ็นเซอร์กรดนิวคลีอิกสามารถกระตุ้นการผลิตอินเตอร์เฟอรอนแอลฟาจากเดนไดรติกเซลล์ และส่งเสริมความรุนแรงของโรคเอสแอลอี Stimulator of interferon genes (Sting) เป็นตัวรับดีเอ็นเอและส่งสัญญาณต่อเพื่อให้เกิดการสร้างอินเตอร์เฟอรอนแอลฟา ความผิดปกติของ Sting สามารถก่อให้เกิดโรคหลอดเลือดอักเสบในเด็กซึ่งมีอาการคล้ายโรคเอสแอลอี อย่างไรก็ตามการศึกษาการทำงานของ Sting ในสัตว์ทดลองของโรคแพ้ภูมิตนเองยังมีความขัดแย้งกันอยู่ เพื่อที่จะค้นหาว่า Sting มีส่วนร่วมในการเกิดโรคลูปัส, หนูลูปัส Fcgr2b-deficient จะถูกทำการผสมพันธุ์กับหนู Sting-deficient และหนูที่ได้จากการผสมนี้จะถูกติดตามอาการของโรคลูปัส จากผลการศึกษาพบว่า หนูลูปัส Fcgr2b-deficient สามารถมีชีวิตอยู่ได้นานขึ้น มีการสร้างแอนติบอดีต่อเนื้อเยื่อตนเองลดลง และ เกิดไตอักเสบลดลง ความรู้จากการศึกษานี้เป็นหลักฐานยืนยันแนวคิดสำหรับการวางแผนที่จะยับยั้ง Sting ในการรักษาโรคแพ้ภูมิตนเองในอนาคต

Autoimmune diseases occur when the immune cells react against self-antigens and subsequently lead to inflammation in the tissues. The interactions between genetics and environmental triggers regulate the phenotypes and outcome of the diseases. Type I interferon has been shown as one of the most crucial cytokines involving in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and

rheumatoid arthritis (RA). SLE is a chronic systemic autoimmune disease which can result in autoantibody production and fatal glomerulonephritis. Activation via nucleic acid sensors can induce the production of type I interferon from dendritic cells and promote SLE severity. Stimulator of interferon genes (Sting) is a cytoplasmic DNA sensor that signals downstream to enhance type I interferon production after its activation. Recently, it was shown that a gain mutation in the STING gene resulting in over-activity of the IFN pathway can cause familial inflammatory syndrome with lupus-like manifestations in humans. However, the functional studies of Sting in different autoimmune mouse models suggest the conflicting roles of Sting in the pathogenesis of autoimmune diseases. In order to determine if Sting participates in lupus pathogenesis, the Fcgr2b-deficient mice (lupus mouse model) were bred with Sting-deficient mice to create the double-deficient mice. In the absence of Sting, the Fcgr2b-deficient mice do not develop fatal glomerulonephritis and autoantibodies. The original knowledge from this study is a proof of concept for targeting Sting as a future promising treatment in autoimmune diseases.

Fc gamma receptor IIb deficient mice, a Systemic Lupus Erythematosus mouse model, susceptible to cecal ligation and puncture sepsis after preconditioned with double separated doses of endotoxin due to the prominent macrophage exhaustion

การติดเชื้อแทรกซ้อนและซ้ำซ้อนพบได้บ่อยในผู้ป่วย เอสแอลอี ซึ่งเป็นสาเหตุการตายที่สำคัญในผู้ป่วย ผู้วิจัยเลียนแบบภาวะการติดเชื้อซ้ำซ้อน โดยการฉีด endotoxin 2 ครั้ง ห่างกัน 5 วัน แล้วจึงทำการผ่าตัดเจาะและ ผูกลำไส้ใหญ่เพื่อกระตุ้นการติดเชื้อในกระแสเลือดที่ 24 ชั่วโมงถัดไป ในหนูทดลองที่ขาดตัวรับชนิด FcGR1Ib ซึ่งเป็น โมเดลของโรคเอสแอลอี โมเดลหนึ่ง พบว่าหนูทดลองกลุ่มเอสแอลอีเมื่อเทียบกับหนู wild type นั้น มีการ

ตอบสนองด้วยการสร้างไซโตไคน์หลังการฉีด endotoxin ครั้งแรกสูงมาก แต่ต่ำมากหลังการฉีดครั้งที่ 2 เข้าได้กับการเกิด immune-exhaustion ซึ่งพบว่าการกระตุ้นให้เกิดการติดเชื้อในกระแสเลือดหลังจากกระตุ้นด้วย endotoxin 2 ครั้งนั้น ส่งผลให้เกิดการติดเชื้อที่รุนแรงมากในหนูเอสแอลอี (พิจารณาจากค่าที่บ่งบอกการทำงานของตับและไตจากการเจาะเลือด จากระดับไซโตไคน์ จากจำนวนแบคทีเรียที่พบในอวัยวะต่างๆและอัตราการตาย) เนื่องจากแมคโครเฟส เป็นเซลล์ที่มีความสำคัญอย่างยิ่งในการต่อสู้กับการติดเชื้อ จึงได้ทำการทดลองในเซลล์ดังกล่าวพบว่า แมคโครเฟสของหนูเองแอลอีเมื่อเปรียบเทียบกับหนูปกติตอบสนองต่อ endotoxin ในครั้งแรก โดยการสร้างไซโตไคน์ในระดับที่สูงมาก แต่มีระดับต่ำมากคล้ายกับที่พบในสัตว์ทดลองหลังจากการกระตุ้นครั้งที่สอง เข้าได้กับภาวะ macrophage paralysis

ดังนั้น immune-exhaustion ในสัตว์ทดลองน่าจะเกิดจาก macrophage paralysis ผลการทดลองแสดงให้เห็นความสำคัญของการติดเชื้อซ้ำซ้อนในผู้ป่วยเอสแอลอี โดยเฉพาะอย่างยิ่งในผู้ที่มีความผิดปกติของยีน FcGR11b การค้นหาความผิดปกติของ FcGR11b ในผู้ป่วยโรคเอสแอลอีอาจจะมีบทบาทในการเฝ้าระวังและป้องกันการติดเชื้อในกระแสเลือดในผู้ป่วยเองแอลอีอันเป็นสาเหตุการตายที่สำคัญของโรค

Repeated bacterial infection in patients with Systemic Lupus Erythematosus (SLE) is common and sepsis is the leading causes of death. Despite proper responses to a single bacterial infection, the repeated infection might lead to immune exhaustion and severe sepsis. Then the bacterial susceptibility was tested with cecal ligation and puncture (CLP) after immune exhaustion induced by the 2-separated-doses of endotoxin (LPS) in FcGR11b^{-/-} mice and wild type (WT) control.

In the comparison with wild type group, the prominent serum cytokine after 1st LPS injection followed by the apparently lower cytokines after 2nd LPS administration, cytokine exhaustion, was demonstrated in FcGR11b^{-/-} mice. Subsequently, CLP was conducted after double doses of LPS preconditioning to test the

immune suppression. Indeed, a higher mortality rate and a more severe sepsis (bacterial burdens, serum cytokines and organs injury) at 18h of CLP demonstrated in FcGRIIb^{-/-} mice. Because macrophages are the major immune cells responsible for sepsis immune responses, we tested *in vitro*. Interestingly, the stimulation with separated 2 doses of LPS in bone marrow-derived macrophage from FcGRIIb^{-/-} mice showed the higher cytokines responses after the 1st LPS stimulation in comparison with WT cells but the cytokines level were lower than WT cells after the 2nd LPS stimulation, supplementary to the *in vivo* results.

In conclusion, macrophage exhaustion was easier inducible in FcGRIIb^{-/-} cells in parallel to the immune paralysis, highly susceptible to CLP, in FcGRIIb^{-/-} mice compare with wild type group. These implied the importance of the repeated infections in patients with SLE, especially with FcGRIIb polymorphisms.

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CHAPTER I

Project Outputs Summary

ARTICLE PUBLICATIONS

Original Articles

Markus M. Rinschen, Thomas Benzing, Kavee Limbutara and Trairak Pisitkun⁴ Proteomic analysis of the kidney filtration barrier—Problems and perspectives. *Proteomics Clin. Appl.* 2015, 9, 1053–1068

Review Article

CHAPTER II

Investigation of aberrant signaling in immune cells of SLE mouse model

“การศึกษาการควบคุมของวิถีสัญญาณอินเตอร์เฟียร์รอนผ่านเซ็นเซอร์ของไวรัสดีเอ็นเอในเซลล์ภูมิคุ้มกันของหนูโรคลูปัสด้วยวิธีโปรตีโอมิกส์”

Introduction

Fcgr2b เป็น Fc receptor ที่ทำหน้าที่ควบคุมและยับยั้งการทำงานของเซลล์ในร่างกายเพื่อป้องกันไม่ให้เซลล์ที่ถูกกระตุ้นนั้นตอบสนองต่อตัวกระตุ้นและทำงานเพิ่มขึ้นเพียงอย่างเดียวโดยที่ไม่สามารถกลับเข้าสู่ภาวะปกติได้เมื่อหมดตัวกระตุ้นแล้ว ดังนั้นในหนูที่ปราศจาก Fcgr2b (Fcgr2b^{-/-}) จึงมีการทำงานของเซลล์ที่มากผิดปกติโดยเฉพาะ B เซลล์และ macrophage เซลล์ ซึ่งเป็นผลทำให้เกิดโรค autoimmune ขึ้นได้ (1) ในหนูลูปัส Fcgr2b^{-/-} จะมีอาการแสดงของโรคใกล้เคียงกับผู้ป่วย SLE อย่างมาก (1) และในหนู Fcgr2b^{-/-} ที่มีอายุน้อยก็สามารถทำให้เกิดอาการเหมือนกับโรค Rheumatoid arthritis โดยการฉีด collagen (collagen-induced arthritis) นอกจากนี้ความผิดปกติของยีน Fcgr2b ก็สามารถพบได้ในผู้ป่วยชาวเอเชียรวมทั้งคนไทยที่เป็น SLE (2-4)* ดังนั้นโมเดลนี้จึงเป็นโมเดลที่เหมาะสมสำหรับใช้ในการศึกษากลไกการเกิดโรค autoimmune disease ทั้งโรค SLE และ Rheumatoid arthritis โดยที่ผู้วิจัยเองได้มีประสบการณ์ในการทำงานวิจัยเพื่อที่จะศึกษากลไกการเกิดโรคในหนู Fcgr2b^{-/-} มาแล้ว โดยพบว่า interleukin-17 signaling pathway มีความสำคัญต่อทั้งการเกิด lupus nephritis ในหนูลูปัส Fcgr2b^{-/-} (5) และต่อการเกิด rheumatoid arthritis ในโมเดล collagen-induced arthritis ในหนู Fcgr2b^{-/-} เช่นกัน (6) นอกจากนี้เรายังสามารถใช้หนูลูปัส Fcgr2b^{-/-} ในการวิจัยเพื่อศึกษากลไกการเกิดโรค SLE และนำความรู้ที่ได้นั้นมาประยุกต์ใช้ในการรักษาผู้ป่วยโรค SLE ต่อไป

การแสดงผลของ TLR9 และ TLR7 มีผลต่อการทำให้เกิดโรคภูมิต้านทานและการสร้าง autoantibody (7-9) นอกจากนี้ TLRs แล้ว, การเพิ่มมากขึ้นของ RNA-sensing โมเลกุลอย่างเช่น MDA5 ก็สามารถกระตุ้นให้หนูที่เป็นโรคภูมิต้านทานอยู่แล้วมีอาการที่รุนแรงขึ้นได้ (10) และความผิดปกติของ DNA metabolism ในหนูที่ขาดยีน Tlx-1 (11) และ DNase (12, 13) ก็ทำให้เกิดโรคภูมิต้านทานได้เหมือนกัน นอกจากนี้ในผู้ป่วย SLE ก็ตรวจพบว่ามี การสร้างแอนติบอดีต่อ DNA-sensing โมเลกุล IFI16, เชื้อไวรัส HHV8 และ CMV (14-16) อย่างไรก็ตามเป็นที่ทราบกันว่าอาการของโรค SLE จะเพิ่มความรุนแรงขึ้นอย่างมีนัยสำคัญในระหว่างการติดเชื้อจากไวรัสต่างๆ เช่น CMV และ EBV (14, 17) จะเห็นได้ว่าข้อมูลเหล่านี้บ่งชี้ให้เห็นถึงความเชื่อมโยงระหว่างการติดเชื้อไวรัส, เซ็นเซอร์ของกรดนิวคลีอิกในเซลล์และการเกิดโรคภูมิต้านทาน แต่ถึงกระนั้นปัจจุบันก็ยังไม่มีการศึกษาที่ทำให้ทราบถึงบทบาทและกลไกของ DNA-sensing โมเลกุลที่อยู่ใน cytoplasm ของเซลล์ว่ามีผลต่อการเกิดโรคภูมิต้านทานหรือไม่ และจนถึงปัจจุบันความเข้าใจในกลไกที่ทำให้เกิดปรากฏการณ์นี้ก็ยังไม่ทราบชัดเจน ซึ่งถ้าเราสามารถทำงานวิจัยที่นำมาซึ่งความเข้าใจในกลไกการเกิดโรคนี้ได้ ข้อมูลนี้อาจนำไปสู่ความรู้ขั้นสูงในการพัฒนาวัคซีนรักษาโรคในการรักษาผู้ป่วยโรคภูมิต้านทานในอนาคตโดยทั่วไป DNA-sensing โมเลกุลที่ถูกกระตุ้น โดย Interferon (IFNs) จะส่งสัญญาณผ่าน

ทางโปรตีน adaptor ที่เรียกว่า STING (Stimulator of interferon genes) (18-20) นอกจากนี้ STING ยังเป็นโมเลกุลที่จำเป็นสำหรับการรวมตัวของไวรัสและ membrane ของ host ซึ่งขบวนการนี้จะกระตุ้นให้เกิดการหลั่ง Interferon (21) ดังนั้น STING จึงเป็น โมเลกุลตัวแทนสำหรับการศึกษาถึงบทบาทของ DNA-sensing pathway ว่ามีผลต่อการเกิดโรคภูมิต้านทานหรือไม่

Materials and Methods

Animal and animal models

Fcgr2b^{-/-} mice on C57BL/6 background were provided by Bolland S. (NIH, Maryland, USA). Sting^{-/-} mice were provided by Paludan (Aarhus University, Aarhus, Denmark). Wild type mice were purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. The Fcgr2b^{-/-} mice were bred with Sting^{-/-} mice to create double deficient mice and their littermate controls. The animal protocols were approved by Faculty of Medicine, Chulalongkorn University followed the National Institutes of Health (NIH) criteria.

Survival study

The double deficient mice will be aged and observed the survival rates compared to their littermates. If the mice can survive up to 12 months, the mice will be euthanized to collect the tissues (kidney, spleen, bone marrow, and sera) for further analysis.

Flow cytometry analysis

The collected spleens were harvested and incubated with collagenase D at 37 c for 30 minutes to isolate splenocyte. The splenocytes were stained with flow antibody. The flow cytometry analysis was performed using BD LSR-II and FlowJo software. The dead cells were excluded from the analysis.

Autoantibody testing

The collected sera of the mice at the age of 6 months were tested for anti-nuclear antibody (Hep-2 cells; immunofluorescence) and anti-dsDNA (ELISA). The sera were diluted into different dilution factors.

Histopathology

The kidneys were fixed in 4%paraformaldehyde and subsequently were stained with H&E and PAS.

The pathology grading will be blinded analysis by the experience nephrologist.

Real-time PCR

The RNA from kidneys was isolated using Trizol. The DNase-treated RNA was purified using RNeasy isolation kit. The conversion of RNA to cDNA using iScript RT Supermix (Biorad) was performed.

The gene expression profiles were tested using SsoAdvanced Universal SYBR Green Supermix. The sequences of primers tested are followed:

Statistical analysis

Significant difference of survival rates is tested by Log-rank test. The comparison between groups is examined by T-test.

Results

Increase of survival rates of the Fcgr2b-deficient mice in the absence of Sting

The Fcgr2b-deficient mice start to die at the age of 6 months and the survival rates drop to 22.2% by 12 month old while the survival rates of double deficient mice are 77.7% ($p < 0.001$). The effect of one allele of Sting to survival rates of Fcgr2b-deficient mice does not show significant difference ($p = 0.6$) (Figure 1).

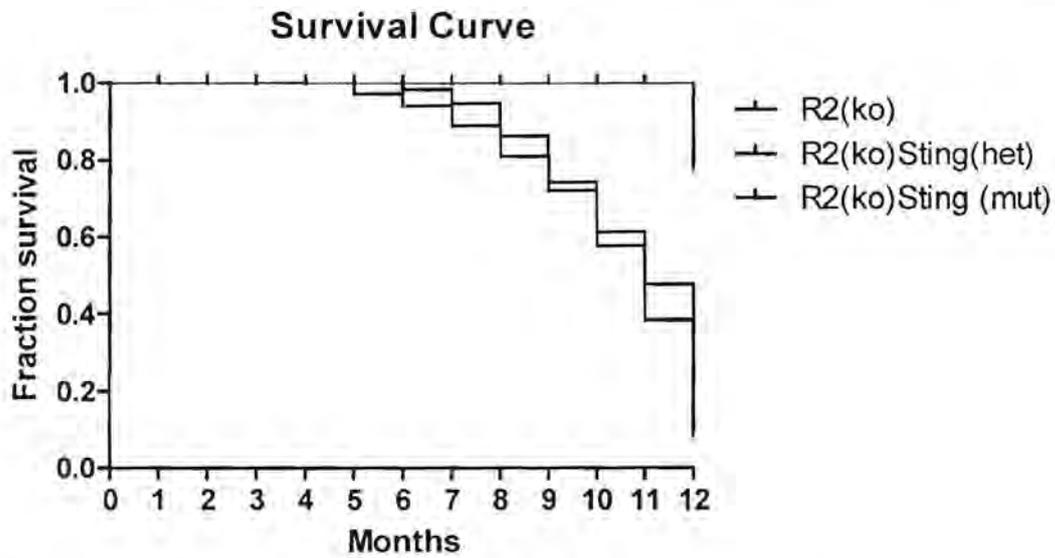


Figure 1 Increase of survival rates of the Fcgr2b-deficient mice in the absence of Sting

The survival of the mice was observed until 12 month old. The absence of Sting increase survival of Fcgr2b^{-/-} mice ($p < 0.001$, N=9 per group).

Sting deficiency diminishes fatal glomerulonephritis in the Fcgr2b-deficient mice

The kidney staining of Fcgr2b-deficient mice shows inflammatory cell infiltrations, enlarged glomeruli and crescentic glomeruli. In the absence of Sting, the Fcgr2b-deficient mice do not develop glomerulonephritis (Figure 2).

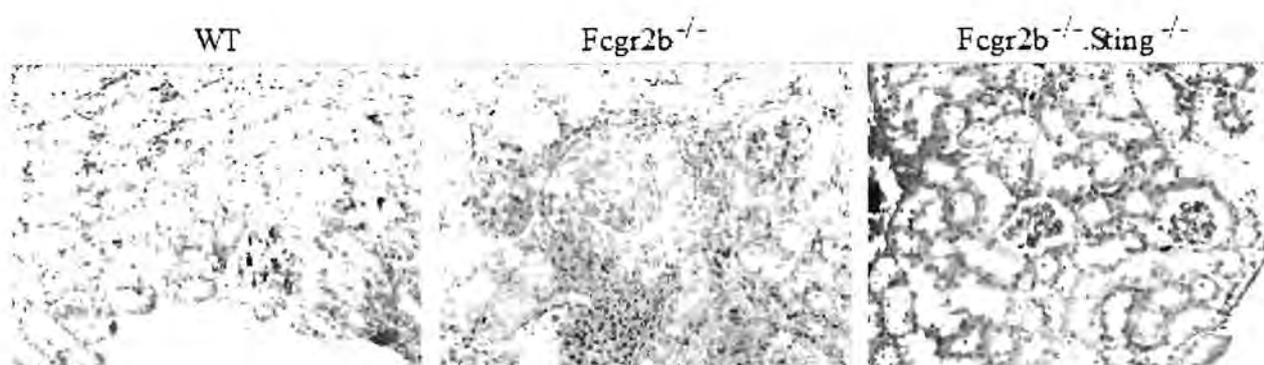


Figure 2 Sting deficiency diminishes fatal glomerulonephritis in the Fcgr2b-deficient mice

Decrease autoantibody production in the double deficient mice

Antinuclear antibody (ANA) and anti-dsDNA production decreases in the Sting and Fcgr2b double deficient mice (Figure 3A, 3B).

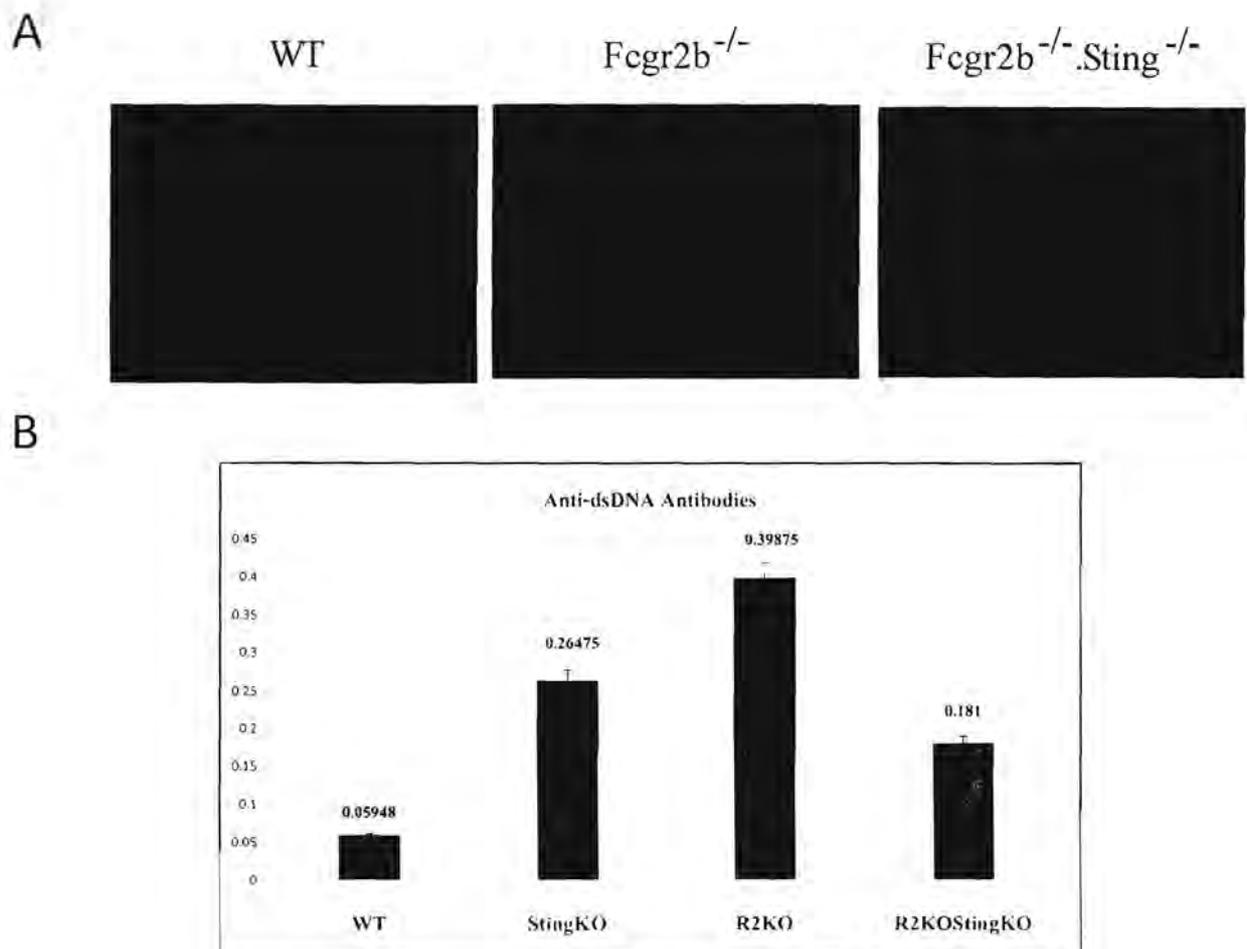


Figure 3 Decrease autoantibody production in the double deficient mice

The sera of the mice were collected at the age of 6 months to test for antinuclear antibody (Figure 3A, representative of mice (N=3/group) and anti-dsDNA (Figure 3B, N=6/group, $p < 0.01$)

Decrease CXCL10 expression in the kidneys of the double deficient mice

Sting-mediated signaling induces type I interferon production and leads to the increase of interferon inducible gene expression. We determined whether the interferon signature genes in the kidneys will diminish in the Fcgr2b-double deficient mice. Surprisingly, only CXCL10 expression was decreased in the double-deficient mice compared to Fcgr2b-deficient mice, but the MX1, ISG15 and IFN α expression were increased (Figure 4).

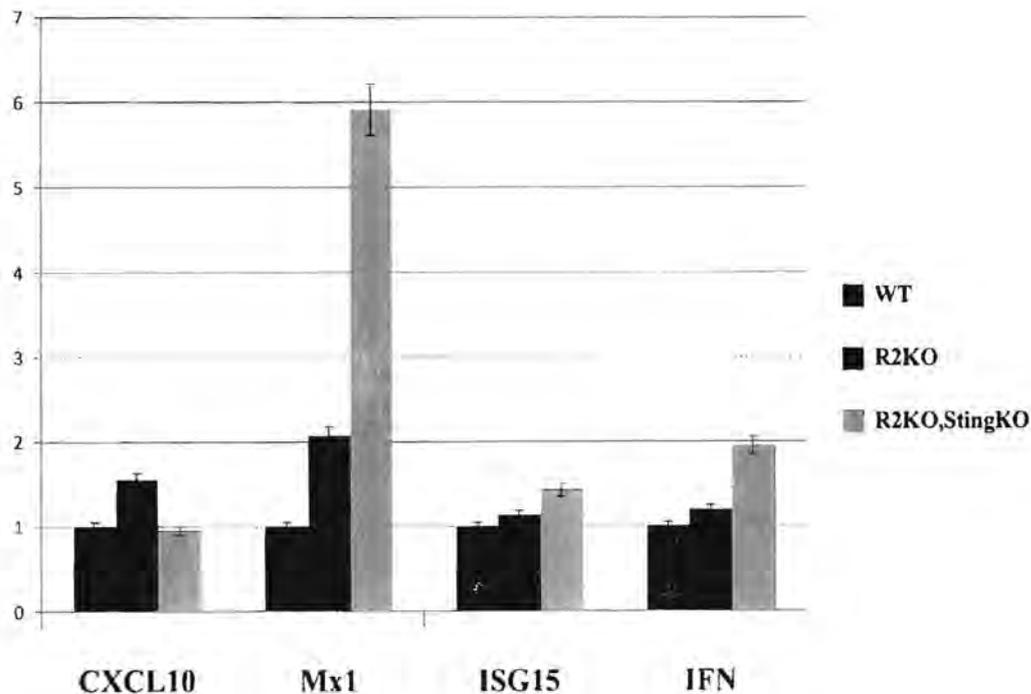


Figure 4 Decrease CXCL10 expressions in the kidneys of the double deficient mice

Interferon inducible gene expression was tested from the kidneys of wild type, *Fcgr2b*^{-/-}, and *Fcgr2b*^{-/-}*Sting*^{-/-} mice. Only CXCL10 mRNA expression was reduced in the group of double-deficient mice compared to *Fcgr2b*-deficient mice (N=5/group, p<0.01).

Discussion

The data from this study confirm the hypothesis that *Sting*-mediated signaling participates in the pathogenesis of the *Fcgr2b*-deficient mice. The blocking *Sting* pathway may alleviate lupus phenotypes and probably be worth to develop therapeutic drug target. Unexpectedly, most of the interferon-inducible gene expressions do not decrease, except CXCL10. Contrary, the increase expression of *Mx1* and *IFN α* suggests the interferon-independent mechanisms of *Sting* signaling in lupus development of *Fcgr2b*-deficient mice. This intriguing effect of *Sting* signaling is worth to explore in details to better understand the immunological mechanisms of *Sting* in autoimmune development and other inflammatory diseases.

สรุปและเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

In order to understand the immunological importance of Sting in lupus Fcgr2b-deficient mice, the flow cytometry will be analyzed from the splenocytes of affected mice and their controls. Furthermore, the in vitro culture of bone-marrow derived dendritic cells with Sting ligands will be performed and tested for the differentiation, activation stage and cytokine production. In addition, the proteomic analysis of kidney tissue from these mice will be performed to discover the new unbiased Sting-signaling pathway.

The translational knowledge from this study will lead to design therapeutic drug for SLE patients.

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Appendix

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7.3 งานวิจัยที่กำลังทำ : ชื่อข้อเสนอการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยแล้วแล้วประมาณร้อยละเท่าใด

ชื่อข้อเสนอการวิจัย Systems biology of aberrant targeting and polarization of membrane proteins in disease

แหล่งทุน Lundbeck Foundation (2012): total award 500,000 Danish krone

การวิจัยแล้วประมาณร้อยละ 25

2. ผู้ร่วมโครงการวิจัย

1. ชื่อ - นามสกุล (ภาษาไทย) ประภาพร พิสิษฐ์กุล
ชื่อ - นามสกุล (ภาษาอังกฤษ) Prapaporn Pisitkun
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ตำแหน่งปัจจุบัน อาจารย์
เวลาที่ใช้ทำวิจัย 40 (ชั่วโมง : สัปดาห์)

3. ตำแหน่ง อาจารย์

4. ภาควิชาภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

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6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

อิมมูโนวิทยา โรคอโตอิมมูน การวิจัยในสัตว์ทดลอง

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น

7.1 ผู้อำนวยการแผนงานวิจัย :-

7.2 หัวหน้าโครงการวิจัย:-

7.3 ผู้ร่วมงานวิจัย:-

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CHAPTER III

Fc gamma receptor IIb deficient mice, a Systemic Lupus Erythematosus mouse model, susceptible to cecal ligation and puncture sepsis after preconditioned with double separated doses of endotoxin due to the prominent macrophage

“หนูทดลองโมเดลโรคเอสแอลอีโดยการขาดตัวรับ FcGRIIb เกิดความรุนแรงของการติดเชื้อในกระแสเลือดด้วยโมเดลการผูกและเจาะลำไส้ในหนูทดลองสูงขึ้นเนื่องจากภาวะ Macrophage exhaustion”

Introduction

Systemic Lupus Erythematosus (SLE), the autoimmune disease with multi-factorial pathogenesis (1, 2) leads to multi-organs injury, showed a higher prevalence in Asia in comparison with other regions of the world (3-5). The defect of Fc gamma receptor IIb (FcGRIIb), the only inhibitory signaling receptors in the FcGR family, is one of the genetic susceptibility to SLE (2, 6). Interestingly, FcGRIIb polymorphisms also demonstrated the high prevalence in Asia which might due to the protective effect of the gene for malarial infection (7). Coincidentally, the association with FcGRIIb polymorphisms in patients with SLE in Asia Pacific region is also common (3-5, 8). Perhaps FcGRIIb polymorphisms could protect malaria in this region but, on other side of the coin, people with this immunological defect might easier develop SLE. In any case, sepsis, the systemic immune responses to the severe infection, is one of the important causes of death in patients with SLE (9). Indeed, the high susceptibility to bacterial sepsis in patients with SLE is well-known (9-13). However, there are debates whether the susceptibility to infection in patients with SLE is due to the *de*

novo defects of immune response or immunosuppressive drugs. Unfortunately, the data on untreated symptomatic patients with SLE is very limited (14). Hence, the studies of infection in FcGRIIb^{-/-} mice, one of the established SLE mouse model (6), could be resemble to untreated patients with SLE, especially with FcGRIIb polymorphisms.

FcGRs binds with Fc portion of immunoglobulin mediate antigen uptake and cellular responses (15). In the mouse, FcGRs are classified into three activation receptors (FcGRI, FcGRIII, FcGRIV) and only one inhibitory receptor (FcGRIIb) (16). The deficiency of all classes of FcGR in mice (FcGR^{-/-}) protected from sepsis (17) and FcGRIIb^{-/-} mice response well to the gram positive bacterial infection due to the effective bacterial killing (18). Nevertheless, a more severe sepsis with the cytokines storm demonstrated in FcGRIIb^{-/-} mice with the bacterial antigen preconditioning before bacterial administration. This data supported the overshoot cytokines responses after the repeated antigen exposures due to the lack of inhibitory signaling in these mice (18). On the other hand, FcGRIIb^{-/-} mice are protected from Plasmodium and Mycobacterium infection (7, 19), the inhibitory signaling defect seems to enhance the activating signaling and show a benefit in these infections. However, the susceptibility of FcGRIIb^{-/-} mice to polymicrobial sepsis is never tested.

Interestingly, sepsis-induced immune exhaustion or immunoparalysis, the high susceptibility to secondary infection after sepsis, has been recognized as an important sepsis complication (20, 21) and was demonstrated by several mouse and human models (22-26). In contrast, the preconditioning of LPS, a single or multiple doses, for 24h before CLP ameliorates sepsis severity in wild type mice has been showed in previous publications (27, 28). Despite the demonstrated protective effect to sepsis after 1 day of LPS preconditioning, we hypothesized that the immune exhaustion after LPS administration might existed in the

earlier period and the repeated endotoxin exposure might mimic the repeated infection in patients. Subsequently, we selected the preconditioning with double doses of LPS with 5 days separation followed by CLP at 12h after the 2nd dose of LPS to demonstrate the immune exhaustion in our models. Of note, half-life of the important LPS-induced cytokines (TNF- α , IL-6 and IL-10) is approximately 0.5-1.5h (29), then at 12h, approximately 8 times of the half-life, should be adequate for avoiding the effect of these cytokines to the subsequent CLP surgery.

On the other hand, the lower macrophage immune responses, especially cytokines production, after repeated LPS activation is demonstrated, and was known by several terms such as “macrophage paralysis” or “macrophage exhaustion” or “macrophage tolerance” or “endotoxin tolerance” (30-32). Although, the association between immunoparalysis and macrophage exhaustion is not clearly demonstrated (31), it is possible that macrophage exhaustion might cause ineffective organisms clearance and increase infection susceptibility compatible with the definition of “immunoparalysis”.

Indeed, macrophage contains both activating and inhibitory FcGRs which competing for immune complex ligands and the direction of this balance determines the direction of the cell responses (16, 33). We hypothesize that the defect in the inhibitory signaling of FcGRIIb^{-/-} mice might result in a prominent response but is easier exhausted after repeated stimulation. Then we test immunoparalysis of FcGRIIb^{-/-} mice *in vivo* and macrophage exhaustion *in vitro*, respectively.

Materials and Methods

Animal and animal models

FcGR1b^{-/-} mice on C57BL/6 background were provided by Bolland S. (NIH, Maryland, USA). Other mice were purchased from the National Laboratory Animal Center, Nakompathom, Thailand. Female, 8- and 24-week-old C57BL/6 mice were used in the experiments. The animal protocols were approved by Faculty of Medicine, Chulalongkorn University followed the National Institutes of Health (NIH) criteria.

Cecal ligation and puncture model

Polymicrobial sepsis was induced by Cecal ligation and puncture model (CLP) slightly modified from the previous publication (29). Briefly, cecum were ligated at 10 mm from cecal tip with silk 2-0, punctured twice with a 21-gauge needle then gently squeezed to expel a small amount of fecal materials through an abdominal incision under isoflurane anesthesia. The incisions were closed with 2 layers by nylon 4-0 and normal saline (NSS) at 2 ml/kg was administered subcutaneously for the fluid replacement.

Cecal ligation and puncture with endotoxin pre-conditioning model

Because LPS induced-immuno-suppression is demonstrated (34, 35) and used as a sepsis-induced immunoparalysis model (26), we follow the principle in our experiments. In our model, the immunoparalysis, a condition susceptible to an infection, was tested by the severity of polymicrobial infection from CLP surgery. Endotoxin (LPS) of *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis, USA) was administered intraperitoneally at 5 days (-120h) and 12h (-12h) before CLP surgery at the dose of 0.8 g/kg (approximately 20 µg per 25 g mouse) and 4 g/kg (approximately 100 µg per 25 g mouse), respectively. Subsequently, CLP was performed as previously mentioned.

To measure inflammatory cytokines after LPS injection, 50 μ l of blood was collected through tail vein nicking at 0h (2h before LPS administration) and at 1, 3 and 6h after. In addition, in separated experiments, blood from tail vein nicking was also collected before CLP (0h) and at 3h and 6h to measure time-courses of bacterial burdens and serum cytokines after CLP surgery. Otherwise, blood was collected through cardiac puncture at sacrifice time under isoflurane anesthesia at 18h or 96h after CLP for sepsis injury analysis or survival test, respectively.

Blood chemistry, supernatant media analysis and urine protein

For the natural history of FcGR11b^{-/-} mice, serum from tail vein nicking and spot urine was collected once a month from 2 to 12-month-old. Serum and urine creatinine were measured by (QuantiChrom Creatinine Assay, DICT-500, BioAssay, CA, USA). Spot urine protein was measured by Bradford protein assay. Urine protein creatinine index (UPCI), a representative of 24h urine protein, were measured from spot urine by equation; urine protein/urine creatinine.

Serum cytokines after LPS were measured by Luminex-based multiplex technology multi-analysis panels 8-plex cytokines assay (BioPlex, Bio-RAD, CA, USA) to explore the panel of pro and anti-inflammatory cytokines (TNF- α , IL-6, IL-1 β , IFN- γ and IL-2, IL-4, IL-5, IL-10, respectively) according to the manufacturer's protocol. Then the selected important cytokines (TNF- α , IL-6, IL-10) were measured by ELISA assay (ReproTech, NJ, USA) in supernatant media and in mouse serum after CLP surgery. Organs injury was determined by blood urea nitrogen (QuantiChrom Urea Assay, DIUR-500, BioAssay), serum creatinine (Scr) (QuantiChrom Creatinine Assay, DICT-500, BioAssay), alanine transaminase (ALT) (EnzyChrom ALT assay, EALT-100, BioAssay) and lactate dehydrogenase (LDH) (EnzyChrom LDH assay,

EDLC-100, BioAssay). Blood bacterial burdens were determined by plating a serial volume of blood into blood agar (Oxoid, Hampshire, UK) at 37°C then counted bacterial colonies after 24h of incubation. For blood polymorphonuclear cell (PMN) and mononuclear cell count, 5 μ l of blood mixed in 85 μ l of 3% acetic acid for the hemolytic reaction and total leukocytes was counted by a hemocytometer. In parallel, blood smeared on a glass slide was stained by Wright stain and counted with $\times 100$ magnification in 100 fields to determine the percentage of PMN and mononuclear cells. The total number of cells was calculated by total leukocyte count from hemocytometer multiplied by the percentage of cells from the Wright stain glass slide.

Anti-dsDNA antibodies

Anti-dsDNA antibodies were measured by coating ELISA plates with salmon sperm DNA as published previously (36). In short, salmon sperm DNA (Life Technologies, Invitrogen™, MA, USA) passage through a 45-mm filter (Minisart, Sartorius, Germany) for selecting double stranded DNA then coated into ELISA plate with the dose at 100 μ g/plate. The plates were dry, blocked and incubated with serial dilutions of serum for 1 hour at 37°C then peroxidase conjugated Fab'2 goat anti-mouse IgG 1/2,000 in 1% bovine serum albumin (BSA) in phosphate buffer solution (PBS) followed by TMB peroxidase substrate (Biolegend, California, USA). The plate was developed in the dark room for 10 min then added TMB stop solution and read with microplate photometers with a wavelength at 410 nm.

Bone Marrow Derived Macrophages

Macrophages were derived from bone marrows (BM) follow the established procedure (37). In short, BM cells from FcGR1b^{-/-} and wild type mice obtained from femurs were centrifuged at 1,000 rpm in 4 °C for 10 min. Then cell were incubated in high glucose DMEM supplement with 10% fetal bovine serum (FBS), 1%

penicillin/streptomycin, HEPES with sodium pyruvate and 20% L929-conditioned media in a humidified 5% CO₂ incubator at 37 °C for 7 days. The cells were harvested at the end of the culture period using very cold PBS and confirmed macrophage phenotype with anti-F4/80 and anti-CD11c antibodies (BioLegend, CA, USA).

Macrophage endotoxin tolerance protocol

Macrophage endotoxin tolerance protocol followed the protocols from the previous publications (38, 39). Briefly, endotoxin (LPS) *Escherichia coli* 026:B6 (Sigma) at 10 or 100 ng/ml was used to activate macrophage 1×10^5 cells/well in 96 well polystyrene tissue culture plate. To see the difference between single or double LPS stimulations, 2 groups of experiments were performed. For the single LPS stimulation (N/LPS), there was no endotoxin at the 1st 24h of the incubation then the plate was washed with phosphate buffer solution (PBS), refilled fresh media and treated with LPS at 10 ng/ml (N/LPS10) or 100 ng/ml (N/LPS100). For the double LPS stimulation (LPS/LPS), LPS at 10 or 100 ng/ml was treated for the 1st 24h and treated with the 2nd dose of LPS at 10 ng/ml or 100ng/ml as indicated. The culture supernatant was collected at 1, 2, 4, 6 and 24h after the 2nd LPS incubation in all groups and stored at -80 °C until cytokine determination by ELISA assays (ReproTech). After the incubation, cell viability was measured by MTS assay (One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) according to the manufacturer's instruction (40). In short, 20 μ l of MTS was added to the culture plates for 2h at 37°C in 5% CO₂ incubator then read with microplate photometers with a wavelength at 450 nm. All *in vitro* experiments demonstrated cell viability more than 95% (data not showed).

Macrophage intracellular killing activity and phagocytosis protocol

The protocol followed the previous publication (41). BM derived macrophage at 1×10^5 cells in 200 μ l of DMEM per well were dispensed into the flat bottom 96-well plate and incubated at 37 °C in a humidified 5% (v/v) CO₂ incubator for 24 h, before gently washing with culture media to remove non-adherent cells. Subsequently, the cells with endotoxin (LPS) *Escherichia coli* 026:B6 (Sigma) at 10 or 1,000 ng/ml, LPS10 or LPS1000, respectively, and incubated with 1×10^7 CFU of *E. Coli* per well.

Then after 15 min of incubation, supernatants were aspirated and cells were washed gently with DMEM to remove un-ingested microorganisms. The supernatant and well washing fluids, containing the non-phagocytized *E. Coli*, were combined, plated in serial dilutions on Tryptic soy agar plates and counted for bacterial colonies for the representative of the non-phagocytic bacteria which reversed correlated with the phagocytic activity. On the other hand, the cellular part, phagocytosed macrophage, was further incubated with 200 μ l of DMEM for 2h to determine intracellular bacterial killing activity. The wells were gently scraped and washed with 200 μ l distilled H₂O to induce cell lysis and the serial dilution of the lysate were plated on Tryptic soy agar, incubated at 37 °C for 16 h and determine the bacterial colony count. The number of bacteria from the cell lysate represented the intracellular killing activity.

Statistical analysis

Data are shown as the mean \pm SE and differences between groups were examined for statistical significance using the unpaired Student t-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with 2 and 3 groups, respectively. Survival analyses were evaluated using the log-rank test by observation and recorded every 6–24h then all mice were sacrifice at 96h

after CLP. *P* values < 0.05 were considered statistically significant. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

Fc gamma receptor IIb deficient mice susceptible to cecal ligation and puncture sepsis in symptomatic SLE mice and asymptomatic SLE group preconditioned with LPS

At 24-weeks-old, FcGRIIb^{-/-} mice showed increased anti-dsDNA with proteinuria but normal kidney function as evaluated by serum creatinine (Scr) (Fig 1). This natural history allows the experiments in 2 groups of mice in correspondence with patients with SLE; asymptomatic genetic prone group (8-weeks-old) and symptomatic proteinuria group (24-weeks-old). To see the susceptibility to bacterial sepsis without LPS preconditioning in these 2 groups, CLP surgery was performed in comparison with age-matched wild type control. In the absence of LPS, only symptomatic, but not asymptomatic, FcGRIIb^{-/-} mice showed higher sepsis mortality rate compare with wild type (Fig 2A, B) supports the correlation between SLE disease activity and infection susceptibility (9).

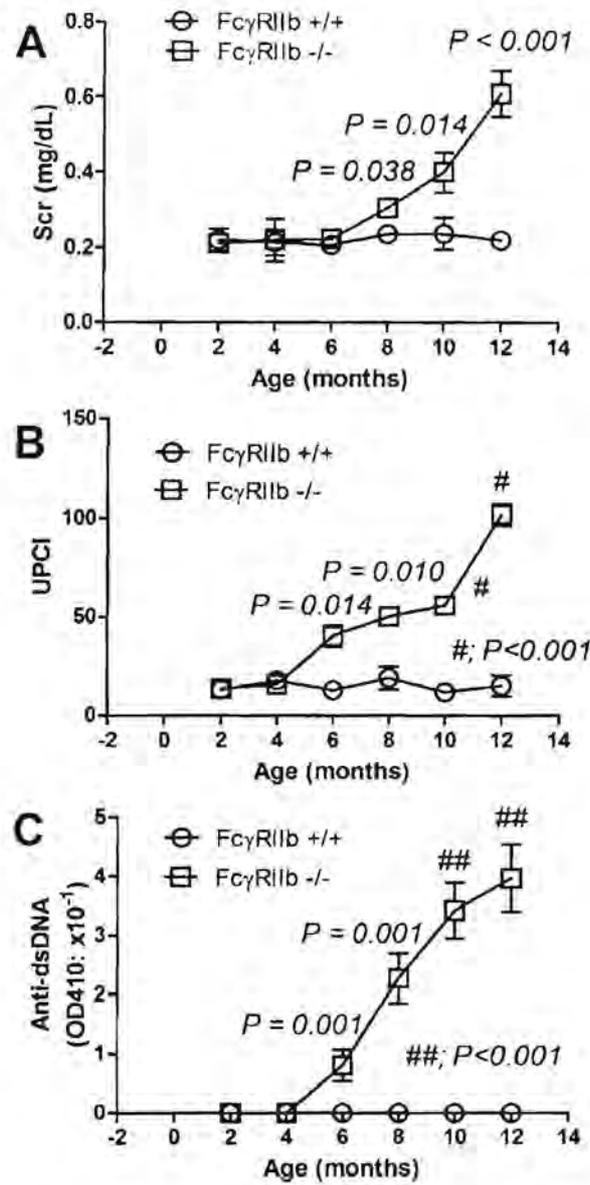


Figure 1. The natural history of Fc γ RIIb $^{-/-}$ and wild type (Fc γ RIIb $^{+/+}$) mice as determined by serum creatinine (Scr), urine protein creatinine index (UPCI) and anti-dsDNA ($n=4-5$ /groups).

Then we test the effect of LPS preconditioning in asymptomatic mice and determine the severity of immunoparalysis by the mortality rate of CLP surgery (20, 21).

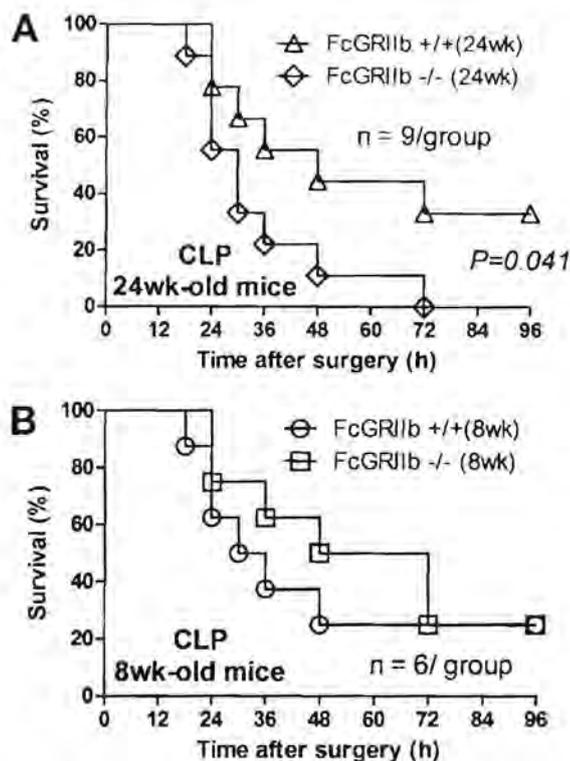


Figure 2. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery in asymptomatic *FcGRIIb*^{-/-} mice (8-wk-old) (A) and symptomatic, proteinuria positive but normal serum creatinine, *FcGRIIb*^{-/-} mice (24-wk-old) (B) in comparison with age-matched wild type mice (*FcGRIIb*^{+/+}).

Although the high CLP mortality rate found in both wild type and *FcGRIIb*^{-/-} mice after LPS-preconditioning, *FcGRIIb*^{-/-} showed the higher mortality rate. Whereas all wild type and *FcGRIIb*^{-/-} mice die within 72h and 36h, respectively, in CLP with LPS, the survival rate at 30% and 22% found in wild type and *FcGRIIb*^{-/-} mice, respectively, in CLP alone (Fig 3A, B). These results supported the immunoparalysis occur in both wild type and *FcGRIIb*^{-/-} mice after LPS preconditioning but *FcGRIIb*^{-/-} mice showed the more severe immunoparalysis (Fig 3C).

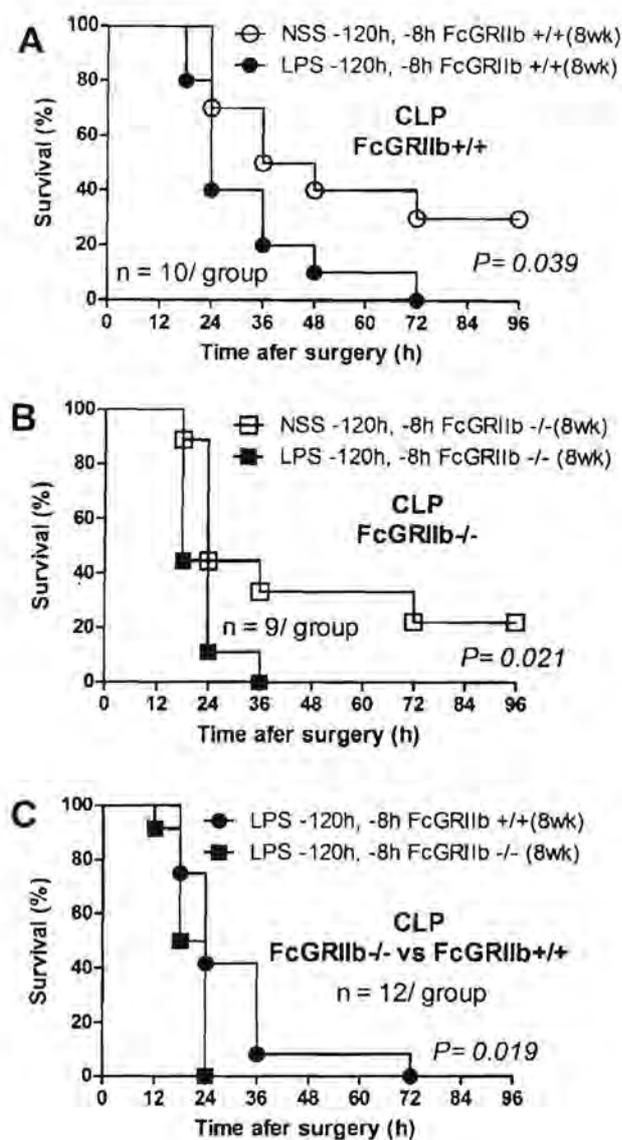


Figure 3. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery preconditioning with 2 separated doses of LPS at 120h and 12h prior to CLP. CLP in endotoxin preconditioning model, in comparison with normal saline (NSS) placebo injection within wild type mice ($Fc\gamma RIIB^{+/+}$) (A) and $Fc\gamma RIIB^{-/-}$ group (B) were showed. Survival analysis of CLP in endotoxin induced immunoparalysis model between wild type ($Fc\gamma RIIB^{+/+}$) and $Fc\gamma RIIB^{-/-}$ mice (C) was also demonstrated.

The cytokine responses after LPS injections and after superimposed by cecal ligation and puncture

The luminex-based multiplex system was used to explore the difference in the cytokines responses after LPS administration between asymptomatic FcGRIIb^{-/-} mice versus wild type. Among the pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β and IFN- γ), we found that most of the pro-inflammatory cytokines, except for TNF- α , were significantly higher in FcGRIIb^{-/-} mice at 1h after the 1st dose of LPS administration (Fig 4A-C). TNF- α , IL-6, IL-1 β and IFN- γ at 1h after LPS administration in FcGRIIb^{-/-} mice and wild type were 21,851 \pm 3,200, 3,525 \pm 117, 350 \pm 38, 21 \pm 2 pg/ml and 12,453 \pm 3,925, 2,301 \pm 157, 54 \pm 11, 3 \pm 1 pg/ml, respectively. In parallel, for the anti-inflammatory cytokines (IL-2, IL-4, IL-5 and IL-10), all of these cytokines, except for IL-5, were higher in FcGRIIb^{-/-} mice at the 1st h after the 1st dose of LPS (Fig 4D-F). In detail, IL-2, IL-4, IL-5 and IL-10 at 1h after LPS administration in FcGRIIb^{-/-} mice and wild type were 26.2 \pm 3.4, 10.8 \pm 1.1, 34.5 \pm 3.5, 958 \pm 106 pg/ml and 12.6 \pm 3.3, 3 \pm 0.4, 27.1 \pm 3.6, 575 \pm 104 pg/ml, respectively. These results supported the prominent cytokines responses in FcGRIIb^{-/-} mice reported previously (18). Interestingly, at 1h and/or 2h of the 2nd LPS administration with the 5 times higher dose of LPS, all of these cytokines, except for IL-1 β and IL-5, were significantly lower than the 1st administration in FcGRIIb^{-/-} mice (Fig 4). In contrast, in wild type mice, only IFN- γ , IL-2, IL-4 and IL-10 were lower and IL-1 β was higher in some time-point of 2nd LPS administration compared with the matched time-point of the 1st LPS injection (Fig 4). The endotoxin tolerance, determined by the lower cytokines responses after 2nd dose of LPS, was easier demonstrated in FcGRIIb^{-/-} mice compare with wild type. Moreover, the severity of endotoxin tolerance, determined by the cytokine level difference after matched time-point of 1st and 2nd LPS administration, was higher in FcGRIIb^{-/-} mice (Fig 4 inset graph).

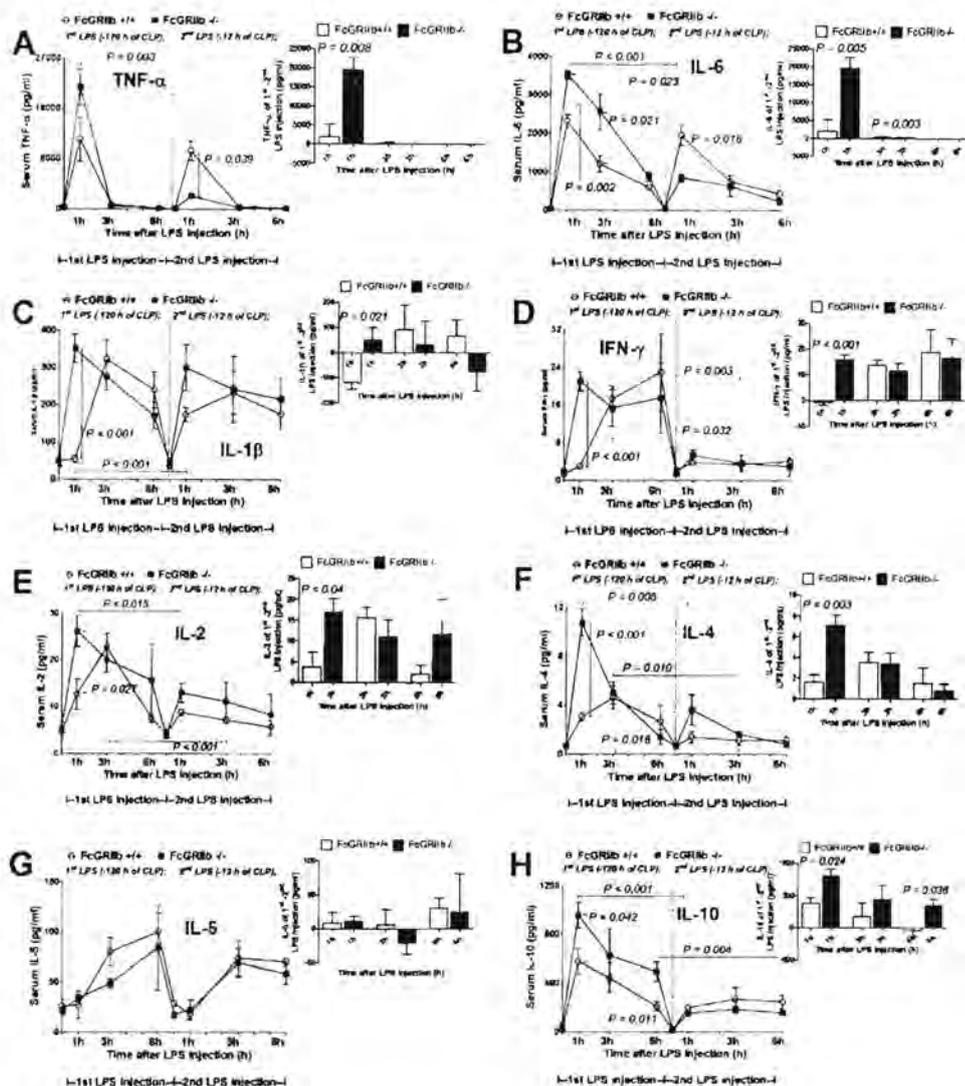


Figure 4. Serum cytokines in wild type (*FcGRIIb*^{+/+}) or *FcGRIIb*^{-/-} mice after at 1, 3 and 6h after 1st LPS injection (0.8 g/kg) and 2nd LPS injection (4 g/kg) as measured by TNF- α (A), IL-6 (B), IL-1 β (C), IFN- γ (D), IL-2 (E), IL-4 (F), IL-5 (G) and IL-10 (H) was demonstrated. To emphasize the difference of serum cytokines after 1st and 2nd doses of LPS, the delta change of serum cytokine response at the matched-time=points after both LPS injection was showed as inset graph. (n= 5-7 per group)

It is interesting to note that, even with the 5 times higher LPS dose of 2nd LPS administration, most cytokines level was lower than the 1st dose implied endotoxin tolerance status in both FcGRIIb^{-/-} and wild type mice. Subsequently, we tested the severity of polymicrobial infection in these mice with CLP surgery and selected to explore only frequently mentioned sepsis cytokines (TNF- α , IL-6 and IL-10) *in vivo*.

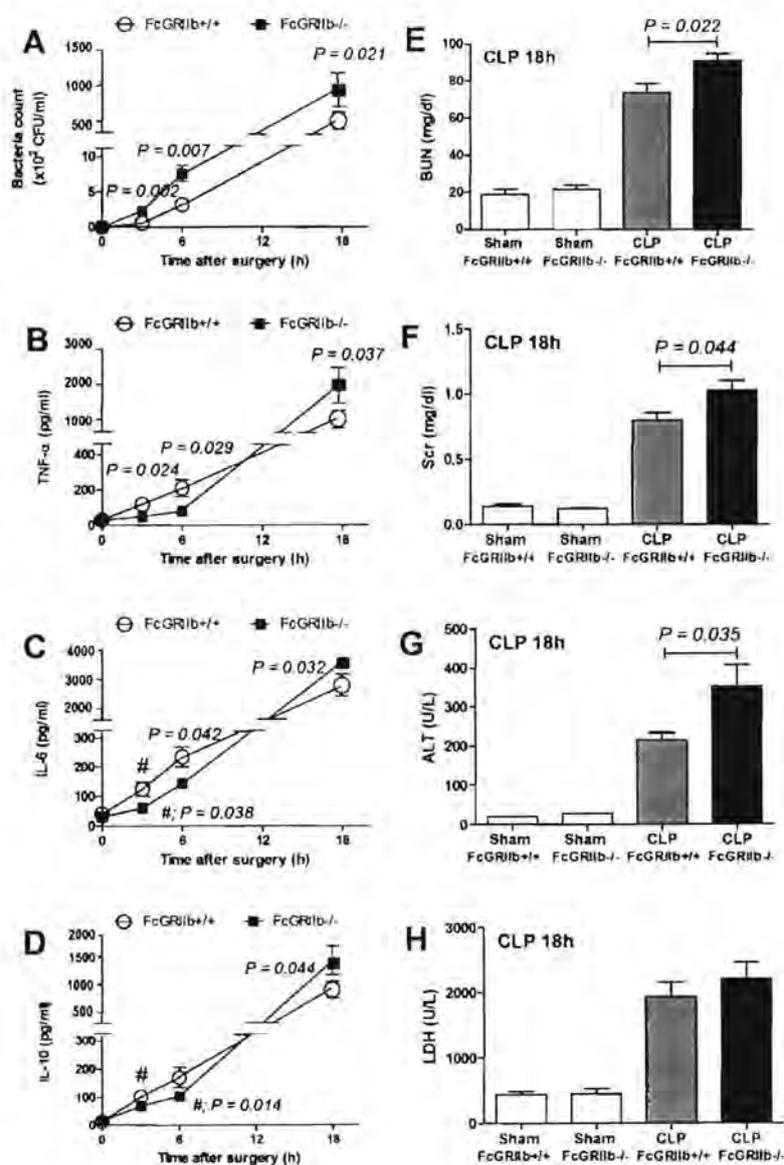


Figure 5. The time-course of bacterial burdens after cecal ligation and puncture (CLP) in blood bacterial burdens (A), mononuclear cell (B), PMN (C), TNF- α (D), IL-6 (E), IL-10 (F) and the severity of organs

injury after 18h of wild type (FcGRIIb+/+) and FcGRIIb-/- as demonstrated by serum creatinine (Scr) (G) and alanine transaminase (ALT) (H) was showed. (n= 5-7/ time-point and group)

Interestingly, bacterial burdens in FcGRIIb-/- mice were higher than wild type in all selected time-points (3, 6 and 18h) after CLP (Fig 5A). Blood bacterial count ($\times 10^2$ CFU/ml) in FcGRIIb-/- mice and wild type at 3, 6 and 18h were 3.1 ± 0.3 , 7.6 ± 1.1 , 964 ± 137 and 0.6 ± 0.2 , 3.3 ± 0.6 , 518 ± 73 , respectively.

Moreover, pro-inflammatory cytokines (TNF- α and IL-6) and anti-inflammatory cytokine (IL-10) were higher in wild type mice at 3 and 6h and only 3h after CLP, respectively (fig 5B-D). Serum TNF- α , IL-6 and IL-10 at 3h and 6h in FcGRIIb-/- versus wild type were 48 ± 9 , and 80 ± 7.1 , 69 ± 9 and 145 ± 18 , 71 ± 6 and 104 ± 4 pg/ml versus 120 ± 24 and 211 ± 49 , 124 ± 24 and 235 ± 33 , 102 ± 7 and 170 ± 36 pg/ml, respectively. In contrast, at 18h after CLP, all of these cytokines and most of the organs injury biomarkers (Scr for kidney injury and ALT for liver injury) were higher in FcGRIIb-/- mice (Fig 5B-H). Despite the prominent responses to LPS in FcGRIIb-/- mice mentioned earlier, cytokines responses at the early phase of CLP after LPS preconditioning were stunted in comparison with wild type implied the more severe immunoparalysis.

Perhaps, severe immunoparalysis at the early phase of sepsis might associate with the higher bacterial burdens leading to the higher sepsis severity (Fig 5) and mortality rate (Fig 3C) in FcGRIIb-/- mice.

Bone marrow derived macrophage of FcGRIIb-/- mice showed higher cytokine responses in the single incubation of LPS but lower responses in the double incubation of LPS

The previous results demonstrated that FcGRIIb-/- mice, an inhibitory signaling deficiency, showed a very high initial response, but subsequently follow with a more apparent exhaustion after LPS stimulation.

Because macrophage might be responsible for the exhaustion *in vivo* then we tested FcGRIIb^{-/-} macrophages response to endotoxin incubations *in vitro* in comparison with wild type cell.

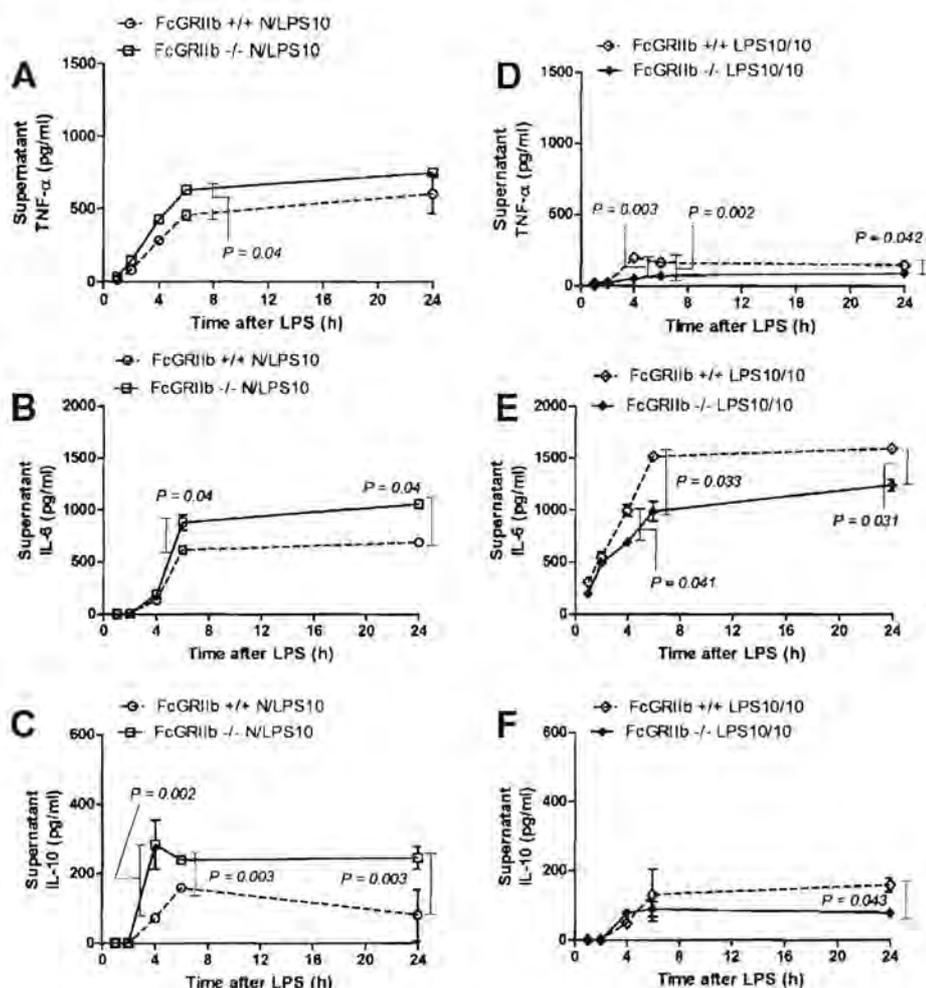


Figure 6. The cytokine responses in supernatant media from macrophages of FcGRIIb^{-/-} or wild type (FcGRIIb^{+/+}) after activated with only once low dose LPS (N/LPS10) as measured by TNF- α (A), IL-6 (B), IL-10 (C) and after activated with twice low dose LPS (LPS10/10) as measured by TNF- α (D), IL-6 (E), IL-10 (F) was showed. (Separated experiments were done in triplicate)

In parallel with the *in vivo* results, higher cytokines, at least in some time-points (3-24h), found in the supernatant of FcGRIIb^{-/-} macrophages with the single low dose LPS (non LPS at the 1st 24h of the incubation followed by LPS dose at 10 μ g/ml; N/LPS10) (Fig 6A-C). Then TNF- α and IL-10, but not IL-6, was lower in the double low dose of LPS stimulation (LPS 10 μ g/ml for 24h then washed and add the same 2nd dose; LPS10/10) (Fig 6D-F). Then, a higher dose of LPS was used. Once again, FcGRIIb^{-/-} macrophages showed the higher responses than wild type in the single high dose of LPS (N/LPS100) (Fig 7A-C). But the cytokines responses of macrophages primed with the high dose of LPS seems to depend on doses of the 2nd LPS. In high LPS followed by low dose LPS (LPS100/10), all cytokines were detected at the low level (Fig 7D-F) and the difference between wild type and FcGRIIb^{-/-} cells were subtle. But the apparent lower TNF- α and IL-10 in FcGRIIb^{-/-} cells appeared again with the higher 2nd dose of LPS (LPS100/100) (Fig 7G-I). To clarify the macrophage exhaustion, the lower cytokines level after double LPS exposure compare with single LPS exposure, the cytokines level after single and double LPS exposure at 6 and 24h was demonstrated (Fig 8). With the double low dose of LPS (LPS10/10), macrophage exhaustion could be demonstrated with only the lower TNF- α in wild type cell but lower both TNF- α and IL-10 in FcGRIIb^{-/-} macrophages (Fig 8A-C).

Interestingly, macrophage exhaustion, lower cytokine in double LPS exposure compare with single LPS, could be demonstrated only by TNF- α and IL-10 in FcGRIIb^{-/-} cells but with TNF- α alone for wild type cell with the double low dose of LPS (LPS10/10) (Fig 8A-C). On the other hand, with the higher dose of LPS stimulation, the exhaustion could be demonstrated in all cytokines in FcGRIIb^{-/-} cells but only in some condition in wild type cell (Fig 8D-F).

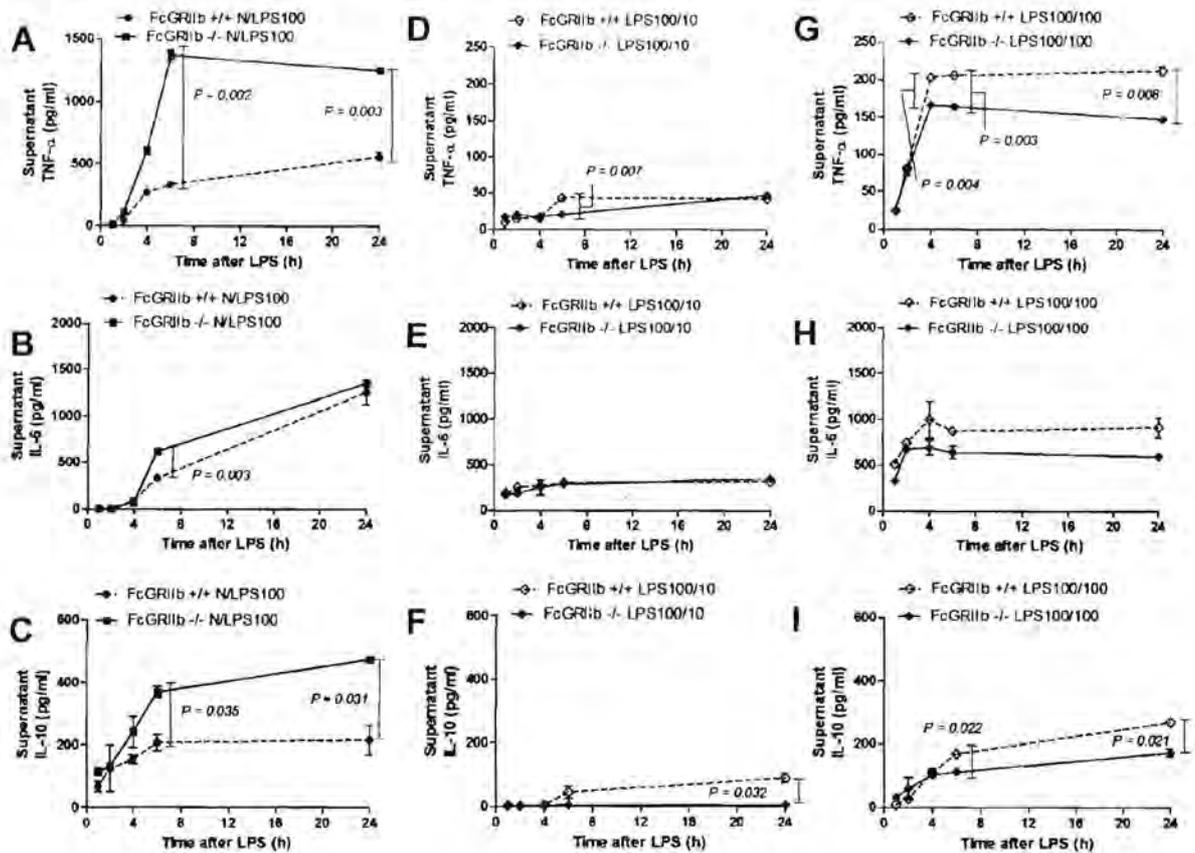


Figure 7. The cytokine responses in supernatant media from macrophages of *FcGR11b*^{-/-} or wild type (*FcGR11b*^{+/+}) after activated with only once high dose LPS (N/LPS100) as measured by TNF- α , IL-6, IL-10 (A-C) and double LPS doses, low and high dose LPS (LPS100/10 and LPS100/100, respectively), (D-F) were demonstrated. (Separated experiments were done in triplicate)

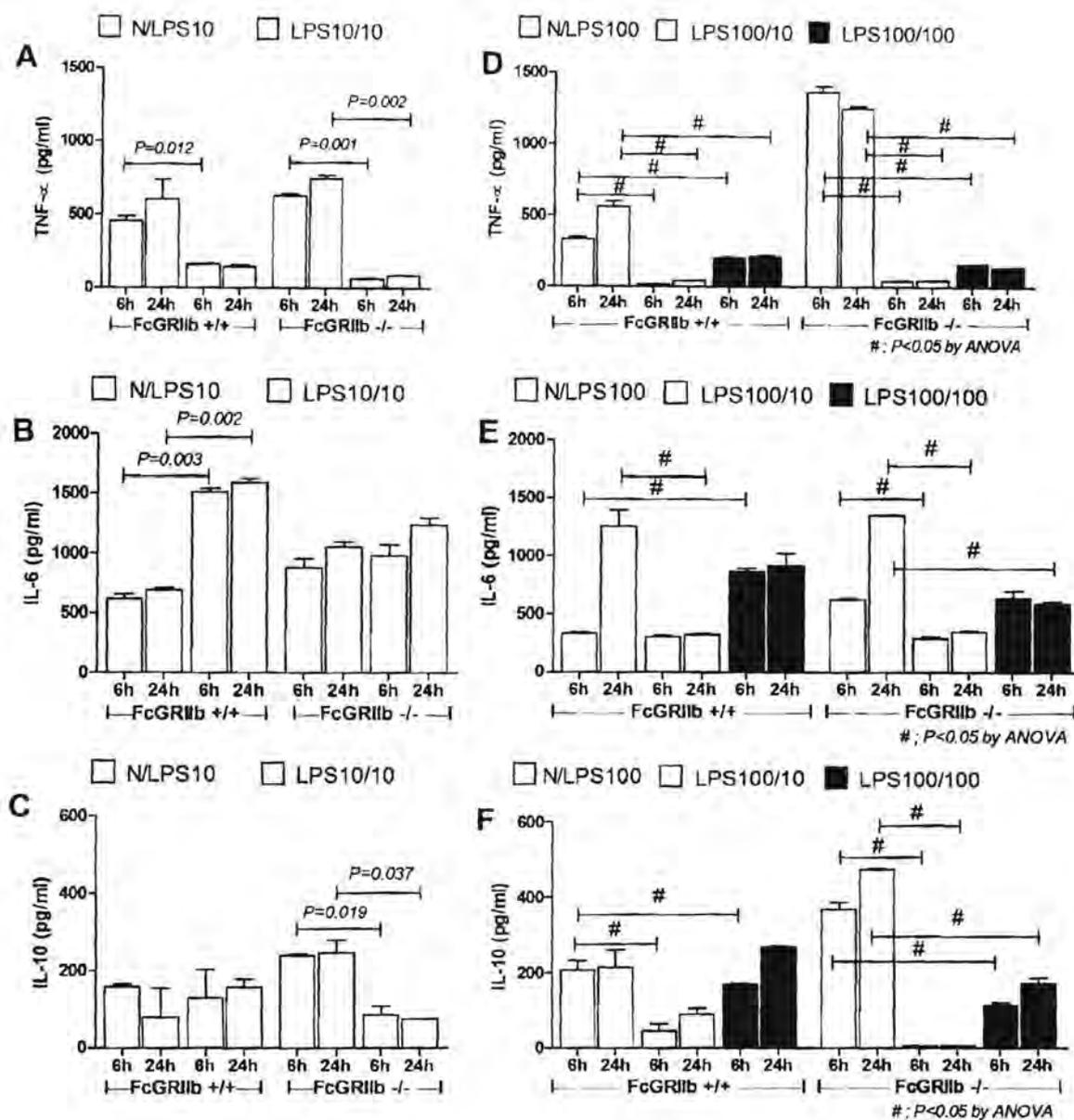


Figure 8. The macrophage endotoxin tolerance emphasized by the difference in cytokine responses (TNF- α , IL-6, IL-10) in supernatant media from macrophages of FcGR11b^{-/-} or wild type (FcGR11b^{+/+}) after activated with only once low dose (N/LPS10) versus twice low dose of LPS (LPS10/10) (A-C) and the difference among only once high dose (N/LPS100) versus twice high and then low dose of LPS (LPS100/10) versus twice high dose of LPS (LPS100/100) (D-F) were demonstrated. (Separated experiments were done in triplicate)

Macrophage exhaustion could not be demonstrated by IL-6 responses both in wild type and FcGRIIb^{-/-} cells with double low dose LPS stimulation. In parallel, with the comparison among single high dose LPS (N/LPS100) with double LPS dose with low and high 2nd LPS dose (LPS100/10 and LPS100/100, respectively), macrophage exhaustion could be shown by all cytokines despite a less prominent in IL-6 response of the wild type cell (Fig 8D-F). It seems the macrophage exhaustion occur in both knock-out and wild type cells but more prominent in FcGRIIb^{-/-} cells.

In contrast, the phagocytosis and killing activity of FcGRIIb^{-/-} macrophages were better than wild type cells and there was a non-significant exhaustion in phagocytosis and killing activity after LPS stimulation (Fig 9). Although, the killing activity of FcGRIIb^{-/-} macrophage should be able to control sepsis severity, perhaps the more severe bacterial sepsis severity in FcGRIIb^{-/-} mice might due to the lower number of mononuclear cell after sepsis (Fig 5).

Discussion

FcGRIIb deficiency is one of the genetic defects of SLE and FcGRIIb polymorphism with a lower gene activity commonly reported in patients with SLE in Asia (3-5, 8). Additionally, sepsis is the leading cause of death in patients with SLE (9). We showed a high susceptibility to CLP in symptomatic SLE mice but not in asymptomatic group in comparison with age-match wild type control. In asymptomatic genetic prone mice, the high susceptibility to CLP was showed only after with repeated endotoxin induced immunoparalysis. The more severe macrophage paralysis in FcGRIIb^{-/-} macrophages might responsible for the more immunoparalysis in mice leading to higher sepsis severity after CLP surgery.

The susceptibility to bacterial infection in patients with SLE is associated with several factors; immunosuppressive drugs, activity of disease, organs involvement, etc (9). Studies on FcGRIIb^{-/-} mice allow for exploring SLE without several confounding factors, especially immunosuppressive drugs. As expected, the higher mortality rate of sepsis was showed in 24-wk-old FcGRIIb^{-/-} mice, positive proteinuria but normal Scr classified into symptomatic SLE group, in comparison with age-matched wild type mice. These results supported the association between SLE disease activity and bacterial infection susceptibility reported previously (9). Interestingly, the mortality rate of sepsis without LPS preconditioning in asymptomatic 8-wk-old FcGRIIb^{-/-} mice, positive anti-dsDNA without proteinuria, did not different to wild type mice supported the effective immune responses to bacterial infection previously published (18).

More prominent endotoxin-induced immunoparalysis in FcGRIIb^{-/-} mice demonstrated by the high mortality rate of CLP sepsis

The immunoparalysis was induced by the double separate LPS administration and the severity of immunoparalysis, the condition with a more susceptible to infection, was determined by the severity of CLP sepsis (20). With this model, there was a higher mortality rate of CLP after LPS preconditioning compare with NSS placebo control within either 8-wk-old FcGRIIb^{-/-} or wild type mice demonstrated immunoparalysis occur in both groups. Nevertheless, FcGRIIb^{-/-} mice showed a higher mortality rate than wild type implied a more severe immunoparalysis.

Despite immunoparalysis could be demonstrated by several biomarkers (42), cytokines responses are frequently used. Then we tested cytokines responses in panels of pro- and anti- inflammatory cytokines, TNF- α , IL-6, IL-1 β , IFN- γ and IL-2, IL-4, IL-5, IL-10, respectively, with luminex-based measurement in mice with double doses of LPS administration. Most of the cytokines selected to measure were mainly produced by macrophage except for IFN- γ and IL-5 which produced prominently by NK cell or T cell and mast cell, respectively. With double dose of LPS administration by the 2nd dose of the 5 times higher than the 1st dose, all of these cytokines response after the 2nd LPS stimulation were not higher than the responses after the 1st LPS dose. These results suggested immunoparalysis in both FcGRIIb^{-/-} and wild type mice. Interestingly, in comparison with wild type mice, most of the pro- and anti-inflammatory cytokines, except for IL-5, were higher in FcGRIIb^{-/-} mice after the 1st LPS stimulation, implied the vigorous cytokines responses in FcGRIIb^{-/-} mice. Then after 2nd LPS administration, all cytokine except for IL-1 β and IL-5 were lower than the 1st responses in both wild type and but with a more prominent difference, demonstrated by the

cytokine difference between 1st and 2nd LPS administration, in FcGRIIb^{-/-} mice. These results demonstrated a more severe immunoparalysis in FcGRIIb^{-/-} group. Of note, most of these cytokines produced from macrophages except for IL-5 and IFN- γ which produced mainly from mast cell and NK cell, respectively. Although functional FcGRIIb expression on mast cell (43) and murine NK cell (44) were reported, IFN- γ , but not IL-5, response differently between FcGRIIb^{-/-} and wild type mice. These implied the difference of FcGRIIb function between these cells. More experiments needed but out of the scope of this article.

Nevertheless, the double LPS preconditioning seems to affect CLP severity. At the initial time-point of CLP surgery, 12h after 2nd dose of LPS, there was non-difference in TNF- α , IL-6 and IL-10 between LPS preconditioning and NSS control (data not showed) supported by the base-line value before CLP surgery (Figure 5). Interestingly, blood bacterial burdens were higher with the lower of these cytokines in FcGRIIb^{-/-} mice compare with wild type at 3h and 6h after CLP surgery. However, at 18h after CLP the sepsis severity was more severe in FcGRIIb^{-/-} mice as demonstrated by bacterial burdens, cytokines and organs injury. Perhaps, the initial cytokine responses were needed for the initial innate immune responses to control the infection and the loss of the initial control in FcGRIIb^{-/-} mice due to LPS preconditioning induced a more severe sepsis. These results support the importance of the initial bacterial control, especially in patients with SLE and /or FcGRIIb polymorphism.

More prominent immunoparalysis in FcGRIIb^{-/-} macrophage demonstrated by cytokine responses after LPS stimulation

The LPS induction *in vitro* alters macrophage characteristics from classical pro-inflammatory macrophage responses into a less pro-inflammatory stage of the macrophage (38) implied the importance of

macrophage in immunoparalysis. With the double low doses of LPS (LPS10/10), macrophage exhaustion could be demonstrated in both FcGRIIb^{-/-} and wild type but cytokines production after 2nd dose of LPS was lower in FcGRIIb^{-/-} macrophages. Moreover, the exhaustion seems to be more apparent with the higher 1st dose of LPS which needed the higher 2nd dose of LPS to re-stimulate. With the initial high dose of LPS followed by a low dose (LPS100/10), only subtle cytokines responses were demonstrated. But with the larger 2nd LPS dose (LPS100/100), the difference between FcGRIIb^{-/-} and wild type appeared again. However, FcGRIIb^{-/-} macrophages produced less cytokines levels after 2nd dose of LPS either with high or low LPS doses. Then LPS could induce a more apparent macrophage paralysis in FcGRIIb^{-/-} cells resulted in immunoparalysis state in mice which demonstrated by the higher CLP sepsis severity.

In conclusion, we demonstrated the impact of the repeated infection in patients with SLE through the CLP preconditioning with LPS in FcGRIIb^{-/-} mice. Despite a good response to the only once bacterial infection in FcGRIIb^{-/-} mice, the responses to the repeated exposure might be impaired lead to a more severe bacterial burdens and infection. The repeated infection in patients with SLE could be more severe due not only to hyperimmunoglobulin induced hyper-immuneresponse and sepsis (18), but also from immunoparalysis with the higher bacterial burdens as currently demonstrated. In the translational aspect, we suggested that the repeated infection in patients with SLE should be vigorously concerned and the FcGRIIb polymorphism screening in Asian patients with SLE might be a useful clinical practice.

สรุปและเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

The next final part of the project is the experiments to see if macrophage of FcGR IIb knock-out which demonstrated exhaustion but intact killing activity show shorter half-life in comparison with wild type cell. If the last experiment results go along with the hypothesis then FcGR IIb knock-out mice will be susceptible to sepsis due to macrophage exhaustion and shorten half life of macrophage which will be stronger support our in vivo results.

Regarding to the translation, the exploration of FcGR polymorphisms in patients with SLE might be beneficial for the prediction of sepsis and might require a more aggressive antibacterial drug at the beginning of the simple infectious diseases. More studies will be needed.

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Appendix

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Educations:

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2002 Board of Internal Medicine, Thailand

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Master of Science, Chulalongkorn University, Thailand

2005-2010 Visiting fellow at National Institute of Diabetes and Digestive and Kidney Disease,
 National Institutes of Health, Bethesda, MD, USA

2010 PhD (Biomedical Science), Chulalongkorn University, Thailand

Brief Chronology of Employment

2011- present Lecturer, Immunology unit, Department of microbiology, Chulalongkorn
 University, Thailand

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2005- 2010	Visiting Fellow, Kidney Disease Branch, National Institute of Diabetes and Digestive and Kidney Disease, Bethesda, MD, USA
2004-2005	Instructor, Division of Nephrology, Department of Internal Medicine, Faculty of Medicine, Chulalongkorn University, Thailand
1998-1999	General practice, Ongkarag Hospital, Nakornnayok, Thailand
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Publications:

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สัญญาเลขที่ GRB_BSS_๕๕_๕๘_๓๐_๑๓

โครงการวิจัย เรื่อง โครงการวิจัยการศึกษาเชิงระบบของการส่งสัญญาณผิดปกติในเซลล์

ของระบบภูมิคุ้มกันในหนูโรคลูปัส

รายงานการรับ-จ่ายเงิน *

	<u>รายได้ประมาณการ</u>	<u>งบที่เกิดขึ้นจริง</u>
เงินอุดหนุนงบประมาณแผ่นดิน	580,000.00	580,296.35
รวมรายได้	<u>00.00</u>	<u>00.00</u>
<u>รายจ่าย</u>		
<u>หมวดค่าจ้างชั่วคราว</u>		
ค่าจ้างนักวิจัยเต็มเวลาปริญญาตรี		280,000.00
<u>หมวดค่าตอบแทน</u>		
-		00.00
<u>หมวดค่าใช้สอย</u>		
-		00.00
<u>หมวดค่าวัสดุ</u>		
- RNeasy mini kit (50)		12,198.00
- RNase- free DNase set (50)		4,066.00
- RNeasy mini kit (50) and RNases free DNase set (50)		16,264.00
- Custom oligos syn, 6 oligo, 212 mers, 25nmole		3,175.76
- phusion flash high- fidelity PCR mater mix 100 rxn		4,066.00
- Peptide synthesis 16 aa, 80 % purity, 10 mg		15,793.20

- ODN 1585 200 ug		2,389.31
- Custom oligos syn, 6 oligo, 122mers,25nmole		1,827.56
- Custom oligos syn, 6 oligo, 213 mers,25nmole		3,190.74
- Mouse Flt3 ligand protein fragment, 10ug		21,453.50
- Fixable viability dye eFlour 780, 500 test		27,606.00
- tube 5 ml 12*75 mm R-BTM, PS, SNAP CAP, 1000/CS		4,515.40
- Cell strainer 70 UM white 50/CS		4,280.00
- Nucleospin gel and PCR Clean-up, 50 prep/kit		3,745.00
- proteinase k, 100 mg		5,671.00
- CALF THYMUS DNA SOLUTION, 5 ML		17,441.00
- Collagenase D, 500 mg		8,560.00
- Freezer Box, LioBox, Pink 9*9 grid (pk of 100)		8,014.30
- Human activating transcription factor 3(ATF3) ELISA Kit-96 well		-
กระดาษกรอง(ขาว) 200 ชั้น		3,531.00
- QUANTICHROM CREATININE ASSAY KIT		27,830.70
- DNASE I,100 U		3,745.00
- HORSE SERUM,500 ML		10,700.00
- Freezer Box,liobox9x9 grids(pk of 10 ea)		2,568.00
- Tube 15 ml, 50 ml,		14,102.60
- Reagent reservoir, Trifill reservoir		8,506.00
- 40% acrylamide/bis solution		4,815.00
- Creatinine assay kit,100 tests		20,865.00
- Mycoplasma-off,1 litre		9,630.00
- Defibrinated sheep blood 200 cc		3,600.00
<u>หมวดค่าครุภัณฑ์ (ถ้ามี)</u>		
-	00.00	00.00
รวมรายจ่าย		580,296.35

รายรับสูงกว่ารายจ่าย นำส่งส่วนการคลังจุฬาลงกรณ์มหาวิทยาลัย (เอกสารแนบ)

0

ดอกเบี้ย (ถ้ามี)

00.00

รวมจำนวนเงินที่นำส่งส่วนการคลังทั้งสิ้น

0

ขอรับรองว่ารายงานการรับ-จ่ายเงินข้างต้นเป็นความจริงทุกประการ


 (..... ไชยรักษ์ นิสิตกุล/ว.....)

(หัวหน้าโครงการวิจัย)

4 / ๗ / ๒๕๕๙

หมายเหตุ : รายงานตลอดโครงการเมื่อสิ้นสุดโครงการ/ปิดโครงการ

ชุดโครงการย่อย 1

แนวทางในการจัดทำรายงานการวิจัยฉบับสมบูรณ์
ทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล

"รายงานการวิจัยเรื่อง การศึกษาการควบคุมของวิถีสัญญาณอินเตอร์เฟียรอนผ่านเซ็นเซอร์ของไวรัส
ดีเอ็นเอในเซลล์ภูมิคุ้มกันของหนูโรคลูปัสด้วยวิธีโปรตีโอมิกส์"

"Systems-investigation of aberrant signaling in immune cells of SLE mouse model"

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บทคัดย่อภาษาไทย

โรคแพ้ภูมิตนเองเกิดขึ้นเมื่อเซลล์ภูมิคุ้มกันตอบสนองต่อแอนติเจนของตัวเองผิดปกติและนำไปสู่การอักเสบในเนื้อเยื่อ ปฏิสัมพันธ์ระหว่างพันธุกรรมและสิ่งแวดล้อมเป็นส่วนส่งเสริมให้อาการของโรคเป็นมากขึ้น

สารไซโตไคน์ที่ทำให้เกิดโรคเอสแอลอีและโรครูมาตอยด์ที่สำคัญคือ อินเตอร์เฟอรอนแอลฟา

โรคเอสแอลอีเกิดจากภูมิคุ้มกันผิดปกติเรื้อรังซึ่งจะทำให้เกิดการสร้างแอนติบอดีต่อเนื้อเยื่อตนเองและทำให้เกิดไตอักเสบ การส่งสัญญาณผ่านเซ็นเซอร์กรดนิวคลีอิกสามารถกระตุ้นการผลิตอินเตอร์เฟอรอนแอลฟา จากเดนไดรติกเซลล์ และส่งเสริมความรุนแรงของโรคเอสแอลอี Stimulator of Interferon Genes (Sting) เป็นตัวรับดีเอ็นเอและส่งสัญญาณต่อเพื่อให้เกิดการสร้างอินเตอร์เฟอรอนแอลฟา ความผิดปกติของ Sting สามารถก่อให้เกิดโรคหลอดเลือดอักเสบในเด็กซึ่งมีอาการคล้ายโรคเอสแอลอี อย่างไรก็ตาม การศึกษาการทำงานของ Sting ในสัตว์ทดลองของโรคแพ้ภูมิตนเองยังมีความขัดแย้งกันอยู่ เพื่อที่จะค้นหาว่า Sting มีส่วนร่วมในการเกิดโรคลูปัส, หนูลูปัส Fcgr2b-deficient จะถูกทำการผสมพันธุ์กับหนู Sting-deficient และหนูที่ได้จากการผสมนี้จะถูกติดตามอาการของโรคลูปัส จากผลการศึกษาพบว่า หนูลูปัส Fcgr2b-deficient สามารถมีชีวิตอยู่ได้นานขึ้น มีการสร้างแอนติบอดีต่อเนื้อเยื่อตนเองลดลง และ เกิดไตอักเสบลดลง ความรู้จากการศึกษานี้เป็นหลักฐานยืนยันแนวคิดสำหรับการวางแผนที่จะยับยั้ง Sting ในการรักษาโรคแพ้ภูมิตนเองในอนาคต

Abstract

Autoimmune diseases occur when the immune cells react against self-antigens and subsequently lead to inflammation in the tissues. The interactions between genetics and environmental triggers regulate the phenotypes and outcome of the diseases. Type I interferon has been shown as one of the most crucial cytokines involving in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). SLE is a chronic systemic autoimmune disease which can result in autoantibody production and fatal glomerulonephritis. Activation via nucleic acid sensors can induce the production of type I interferon from dendritic cells and promote SLE severity. Stimulator of interferon genes (Sting) is a cytoplasmic DNA sensor that signals downstream to enhance type I interferon production after its activation. Recently, it was shown that a gain mutation in the STING gene resulting in over-activity of the IFN pathway can cause familial inflammatory syndrome with lupus-like manifestations in humans. However, the functional studies of Sting in different autoimmune mouse models suggest the conflicting roles of Sting in the pathogenesis of autoimmune diseases. In order to determine if Sting participates in lupus pathogenesis, the Fcgr2b-deficient mice (lupus mouse model) were bred with Sting-deficient mice to create the double-deficient mice. In the absence of Sting, the Fcgr2b-deficient mice do not develop fatal glomerulonephritis and autoantibodies. The original knowledge from this study is a proof of concept for targeting Sting as a future promising treatment in autoimmune diseases.

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6. คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

SLE, Systemic lupus Erythematosus; Fcgr2b, Fc gamma receptor IIb; Sting; type I interferon,

ส่วนประกอบเนื้อเรื่อง

1. บทนำ (Introduction) ซึ่งกล่าวถึงเนื้อหาของเรื่องที่เคยมีผู้ทำการวิจัยมาก่อน ความสำคัญและที่มาของปัญหา วัตถุประสงค์และขอบเขตการวิจัย วิธีดำเนินการวิจัยโดยสรุปทฤษฎีและ/หรือแนวทางการความคิดที่นำมาใช้ในการวิจัย ประโยชน์ที่คาดว่าจะได้รับ ฯลฯ

Fcgr2b เป็น Fc receptor ที่ทำหน้าที่ควบคุมและยับยั้งการทำงานของเซลล์ในร่างกาย

เพื่อป้องกันไม่ให้เซลล์ที่ถูกกระตุ้นนั้นตอบสนองต่อตัวกระตุ้นและทำงานเพิ่มขึ้นเพียงอย่างเดียวโดยที่ไม่

สามารถกลับเข้าสู่ภาวะปกติได้เมื่อหมดตัวกระตุ้นแล้ว ดังนั้นในหนูที่ปราศจาก Fcgr2b (Fcgr2b^{-/-}) จึงมีการ

ทำงานของเซลล์ที่มากผิดปกติโดยเฉพาะ B เซลล์และ macrophage เซลล์ ซึ่งเป็นผลทำให้เกิดโรค

autoimmune ขึ้นได้ (1) ในหนูลูบัส Fcgr2b^{-/-} จะมีอาการแสดงของโรคใกล้เคียงกับผู้ป่วย SLE อย่างมาก (1)

และในหนู Fcgr2b^{-/-} ที่มีอายุน้อยก็สามารถทำให้เกิดอาการเหมือนกับโรค Rheumatoid arthritis โดยการฉีด

collagen (collagen-induced arthritis) นอกจากนี้ความผิดปกติของยีน Fcgr2b ก็สามารถพบได้ในผู้ป่วยชาว

เอเชียรวมทั้งคนไทยที่เป็น SLE (2-4)* ดังนั้นโมเดลนี้จึงเป็นโมเดลที่เหมาะสมสำหรับใช้ในการศึกษากลไกการ

เกิดโรค autoimmune disease ทั้งโรค SLE และ Rheumatoid arthritis โดยที่ผู้วิจัยเองได้มีประสบการณ์ใน

การทำงานวิจัยเพื่อที่จะศึกษากลไกการเกิดโรคในหนู Fcgr2b^{-/-} มาแล้ว โดยพบว่า interleukin-17 signaling

pathway มีความสำคัญต่อทั้งการเกิด lupus nephritis ในหนูลูบัส Fcgr2b^{-/-} (5) และต่อการเกิด

rheumatoid arthritis ในโมเดล collagen-induced arthritis ในหนู Fcgr2b^{-/-} เช่นกัน (6) นอกจากนี้เรายัง

สามารถใช้หนูลูบัส Fcgr2b^{-/-} ในการวิจัยเพื่อศึกษากลไกการเกิดโรค SLE และนำความรู้ที่ได้นั้นมาประยุกต์ใช้

ในการรักษาผู้ป่วยโรค SLE ต่อไป

การแสดงออกของ TLR9 และ TLR7 มีผลต่อการทำให้เกิดโรคลูบัสและการสร้าง

autoantibody (7-9) นอกจากนี้ การเพิ่มมากขึ้นของ RNA-sensing โมเลกุลอย่างเช่น MDA5 ก็

สามารถกระตุ้นให้หนูที่เป็นโรคลูบัสอยู่แล้วมีอาการที่รุนแรงขึ้นได้ (10) และความผิดปกติของ DNA

metabolism ในหนูที่ขาดยีน Trex-1 (11) และ DNase (12, 13) ก็ทำให้เกิดโรคลูบัสได้เหมือนกัน นอกจากนี้

ในผู้ป่วย SLE ก็ตรวจพบว่าการสร้างแอนติบอดีต่อ DNA-sensing โมเลกุล IFI16, เชื้อไวรัส HHV8 และ

CMV (14-16) อย่างไรก็ตามเป็นที่ทราบกันว่าอาการของโรค SLE จะเพิ่มความรุนแรงขึ้นอย่างมีนัยสำคัญใน

ระหว่างการติดเชื้อจากไวรัสต่างๆ เช่น CMV และ EBV (14, 17) จะเห็นได้ว่าข้อมูลเหล่านี้บ่งชี้ให้เห็นถึงความ

เชื่อมโยงระหว่างการติดเชื้อไวรัส, เซ็นเซอร์ของกรดนิวคลีอิกในเซลล์และการเกิดโรคลูบัส แต่ถึงกระนั้น

ปัจจุบันก็ยังไม่มีการศึกษาที่ทำให้ทราบถึงบทบาทและกลไกของ DNA-sensing โมเลกุลที่อยู่ใน cytoplasm ของเซลล์ว่ามีผลต่อการเกิดโรคภูมิคุ้มกันบกพร่องหรือไม่ และจนถึงปัจจุบันความเข้าใจในกลไกที่ทำให้เกิดปรากฏการณ์นี้ก็ยังไม่ทราบชัดเจน ซึ่งถ้าเราสามารถทำงานวิจัยที่นำมาซึ่งความเข้าใจในกลไกการเกิดโรคนี้ได้ ข้อมูลนี้อาจนำไปสู่ความรู้ขั้นสูงในการพัฒนาวัคซีนรักษาโรคในการรักษาผู้ป่วยโรคภูมิคุ้มกันบกพร่องในอนาคต

โดยทั่วไป DNA-sensing โมเลกุลที่ถูกกระตุ้นโดย Interferon (IFNs) จะส่งสัญญาณผ่าน

ทางโปรตีน adaptor ที่เรียกว่า STING (Stimulator of interferon genes) (18-20) นอกจากนี้ STING ยังเป็นโมเลกุลที่จำเป็นสำหรับการรวมตัวของไวรัสและ membrane ของ host ซึ่งขบวนการนี้จะกระตุ้นให้เกิดการหลั่ง Interferon (21) ดังนั้น STING จึงเป็นโมเลกุลตัวแทนสำหรับการศึกษาถึงบทบาทของ DNA-sensing pathway ว่ามีผลต่อการเกิดโรคภูมิคุ้มกันบกพร่องหรือไม่

2. เนื้อเรื่อง (Main Body) ซึ่งกล่าวถึงรายละเอียดเกี่ยวกับวิธีดำเนินการวิจัย (Materials & Method)

ผลการวิจัย (Results) ฯลฯ

Materials and Methods

Animal and animal models

Fcgr2b^{-/-} mice on C57BL/6 background were provided by Bolland S. (NIH, Maryland, USA). Sting^{-/-} mice were provided by Paludan (Aarhus University, Aarhus, Denmark). Wild type mice were purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. The Fcgr2b^{-/-} mice were bred with Sting^{-/-} mice to create double deficient mice and their littermate controls. The animal protocols were approved by Faculty of Medicine, Chulalongkorn University followed the National Institutes of Health (NIH) criteria.

Survival study

The double deficient mice will be aged and observed the survival rates compared to their littermates. If the mice can survive up to 12 months, the mice will be euthanized to collect the tissues (kidney, spleen, bone marrow, and sera) for further analysis.

Flow cytometry analysis

The collected spleens were harvested and incubated with collagenase D at 37 c for 30 minutes to isolate splenocyte. The splenocytes were stained with flow antibody. The flow cytometry analysis was performed using BD LSR-II and FlowJo software. The dead cells were excluded from the analysis.

Autoantibody testing

The collected sera of the mice at the age of 6 months were tested for anti-nuclear antibody (Hep-2 cells; immunofluorescence) and anti-dsDNA (ELISA). The sera were diluted into different dilution factors.

Histopathology

The kidneys were fixed in 4% paraformaldehyde and subsequently were stained with H&E and PAS. The pathology grading will be blinded analysis by the experience nephrologist.

Real-time PCR

The RNA from kidneys was isolated using Trizol. The DNase-treated RNA was purified using RNeasy isolation kit. The conversion of RNA to cDNA using iScript RT Supermix (Biorad) was performed. The gene expression profiles were tested using SsoAdvanced Universal SYBR Green Supermix. The sequences of primers tested are followed:

Statistical analysis

Significant difference of survival rates is tested by Log-rank test. The comparison between groups is examined by T-test.

Result

Increase of survival rates of the Fcgr2b-deficient mice in the absence of Sting

The Fcgr2b-deficient mice start to die at the age of 6 months and the survival rates drop to 22.2% by 12 month old while the survival rates of double deficient mice are 77.7% ($p < 0.001$). The effect of one allele of Sting to survival rates of Fcgr2b-deficient mice does not show significant difference ($p = 0.6$) (Figure 1).

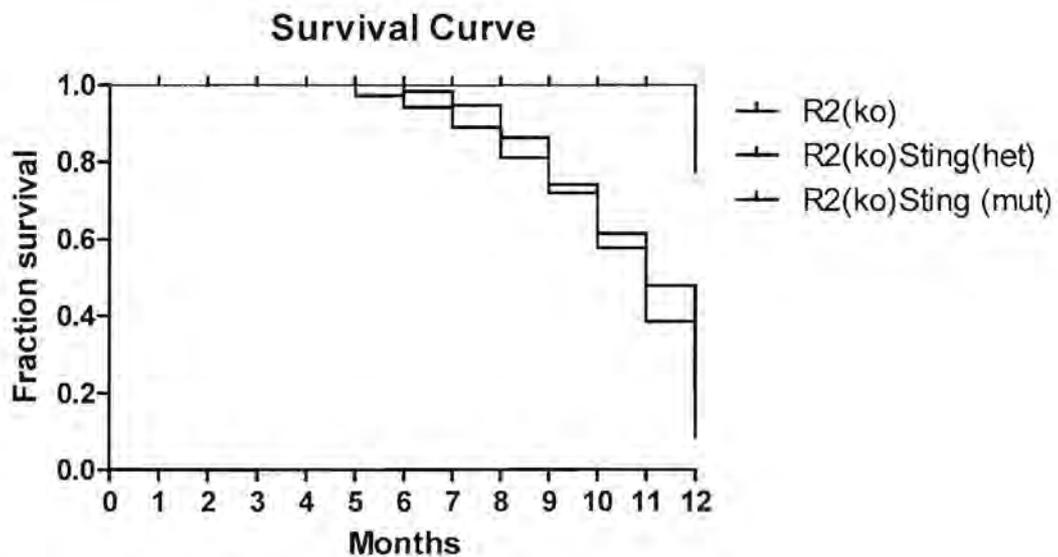


Figure 1 Increase of survival rates of the Fcgr2b-deficient mice in the absence of Sting

The survival of the mice was observed until 12 month old. The absence of Sting increase survival of Fcgr2b^{-/-} mice ($p < 0.001$, $N = 9$ per group).

Sting deficiency diminishes fatal glomerulonephritis in the Fcgr2b-deficient mice

The kidney staining of Fcgr2b-deficient mice shows inflammatory cell infiltrations, enlarged glomeruli and crescentic glomeruli. In the absence of Sting, the Fcgr2b-deficient mice do not develop glomerulonephritis (Figure 2).



Figure 2 Sting deficiency diminishes fatal glomerulonephritis in the Fcgr2b-deficient mice

Decrease autoantibody production in the double deficient mice

Antinuclear antibody (ANA) and anti-dsDNA production decreases in the Sting and Fcgr2b double deficient mice (Figure 3A, 3B).

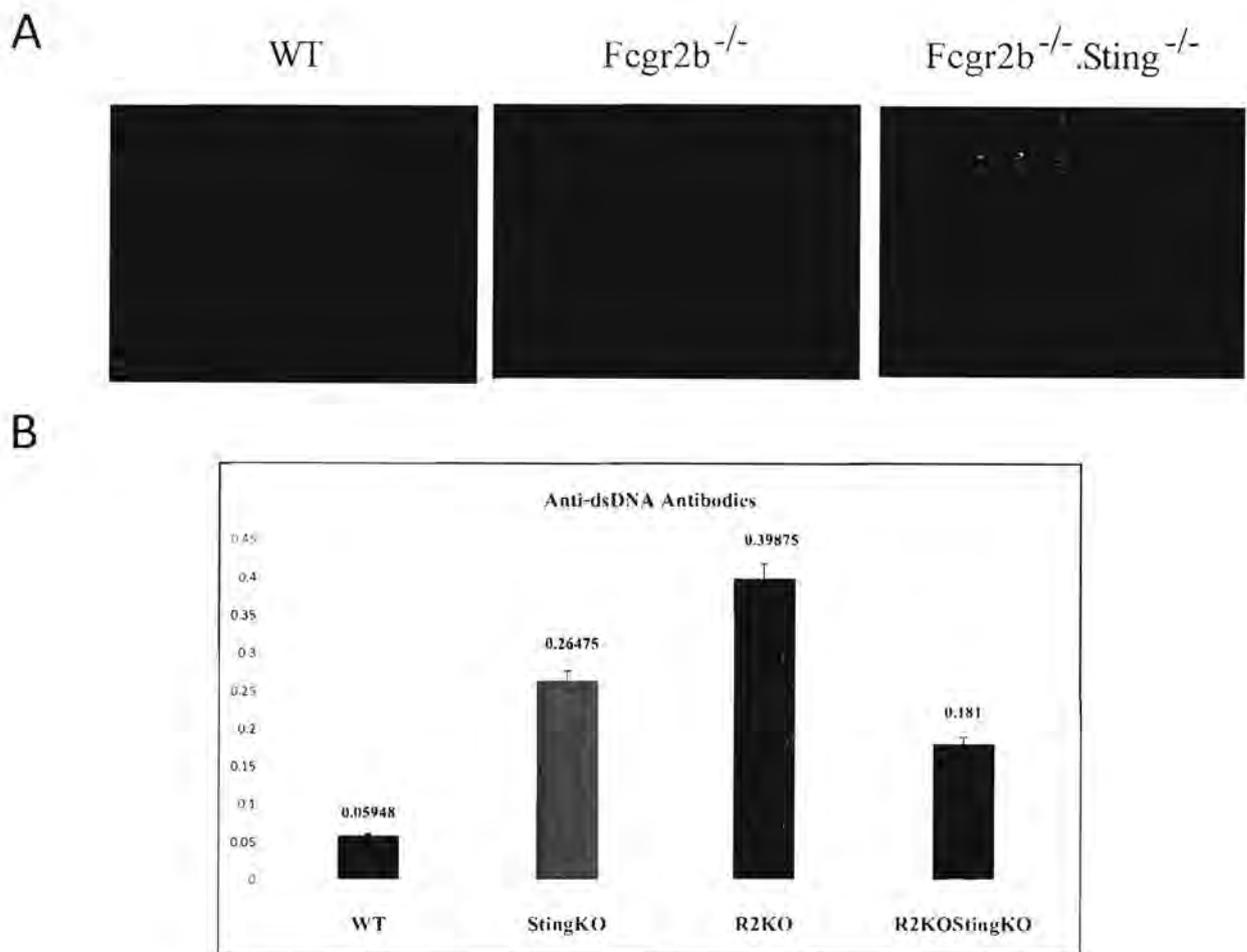


Figure 3 Decrease autoantibody production in the double deficient mice

The sera of the mice were collected at the age of 6 months to test for antinuclear antibody (Figure 3A, representative of mice (N=3/group) and anti-dsDNA (Figure 3B, N=6/group, $p < 0.01$)

Decrease CXCL10 expression in the kidneys of the double deficient mice

Sting-mediated signaling induces type I interferon production and leads to the increase of interferon inducible gene expression. We determined whether the interferon signature genes in the kidneys will diminish in the $Fcgr2b$ -double deficient mice. Surprisingly, only CXCL10 expression was decreased in the double-deficient mice compared to $Fcgr2b$ -deficient mice, but the MX1, ISG15 and IFN α expression were increased (Figure 4).

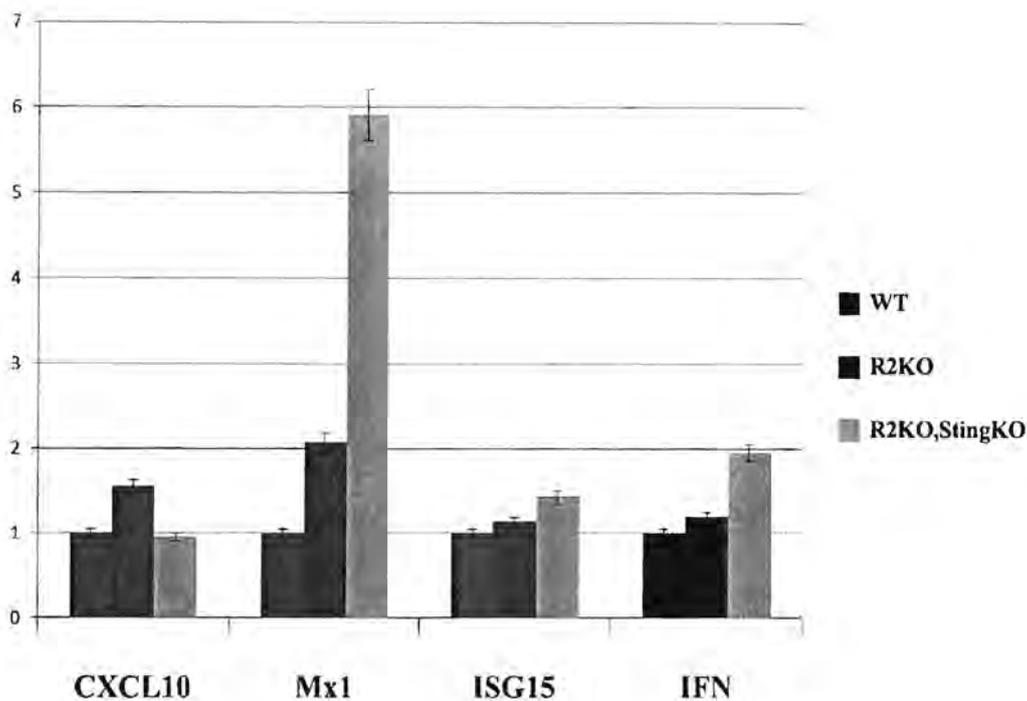


Figure 4 Decrease CXCL10 expressions in the kidneys of the double deficient mice Interferon inducible gene expression was tested from the kidneys of wild type, Fcgr2b^{-/-}, and Fcgr2b^{-/-}Sting^{-/-} mice. Only CXCL10 mRNA expression was reduced in the group of double-deficient mice compared to Fcgr2b-deficient mice (N=5/group, p<0.01).

3. อภิปราย / วิเคราะห์ (Discussion) ผลการทดลอง / ผลการวิจัยที่ได้ทั้งหมด (ทั้งที่เป็นและไม่เป็นไปตามสมมติฐานที่ตั้งไว้)

The data from this study confirm the hypothesis that Sting-mediated signaling participates in the pathogenesis of the Fcgr2b-deficient mice. The blocking Sting pathway may alleviate lupus phenotypes and probably be worth to develop therapeutic drug target. Unexpectedly, most of the interferon-inducible gene expressions do not decrease, except CXCL10. Contrary, the increase expression of Mx1 and IFN α suggests the interferon-independent mechanisms of Sting signaling in lupus development of Fcgr2b-deficient mice. This intriguing effect of Sting signaling is worth to

explore in details to better understand the immunological mechanisms of Sting in autoimmune development and other inflammatory diseases.

4. สรุปและเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

In order to understand the immunological importance of Sting in lupus Fcgr2b-deficient mice, the flow cytometry will be analyzed from the splenocytes of affected mice and their controls. Furthermore, the in vitro culture of bone-marrow derived dendritic cells with Sting ligands will be performed and tested for the differentiation, activation stage and cytokine production. In addition, the proteomic analysis of kidney tissue from these mice will be performed to discover the new unbiased Sting-signaling pathway.

The translational knowledge from this study will lead to design therapeutic drug for SLE patients.

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2. ภาคผนวก (Appendix) ถ้ามี่

3. ประวัตินักวิจัยและคณะ พร้อมหน่วยงานสังกัด

ส่วน ค : ประวัติคณะผู้วิจัย

1. หัวหน้าโครงการ

1. ชื่อ - นามสกุล (ภาษาไทย) นาย ไตรรักษ์ พิสิษฐ์กุล

ชื่อ - นามสกุล (ภาษาอังกฤษ) Mr. Trairak Pisitkun

2. เลขหมายบัตรประจำตัวประชาชน 4100700009959

ตำแหน่งปัจจุบัน อาจารย์

เงินเดือน 65,000 (บาท)

เวลาที่ใช้ทำวิจัย 40 (ชั่วโมง : สัปดาห์)

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2002 Master of Science, Chulalongkorn University, Thailand

1998 Board of Internal Medicine, Thailand

1994 M.D. (First Class Honors), Mahidol University, Thailand

1992 Bachelor of Science, Mahidol University, Thailand

5. สาขาวิชาการที่มีความชำนาญพิเศษ Systems Biology

6. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละผลงานวิจัย

7 ผู้อำนวยการแผนงานวิจัย : ชื่อแผนงานวิจัย -

7.1 หัวหน้าโครงการวิจัย : ชื่อโครงการวิจัย Systems biology of aberrant targeting and polarization of membrane proteins in disease

7.2 งานวิจัยที่ทำเสร็จแล้ว : ชื่อผลงานวิจัย ปีที่พิมพ์ การเผยแพร่ และแหล่งทุน (อาจมากกว่า 1 เรื่อง)

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แหล่งทุน Faculty of Medicine, Chulalongkorn University

7.3 งานวิจัยที่กำลังทำ : ชื่อข้อเสนอการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด

ชื่อข้อเสนอการวิจัย Systems biology of aberrant targeting and polarization of membrane proteins in disease

แหล่งทุน Lundbeck Foundation (2012): total award 500,000 Danish krone

การวิจัยลุล่วงแล้วประมาณร้อยละ 25

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อิมมูโนวิทยา โรคอโตอิมมูน การวิจัยในสัตว์ทดลอง

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น

7.1 ผู้อำนวยการแผนงานวิจัย :-

7.2 หัวหน้าโครงการวิจัย :-

7.3 ผู้ร่วมงานวิจัย :-

7.4 โครงการที่ทำเสร็จแล้ว :

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ชุดโครงการย่อย

สัญญาเลขที่ GRB_BSS_๕๕_๕๘_๓๐_๑๓

โครงการวิจัย เรื่อง การศึกษาการควบคุมของวิถีสัญญาณอินเตอร์เฟียร์รอนผ่านเซ็นเซอร์ของไวรัสดีเอ็นเอใน
เซลล์ภูมิคุ้มกันของหนูโรคอุปนิสัยด้วยวิธีโปรตีโอมิกส์

รายงานการรับ-จ่ายเงิน *

	ประมาณการ	งบที่เกิดขึ้นจริง
รายได้		
เงินอุดหนุนงบประมาณแผ่นดิน	290,000.00	00.00
รวมรายได้	00.00	00.00
รายจ่าย		
<u>หมวดค่าจ้างชั่วคราว</u>		
ค่าจ้างนักวิจัยเต็มเวลาปริญญาตรี		140,000.00
<u>หมวดค่าตอบแทน</u>		
-	00.00	00.00
<u>หมวดค่าใช้สอย</u>		
-	00.00	00.00
<u>หมวดค่าวัสดุ</u>		
- RNeasy mini kit (50)		12,198.00
- RNase- free DNase set (50)		4,066.00
- RNeasy mini kit (50) and RNases free DNase set (50)		16,264.00
- Custom oligos syn, 6 oligo, 212 mers, 25nmole		3,175.76
- phusion flash high- fidelity PCR mater mix 100 rxn		4,066.00
- Peptide synthesis 16 aa, 80 % purity, 10 mg		15,793.20

- ODN 1585 200 ug	2,389.31
- Custom oligos syn, 6 oligo, 122mers,25nmole	1,827.56
- Custom oligos syn, 6 oligo, 213 mers,25nmole	3,190.74
- Mouse Flt3 ligand protein fragment, 10ug	21,453.50
- Fiixable viability dye eFlour 780, 500 test	27,606.00
- tube 5 ml 12*75 mm R-BTM, PS, SNAP CAP, 1000/CS	4,515.40
- Fetal bovine serum 100 ml x 1	4,280.00
- Nucleospin gel and PCR Clean-up, 50 prep/kit	3,745.00
- proteinase k, 100 mg	5,671.00
- DNASE I,100 U	3,745.00
- HORSE SERUM,500 ML	10,700.00
- Defibrinated sheep blood 200 cc	3,600.00
- Eppendorf	1,700.00

หมวดค่าครุภัณฑ์ (ถ้ามี)

-	00.00
รวมรายจ่าย	<u>289,986.94</u>

รายรับสูงกว่ารายจ่าย นำส่งส่วนการคลังจุฬาลงกรณ์มหาวิทยาลัย (เอกสารแนบ)

ดอกเบี้ย (ถ้ามี)

00.00

รวมจำนวนเงินที่นำส่งส่วนการคลังทั้งสิ้น

13.36

ขอรับรองว่ารายงานการรับ-จ่ายเงินข้างต้นเป็นความจริงทุกประการ


.....
(.....โกศลวิมล.....)
(หัวหน้าโครงการวิจัย)
.....4 / ธ.ค. / 2557.....

หมายเหตุ : รายงานตลอดโครงการเมื่อสิ้นสุดโครงการ/ปิดโครงการ

ชุดโครงการย่อย 2

แนวทางในการจัดทำรายงานการวิจัยฉบับสมบูรณ์
ทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล

"รายงานการวิจัย"เรื่อง "หนูทดลองโมเดลโรคเอสแอลอีโดยการขาดตัวรับ FcγRIIb เกิดความรุนแรงของการติดเชื้อในกระแสเลือดด้วยโมเดลการผูกและเจาะลำไส้ในหนูทดลองสูงขึ้นเนื่องจากภาวะ Macrophage exhaustion"

"Fc gamma receptor IIb deficient mice, a Systemic Lupus Erythematosus mouse model, susceptible to cecal ligation and puncture sepsis after preconditioned with double separated doses of endotoxin due to the prominent macrophage exhaustion"

กิตติกรรมประกาศ (Acknowledgement) ให้ระบุได้รับทุนอุดหนุนการวิจัยจาก
เงินอุดหนุนทั่วไปจากรัฐบาล ประจำปีงบประมาณ ๒๕๕๘

บทคัดย่อภาษาไทย

การติดเชื้อแทรกซ้อนและซ้ำซ้อนพบได้บ่อยในผู้ป่วย เอสแอลอี ซึ่งเป็นสาเหตุการตายที่สำคัญในผู้ป่วย ผิวจัยเลียนแบบภาวะการติดเชื้อซ้ำซ้อนโดยการฉีด endotoxin 2 ครั้ง ห่างกัน 5 วัน แล้วจึงทำการผ่าตัดเจาะและผูกกล้ามเนื้อเพื่อกระตุ้นการติดเชื้อในกระแสเลือดที่ 24 ชั่วโมงถัดไป ในหนูทดลองที่ขาดตัวรับชนิด FcGR11b ซึ่งเป็นโมเดลของโรคเอสแอลอีโมเดลหนึ่ง พบว่าหนูทดลองกลุ่มเอสแอลอีเมื่อเทียบกับหนู wild type นั้น มีการตอบสนองด้วยการสร้างไซโตคายต์หลังการฉีด endotoxin ครั้งแรกสูงมาก แต่ต่ำมากหลังการฉีดครั้งที่ 2 เข้าได้กับ การเกิด immune-exhaustion ซึ่งพบว่าการกระตุ้นให้เกิดการติดเชื้อในกระแสเลือดหลังจากกระตุ้นด้วย endotoxin 2 ครั้งนั้น ส่งผลให้เกิดการติดเชื้อที่รุนแรงมากในหนูเอสแอลอี (พิจารณาจากค่าที่บ่งบอกการทำงานของตับและไตจากการเจาะเลือด จากระดับไซโตคายต์ จากจำนวนแบคทีเรียที่พบในอวัยวะต่างๆและอัตราการตาย) เนื่องจากแมคโครเฟส เป็นเซลล์ที่มีความสำคัญอย่างยิ่งในการต่อสู้กับการติดเชื้อ จึงได้ทำการทดลองในเซลล์ดังกล่าวพบว่า แมคโครเฟสของหนูเองแอลอีเมื่อเปรียบเทียบกับหนูปกติตอบสนองต่อ endotoxin ในครั้งแรกโดยการสร้างไซโตคายต์ในระดับที่สูงมาก แต่มีระดับต่ำมากคล้ายกับที่พบในสัตว์ทดลองหลังจากการกระตุ้นครั้งที่สอง เข้าได้กับภาวะ macrophage paralysis

ดังนั้น immune-exhaustion ในสัตว์ทดลองน่าจะเกิดจาก macrophage paralysis ผลการทดลองแสดงให้เห็นความสำคัญของการติดเชื้อซ้ำซ้อนในผู้ป่วยเอสแอลอี โดยเฉพาะอย่างยิ่งในผู้ที่มีความผิดปกติของยีน FcGR11b การค้นหาความผิดปกติของ FcGR11b ในผู้ป่วยโรคเอสแอลอีอาจจะมึบทบาทในการเฝ้าระวังและป้องกันการติดเชื้อในกระแสเลือดในผู้ป่วยเองแอลอีอันเป็นสาเหตุการตายที่สำคัญของโรค

Abstract

Repeated bacterial infection in patients with Systemic Lupus Erythematosus (SLE) is common and sepsis is the leading causes of death. Despite proper responses to a single bacterial infection, the repeated infection might lead to immune exhaustion and severe sepsis. Then the bacterial susceptibility was tested with cecal ligation and puncture (CLP) after immune exhaustion induced by the 2-separated-doses of endotoxin (LPS) in FcGRIIb^{-/-} mice and wild type (WT) control.

In the comparison with wild type group, the prominent serum cytokine after 1st LPS injection followed by the apparently lower cytokines after 2nd LPS administration, cytokine exhaustion, was demonstrated in FcGRIIb^{-/-} mice. Subsequently, CLP was conducted after double doses of LPS preconditioning to test the immune suppression. Indeed, a higher mortality rate and a more severe sepsis (bacterial burdens, serum cytokines and organs injury) at 18h of CLP demonstrated in FcGRIIb^{-/-} mice. Because macrophages are the major immune cells responsible for sepsis immune responses, we tested *in vitro*. Interestingly, the stimulation with separated 2 doses of LPS in bone marrow-derived macrophage from FcGRIIb^{-/-} mice showed the higher cytokines responses after the 1st LPS stimulation in comparison with WT cells but the cytokines level were lower than WT cells after the 2nd LPS stimulation, supplementary to the *in vivo* results.

In conclusion, macrophage exhaustion was easier inducible in FcGRIIb^{-/-} cells in parallel to the immune paralysis, highly susceptible to CLP, in FcGRIIb^{-/-} mice compare with wild type group. These implied the importance of the repeated infections in patients with SLE, especially with FcGRIIb polymorphisms.

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6. คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

SLE, Systemic lupus Erythematosus; FcGR11b, Fc gamma receptor 11b; LPS, endotoxin; CLP, cecalligation and puncture model; UPCI, urine protein creatinine index; LDH, lactate dehydrogenase

ส่วนประกอบเนื้อเรื่อง

1. บทนำ (Introduction) ซึ่งกล่าวถึงเนื้อหาของเรื่องที่เคยมีผู้ทำการวิจัยมาก่อน ความสำคัญและที่มาของปัญหา วัตถุประสงค์และขอบเขตการวิจัย วิธีดำเนินการวิจัยโดยสรุปทฤษฎีและ/หรือแนวทางการคิดที่นำมาใช้ในการวิจัย ประโยชน์ที่คาดว่าจะได้รับ ฯลฯ

Systemic Lupus Erythematosus (SLE), the autoimmune disease with multi-factorial pathogenesis (1, 2) leads to multi-organs injury, showed a higher prevalence in Asia in comparison with other regions of the world (3-5). The defect of Fc gamma receptor 11b (FcGR11b), the only inhibitory signaling receptors in the FcGR family, is one of the genetic susceptibility to SLE (2, 6). Interestingly, FcGR11b polymorphisms also demonstrated the high prevalence in Asia which might due to the protective effect of the gene for malarial infection (7). Coincidentally, the association with FcGR11b polymorphisms in patients with SLE in Asia Pacific region is also common (3-5, 8). Perhaps FcGR11b polymorphisms could protect malaria in this region but, on other side of the coin, people with this immunological defect might easier develop SLE. In any case, sepsis, the systemic immune responses to the severe infection, is one of the important causes of death in patients with SLE (9). Indeed, the high susceptibility to bacterial sepsis in patients with SLE is well-known (9-13). However, there are debates whether the susceptibility to infection in patients with SLE is due to the *de novo* defects of immune response or immunosuppressive drugs. Unfortunately, the data on untreated symptomatic patients with SLE is very limited (14). Hence, the studies of infection in FcGR11b^{-/-} mice, one of the established SLE mouse model (6), could be resemble to untreated patients with SLE, especially with FcGR11b polymorphisms.

FcGRs binds with Fc portion of immunoglobulin mediate antigen uptake and cellular responses (15). In the mouse, FcGRs are classified into three activation receptors (FcGR1, FcGR3, FcGR4) and only one inhibitory receptor (FcGR11b) (16). The deficiency of all classes of FcGR in

mice (FcGR -/-) protected from sepsis (17) and FcGR11b-/- mice response well to the gram positive bacterial infection due to the effective bacterial killing (18). Nevertheless, a more severe sepsis with the cytokines storm demonstrated in FcGR11b-/- mice with the bacterial antigen preconditioning before bacterial administration. This data supported the overshoot cytokines responses after the repeated antigen exposures due to the lack of inhibitory signaling in these mice (18). On the other hand, FcGR11b-/- mice are protected from Plasmodium and Mycobacterium infection (7, 19), the inhibitory signaling defect seems to enhance the activating signaling and show a benefit in these infections. However, the susceptibility of FcGR11b-/- mice to polymicrobial sepsis is never tested.

Interestingly, sepsis-induced immune exhaustion or immunoparalysis, the high susceptibility to secondary infection after sepsis, has been recognized as an important sepsis complication (20, 21) and was demonstrated by several mouse and human models (22-26). In contrast, the preconditioning of LPS, a single or multiple doses, for 24h before CLP ameliorates sepsis severity in wild type mice has been showed in previous publications (27, 28). Despite the demonstrated protective effect to sepsis after 1 day of LPS preconditioning, we hypothesized that the immune exhaustion after LPS administration might existed in the earlier period and the repeated endotoxin exposure might mimic the repeated infection in patients. Subsequently, we selected the preconditioning with double doses of LPS with 5 days separation followed by CLP at 12h after the 2nd dose of LPS to demonstrate the immune exhaustion in our models. Of note, half-life of the important LPS-induced cytokines (TNF- α , IL-6 and IL-10) is approximately 0.5-1.5h (29), then at 12h, approximately 8 times of the half-life, should be adequate for avoiding the effect of these cytokines to the subsequent CLP surgery.

On the other hand, the lower macrophage immune responses, especially cytokines production, after repeated LPS activation is demonstrated, and was known by several terms such as "macrophage paralysis" or "macrophage exhaustion" or "macrophage tolerance" or "endotoxin tolerance" (30-32). Although, the association between immunoparalysis and macrophage exhaustion is not clearly demonstrated (31), it is possible that macrophage exhaustion might cause ineffective organisms clearance and increase infection susceptibility compatible with the definition of "immunoparalysis".

Indeed, macrophage contains both activating and inhibitory FcGRs which competing for immune complex ligands and the direction of this balance determines the direction of the cell responses (16, 33). We hypothesize that the defect in the inhibitory signaling of FcGR1b^{-/-} mice might result in a prominent response but is easier exhausted after repeated stimulation. Then we test immunoparalysis of FcGR1b^{-/-} mice *in vivo* and macrophage exhaustion *in vitro*, respectively.

2. เนื้อเรื่อง (Main Body) ซึ่งกล่าวถึงรายละเอียดเกี่ยวกับวิธีดำเนินการวิจัย (Materials & Method) ผลการวิจัย (Results) ฯลฯ

Materials and Methods

Animal and animal models

FcGR1b^{-/-} mice on C57BL/6 background were provided by Bolland S. (NIH, Maryland, USA). Other mice were purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. Female, 8- and 24-week-old C57BL/6 mice were used in the experiments. The animal protocols were approved by Faculty of Medicine, Chulalongkorn University followed the National Institutes of Health (NIH) criteria.

Cecal ligation and puncture model

Polymicrobial sepsis was induced by Cecal ligation and puncture model (CLP) slightly modified from the previous publication (29). Briefly, cecum were ligated at 10 mm from cecal tip with silk 2-0, punctured twice with a 21-gauge needle then gently squeezed to expel a small amount of fecal materials through an abdominal incision under isoflurane anesthesia. The incisions were closed with 2 layers by nylon 4-0 and normal saline (NSS) at 2 ml/kg was administered subcutaneously for the fluid replacement.

Cecal ligation and puncture with endotoxin pre-conditioning model

Because LPS induced-immuno-suppression is demonstrated (34, 35) and used as a sepsis-induced immunoparalysis model (26), we follow the principle in our experiments. In our model, the immunoparalysis, a condition susceptible to an infection, was tested by the severity of polymicrobial infection from CLP surgery. Endotoxin (LPS) of *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis,

USA) was administered intraperitoneally at 5 days (-120h) and 12h (-12h) before CLP surgery at the dose of 0.8 g/kg (approximately 20 μ g per 25 g mouse) and 4 g/kg (approximately 100 μ g per 25 g mouse), respectively. Subsequently, CLP was performed as previously mentioned.

To measure inflammatory cytokines after LPS injection, 50 μ l of blood was collected through tail vein nicking at 0h (2h before LPS administration) and at 1, 3 and 6h after. In addition, in separated experiments, blood from tail vein nicking was also collected before CLP (0h) and at 3h and 6h to measure time-courses of bacterial burdens and serum cytokines after CLP surgery. Otherwise, blood was collected through cardiac puncture at sacrifice time under isoflurane anesthesia at 18h or 96h after CLP for sepsis injury analysis or survival test, respectively.

Blood chemistry, supernatant media analysis and urine protein

For the natural history of FcGR1b^{-/-} mice, serum from tail vein nicking and spot urine was collected once a month from 2 to 12-month-old. Serum and urine creatinine were measured by (QuantiChrom Creatinine Assay, DICT-500, BioAssay, CA, USA). Spot urine protein was measured by Bradford protein assay. Urine protein creatinine index (UPCI), a representative of 24h urine protein, were measured from spot urine by equation; urine protein/urine creatinine.

Serum cytokines after LPS were measured by Luminex-based multiplex technology multi-analysis panels 8-plex cytokines assay (BioPlex, Bio-RAD, CA, USA) to explore the panel of pro and anti-inflammatory cytokines (TNF- α , IL-6, IL-1 β , IFN- γ and IL-2, IL-4, IL-5, IL-10, respectively) according to the manufacturer's protocol. Then the selected important cytokines (TNF- α , IL-6, IL-10) were measured by ELISA assay (ReproTech, NJ, USA) in supernatant media and in mouse serum after CLP surgery. Organs injury was determined by blood urea nitrogen (QuantiChrom Urea Assay, DIUR-500, BioAssay), serum creatinine (Scr) (QuantiChrom Creatinine Assay, DICT-500, BioAssay), alanine transaminase (ALT) (EnzyChrom ALT assay, EALT-100, BioAssay) and lactate dehydrogenase (LDH) (EnzyChrom LDH assay, EDLC-100, BioAssay). Blood bacterial burdens were determined by plating a serial volume of blood into blood agar (Oxoid, Hampshire, UK) at 37°C then counted bacterial colonies after 24h of incubation. For blood polymorphonuclear cell (PMN) and mononuclear cell count, 5 μ l of blood mixed in 85 μ l of 3% acetic acid for the hemolytic reaction and total leukocytes was counted by a hemocytometer. In parallel, blood smeared on a

glass slide was stained by Wright stain and counted with x100 magnification in 100 fields to determine the percentage of PMN and mononuclear cells. The total number of cells was calculated by total leukocyte count from hemocytometer multiplied by the percentage of cells from the Wright stain glass slide.

Anti-dsDNA antibodies

Anti-dsDNA antibodies were measured by coating ELISA plates with salmon sperm DNA as published previously (36). In short, salmon sperm DNA (Life Technologies, Invitrogen™, MA, USA) passage through a 45-mm filter (Minisart, Sartorius, Germany) for selecting double stranded DNA then coated into ELISA plate with the dose at 100 µg/plate. The plates were dry, blocked and incubated with serial dilutions of serum for 1 hour at 37°C then peroxidase conjugated Fab'2 goat anti-mouse IgG 1/2,000 in 1% bovine serum albumin (BSA) in phosphate buffer solution (PBS) followed by TMB peroxidase substrate (Biolegend, California, USA). The plate was developed in the dark room for 10 min then added TMB stop solution and read with microplate photometers with a wavelength at 410 nm.

Bone Marrow Derived Macrophages

Macrophages were derived from bone marrows (BM) follow the established procedure (37). In short, BM cells from FcGR1b^{-/-} and wild type mice obtained from femurs were centrifuged at 1,000 rpm in 4 °C for 10 min. Then cell were incubated in high glucose DMEM supplement with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, HEPES with sodium pyruvate and 20% L929-conditioned media in a humidified 5% CO₂ incubator at 37 °C for 7 days. The cells were harvested at the end of the culture period using very cold PBS and confirmed macrophage phenotype with anti-F4/80 and anti-CD11c antibodies (BioLegend, CA, USA).

Macrophage endotoxin tolerance protocol

Macrophage endotoxin tolerance protocol followed the protocols from the previous publications (38, 39). Briefly, endotoxin (LPS) *Escherichia coli* 026:B6 (Sigma) at 10 or 100 ng/ml was used to activate macrophage 1x10⁵ cells/well in 96 well polystyrene tissue culture plate. To see the difference between single or double LPS stimulations, 2 groups of experiments were performed. For the single LPS stimulation (N/LPS), there was no endotoxin at the 1st 24h of the incubation then

the plate was washed with phosphate buffer solution (PBS), refilled fresh media and treated with LPS at 10 ng/ml (N/LPS10) or 100 ng/ml (N/LPS100). For the double LPS stimulation (LPS/LPS), LPS at 10 or 100 ng/ml was treated for the 1st 24h and treated with the 2nd dose of LPS at 10 ng/ml or 100ng/ml as indicated. The culture supernatant was collected at 1, 2, 4, 6 and 24h after the 2nd LPS incubation in all groups and stored at -80 °C until cytokine determination by ELISA assays (ReproTech). After the incubation, cell viability was measured by MTS assay (One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) according to the manufacturer's instruction (40). In short, 20 μ l of MTS was added to the culture plates for 2h at 37°C in 5% CO₂ incubator then read with microplate photometers with a wavelength at 450 nm. All *in vitro* experiments demonstrated cell viability more than 95% (data not showed).

Macrophage intracellular killing activity and phagocytosis protocol

The protocol followed the previous publication (41). BM derived macrophage at 1×10^5 cells in 200 μ l of DMEM per well were dispensed into the flat bottom 96-well plate and incubated at 37 °C in a humidified 5% (v/v) CO₂ incubator for 24 h, before gently washing with culture media to remove non-adherent cells. Subsequently, the cells with endotoxin (LPS) *Escherichia coli* 026:B6 (Sigma) at 10 or 1,000 ng/ml, LPS10 or LPS1000, respectively, and incubated with 1×10^7 CFU of *E. Coli* per well.

Then after 15 min of incubation, supernatants were aspirated and cells were washed gently with DMEM to remove un-ingested microorganisms. The supernatant and well washing fluids, containing the non-phagocytized *E. Coli*, were combined, plated in serial dilutions on Tryptic soy agar plates and counted for bacterial colonies for the representative of the non-phagocytic bacteria which reversed correlated with the phagocytic activity. On the other hand, the cellular part, phagocytosed macrophage, was further incubated with 200 μ l of DMEM for 2h to determine intracellular bacterial killing activity. The wells were gently scraped and washed with 200 μ l distilled H₂O to induce cell lysis and the serial dilution of the lysate were plated on Tryptic soy agar, incubated at 37 °C for 16 h and determine the bacterial colony count. The number of bacteria from the cell lysate represented the intracellular killing activity.

Statistical analysis

Data are shown as the mean \pm SE and differences between groups were examined for statistical significance using the unpaired Student t-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with 2 and 3 groups, respectively. Survival analyses were evaluated using the log-rank test by observation and recorded every 6–24h then all mice were sacrifice at 96h after CLP. *P* values < 0.05 were considered statistically significant. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Result

Fc gamma receptor IIb deficient mice susceptible to cecal ligation and puncture sepsis in symptomatic SLE mice and asymptomatic SLE group preconditioned with LPS

At 24-weeks-old, FcGR11b^{-/-} mice showed increased anti-dsDNA with proteinuria but normal kidney function as evaluated by serum creatinine (Scr) (Fig 1). This natural history allows the experiments in 2 groups of mice in correspondence with patients with SLE; asymptomatic genetic prone group (8-weeks-old) and symptomatic proteinuria group (24-weeks-old). To see the susceptibility to bacterial sepsis without LPS preconditioning in these 2 groups, CLP surgery was performed in comparison with age-matched wild type control. In the absence of LPS, only symptomatic, but not asymptomatic, FcGR11b^{-/-} mice showed higher sepsis mortality rate compare with wild type (Fig 2A, B) supports the correlation between SLE disease activity and infection susceptibility (9).

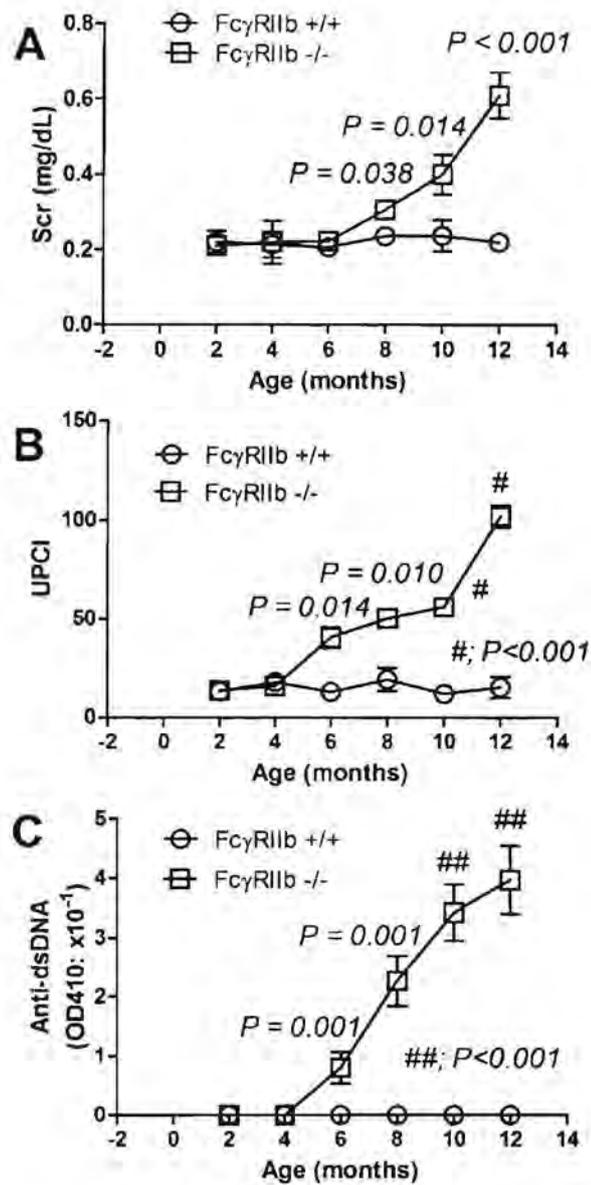


Figure 1. The natural history of Fc γ RIIb $^{-/-}$ and wild type (Fc γ RIIb $^{+/+}$) mice as determined by serum creatinine (Scr), urine protein creatinine index (UPCI) and anti-dsDNA ($n=4-5$ /groups).

Then we test the effect of LPS preconditioning in asymptomatic mice and determine the severity of immunoparalysis by the mortality rate of CLP surgery (20, 21).

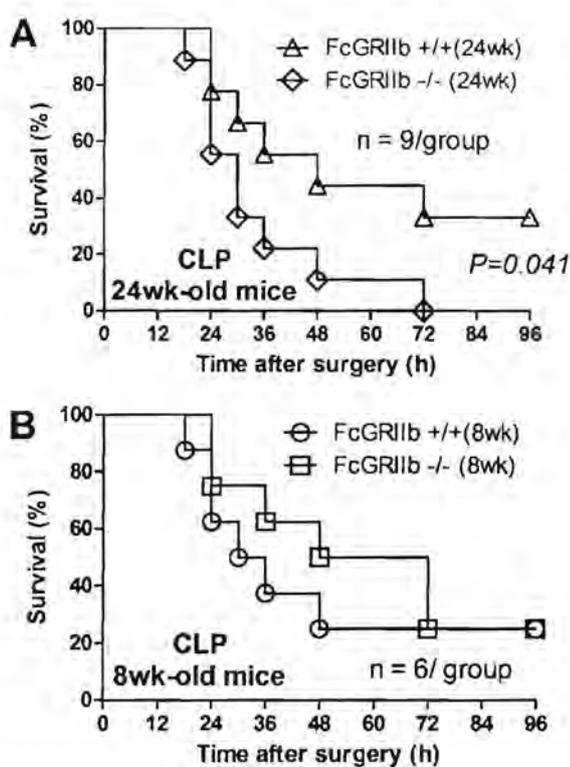


Figure 2. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery in asymptomatic *FcGR11b*^{-/-} mice (8-wk-old) (A) and symptomatic, proteinuria positive but normal serum creatinine, *FcGR11b*^{-/-} mice (24-wk-old) (B) in comparison with age-matched wild type mice (*FcGR11b*^{+/+}).

Although the high CLP mortality rate found in both wild type and *FcGR11b*^{-/-} mice after LPS-preconditioning, *FcGR11b*^{-/-} showed the higher mortality rate. Whereas all wild type and *FcGR11b*^{-/-} mice die within 72h and 36h, respectively, in CLP with LPS, the survival rate at 30% and 22% found in wild type and *FcGR11b*^{-/-} mice, respectively, in CLP alone (Fig 3A, B). These results supported the immunoparalysis occur in both wild type and *FcGR11b*^{-/-} mice after LPS preconditioning but *FcGR11b*^{-/-} mice showed the more severe immunoparalysis (Fig 3C).

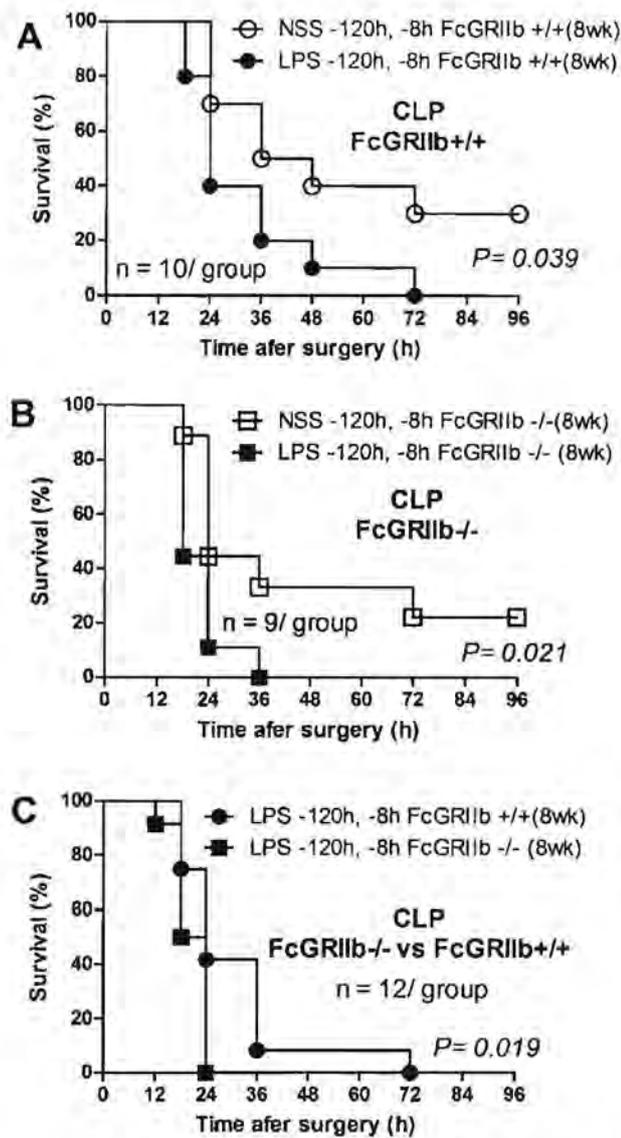


Figure 3. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery preconditioning with 2 separated doses of LPS at 120h and 12h prior to CLP, CLP in endotoxin preconditioning model, in comparison with normal saline (NSS) placebo injection within wild type mice (FcGR11b+/+) (A) and FcGR11b-/- group (B) were showed. Survival analysis of CLP in endotoxin induced immunoparalysis model between wild type (FcGR11b+/+) and FcGR11b-/- mice (C) was also demonstrated.

The cytokine responses after LPS injections and after superimposed by cecal ligation and puncture

The luminex-based multiplex system was used to explore the difference in the cytokines responses after LPS administration between asymptomatic FcGR11b^{-/-} mice versus wild type.

Among the pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β and IFN- γ), we found that most of the pro-inflammatory cytokines, except for TNF- α , were significantly higher in FcGR11b^{-/-} mice at 1h after the 1st dose of LPS administration (Fig 4A-C). TNF- α , IL-6, IL-1 β and IFN- γ at 1h after LPS

administration in FcGR11b^{-/-} mice and wild type were 21,851 \pm 3,200, 3,525 \pm 117, 350 \pm 38, 21 \pm 2 pg/ml and 12,453 \pm 3,925, 2,301 \pm 157, 54 \pm 11, 3 \pm 1 pg/ml, respectively. In parallel, for the anti-

inflammatory cytokines (IL-2, IL-4, IL-5 and IL-10), all of these cytokines, except for IL-5, were

higher in FcGR11b^{-/-} mice at the 1st h after the 1st dose of LPS (Fig 4D-F). In detail, IL-2, IL-4, IL-5 and IL-10 at 1h after LPS administration in FcGR11b^{-/-} mice and wild type were 26.2 \pm 3.4, 10.8 \pm 1.1,

34.5 \pm 3.5, 958 \pm 106 pg/ml and 12.6 \pm 3.3, 3 \pm 0.4, 27.1 \pm 3.6, 575 \pm 104 pg/ml, respectively. These

results supported the prominent cytokines responses in FcGR11b^{-/-} mice reported previously (18).

Interestingly, at 1h and/or 2h of the 2nd LPS administration with the 5 times higher dose of LPS, all of these cytokines, except for IL-1 β and IL-5, were significantly lower than the 1st administration in

FcGR11b^{-/-} mice (Fig 4). In contrast, in wild type mice, only IFN- γ , IL-2, IL-4 and IL-10 were lower

and IL-1 β was higher in some time-point of 2nd LPS administration compared with the matched time-point of the 1st LPS injection (Fig 4). The endotoxin tolerance, determined by the lower

cytokines responses after 2nd dose of LPS, was easier demonstrated in FcGR11b^{-/-} mice compare

with wild type. Moreover, the severity of endotoxin tolerance, determined by the cytokine level

difference after matched time-point of 1st and 2nd LPS administration, was higher in FcGR11b^{-/-} mice

(Fig 4 inset graph).

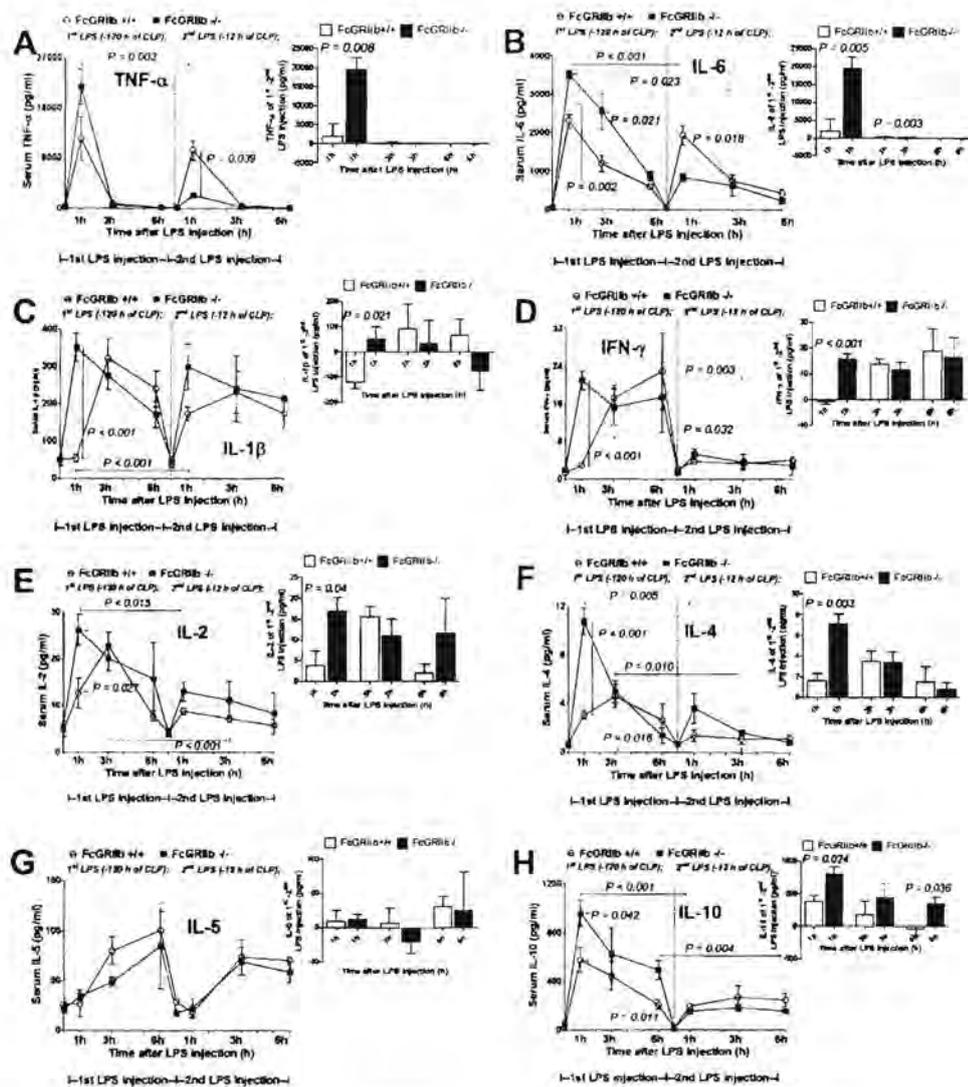


Figure 4. Serum cytokines in wild type (*FcGR11b*^{+/+}) or *FcGR11b*^{-/-} mice after at 1, 3 and 6h after 1st LPS injection (0.8 g/kg) and 2nd LPS injection (4 g/kg) as measured by TNF- α (A), IL-6 (B), IL-1 β (C), IFN- γ (D), IL-2 (E), IL-4 (F), IL-5 (G) and IL-10 (H) was demonstrated. To emphasize the difference of serum cytokines after 1st and 2nd doses of LPS, the delta change of serum cytokine response at the matched-time=points after both LPS injection was showed as inset graph. (n= 5-7 per group)

It is interesting to note that, even with the 5 times higher LPS dose of 2nd LPS administration, most cytokines level was lower than the 1st dose implied endotoxin tolerance status in both

FcGR11b^{-/-} and wild type mice. Subsequently, we tested the severity of polymicrobial infection in these mice with CLP surgery and selected to explore only frequently mentioned sepsis cytokines (TNF- α , IL-6 and IL-10) *in vivo*.

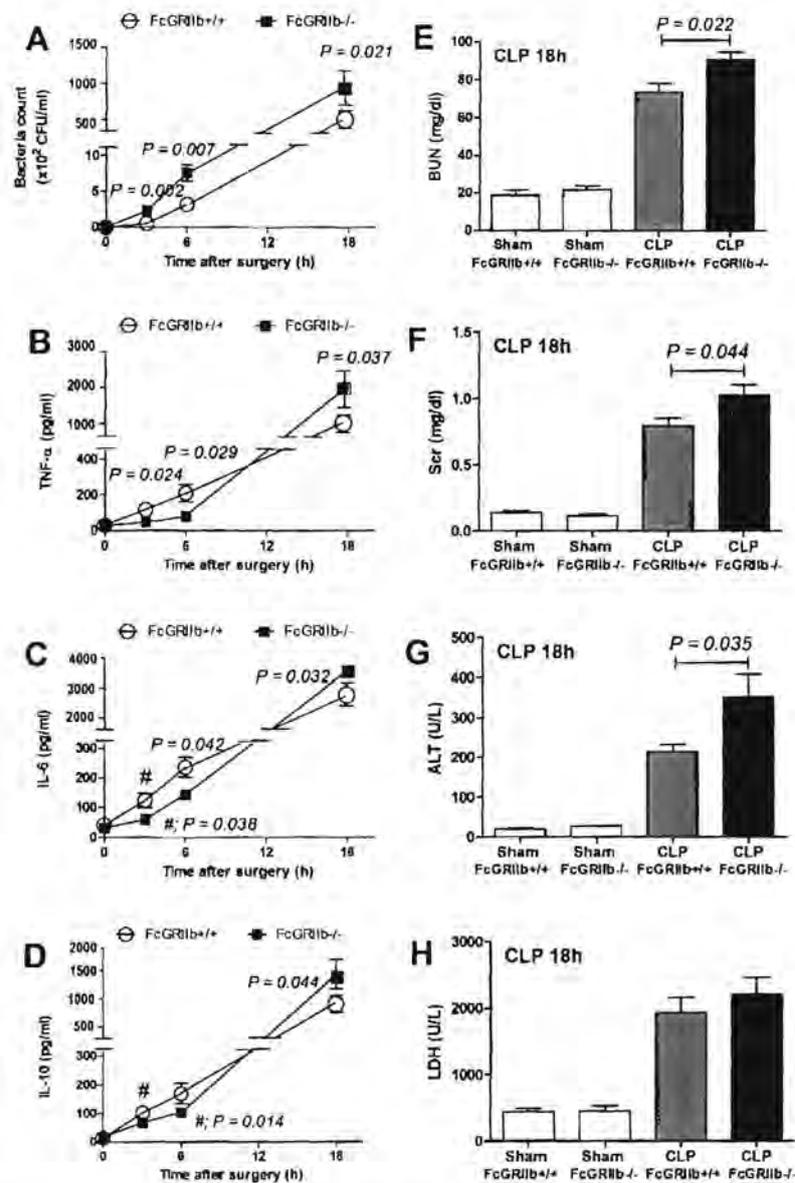


Figure 5. The time-course of bacterial burdens after cecal ligation and puncture (CLP) in blood bacterial burdens (A), mononuclear cell (B), PMN (C), TNF- α (D), IL-6 (E), IL-10 (F) and the severity of organs injury after 18h of wild type (FcGR11b^{+/+}) and FcGR11b^{-/-} as demonstrated by

serum creatinine (Scr) (G) and alanine transaminase (ALT) (H) was showed. (n= 5-7/ time-point and group)

Interestingly, bacterial burdens in FcGR11b^{-/-} mice were higher than wild type in all selected time-points (3, 6 and 18h) after CLP (Fig 5A). Blood bacterial count ($\times 10^2$ CFU/ml) in FcGR11b^{-/-} mice and wild type at 3, 6 and 18h were 3.1 ± 0.3 , 7.6 ± 1.1 , 964 ± 137 and 0.6 ± 0.2 , 3.3 ± 0.6 , 518 ± 73 , respectively.

Moreover, pro-inflammatory cytokines (TNF- α and IL-6) and anti-inflammatory cytokine (IL-10) were higher in wild type mice at 3 and 6h and only 3h after CLP, respectively (fig 5B-D). Serum TNF- α , IL-6 and IL-10 at 3h and 6h in FcGR11b^{-/-} versus wild type were 48 ± 9 , and 80 ± 7.1 , 69 ± 9 and 145 ± 18 , 71 ± 6 and 104 ± 4 pg/ml versus 120 ± 24 and 211 ± 49 , 124 ± 24 and 235 ± 33 , 102 ± 7 and 170 ± 36 pg/ml, respectively. In contrast, at 18h after CLP, all of these cytokines and most of the organs injury biomarkers (Scr for kidney injury and ALT for liver injury) were higher in FcGR11b^{-/-} mice (Fig 5B-H). Despite the prominent responses to LPS in FcGR11b^{-/-} mice mentioned earlier, cytokines responses at the early phase of CLP after LPS preconditioning were stun in comparison with wild type implied the more severe immunoparalysis.

Perhaps, severe immunoparalysis at the early phase of sepsis might associate with the higher bacterial burdens leading to the higher sepsis severity (Fig 5) and mortality rate (Fig 3C) in FcGR11b^{-/-} mice.

Bone marrow derived macrophage of FcGR11b^{-/-} mice showed higher cytokine responses in the single incubation of LPS but lower responses in the double incubation of LPS

The previous results demonstrated that FcGR11b^{-/-} mice, an inhibitory signaling deficiency, showed a very high initial response, but subsequently follow with a more apparent exhaustion after LPS stimulation. Because macrophage might be responsible for the exhaustion *in vivo* then we tested FcGR11b^{-/-} macrophages response to endotoxin incubations *in vitro* in comparison with wild type cell.

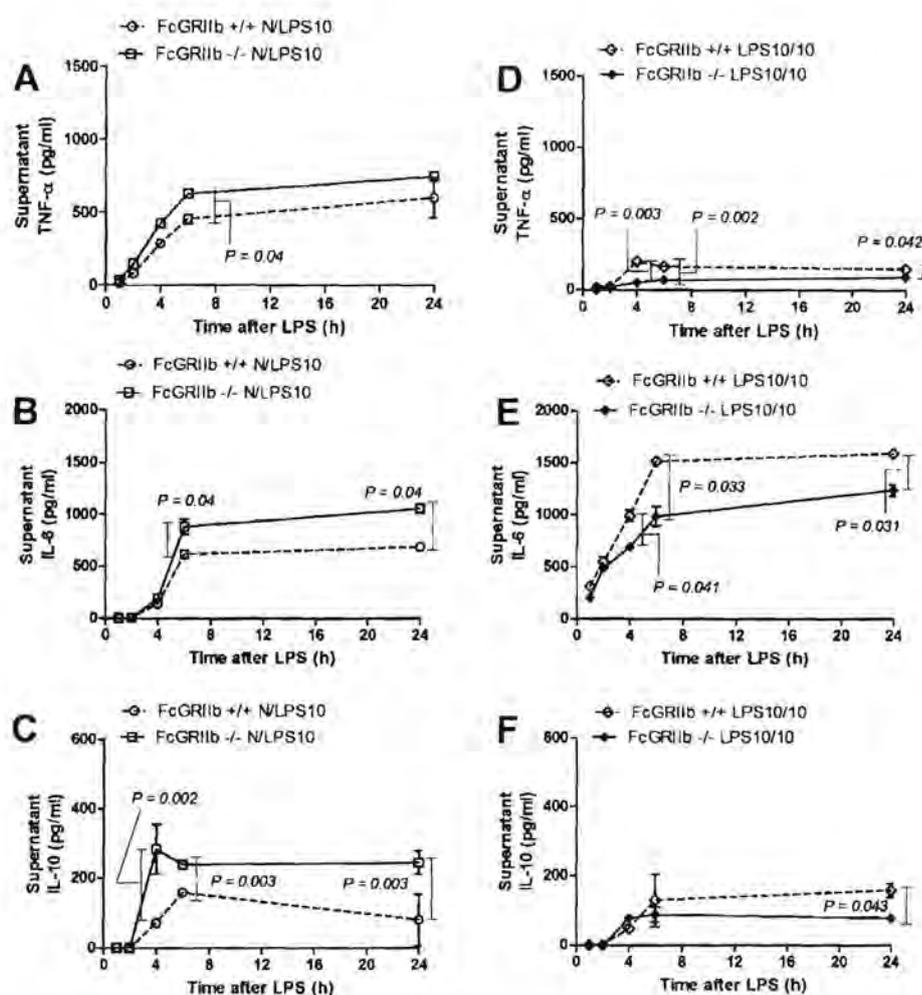


Figure 6. The cytokine responses in supernatant media from macrophages of FcGR11b^{-/-} or wild type (FcGR11b^{+/+}) after activated with only once low dose LPS (N/LPS10) as measured by TNF- α (A), IL-6 (B), IL-10 (C) and after activated with twice low dose LPS (LPS10/10) as measured by TNF- α (D), IL-6 (E), IL-10 (F) was showed. (Separated experiments were done in triplicate)

In parallel with the *in vivo* results, higher cytokines, at least in some time-points (3-24h), found in the supernatant of FcGR11b^{-/-} macrophages with the single low dose LPS (non LPS at the 1st 24h of the incubation followed by LPS dose at 10 μ g/ml; N/LPS10) (Fig 6A-C). Then TNF- α and IL-10, but not IL-6, was lower in the double low dose of LPS stimulation (LPS 10 μ g/ml for 24h then washed and add the same 2nd dose; LPS10/10) (Fig 6D-F). Then, a higher dose of LPS was used. Once again, FcGR11b^{-/-} macrophages showed the higher responses than wild type in the single high dose of LPS (N/LPS100) (Fig 7A-C). But the cytokines responses of macrophages primed with

the high dose of LPS seems to depend on doses of the 2nd LPS. In high LPS followed by low dose LPS (LPS100/10), all cytokines were detected at the low level (Fig 7D-F) and the difference between wild type and FcGR11b^{-/-} cells were subtle. But the apparent lower TNF- α and IL-10 in FcGR11b^{-/-} cells appeared again with the higher 2nd dose of LPS (LPS100/100) (Fig 7G-I). To clarify the macrophage exhaustion, the lower cytokines level after double LPS exposure compare with single LPS exposure, the cytokines level after single and double LPS exposure at 6 and 24h was demonstrated (Fig 8). With the double low dose of LPS (LPS10/10), macrophage exhaustion could be demonstrated with only the lower TNF-a in wild type cell but lower both TNF-a and IL-10 in FcGR11b^{-/-} macrophages (Fig 8A-C).

Interestingly, macrophage exhaustion, lower cytokine in double LPS exposure compare with single LPS, could be demonstrated only by TNF-a and IL-10 in FcGR11b^{-/-} cells but with TNF-a alone for wild type cell with the double low dose of LPS (LPS10/10) (Fig 8A-C). On the other hand, with the higher dose of LPS stimulation, the exhaustion could be demonstrated in all cytokines in FcGR11b^{-/-} cells but only in some condition in wild type cell (Fig 8D-F).

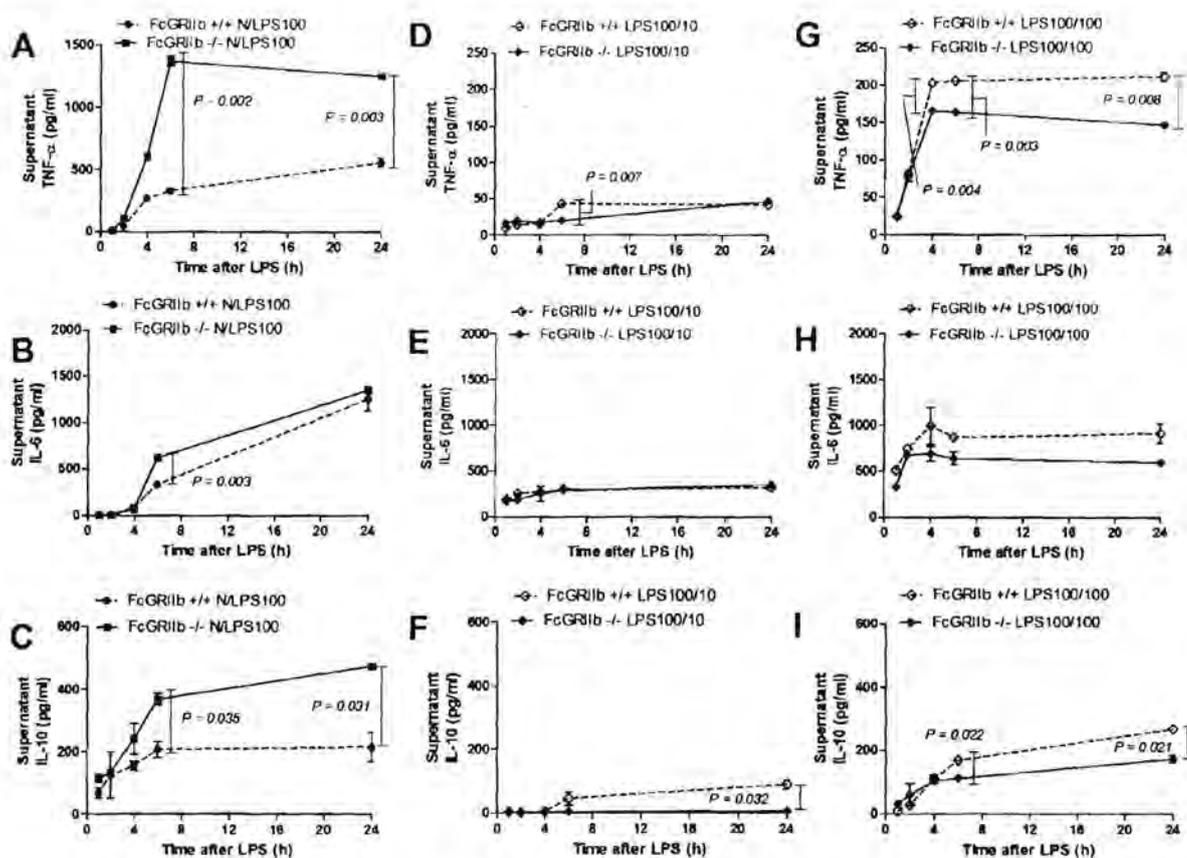


Figure 7. The cytokine responses in supernatant media from macrophages of FcGR11b^{-/-} or wild type (FcGR11b^{+/+}) after activated with only once high dose LPS (N/LPS100) as measured by TNF- α , IL-6, IL-10 (A-C) and double LPS doses, low and high dose LPS (LPS100/10 and LPS100/100, respectively), (D-F) were demonstrated. (Separated experiments were done in triplicate)

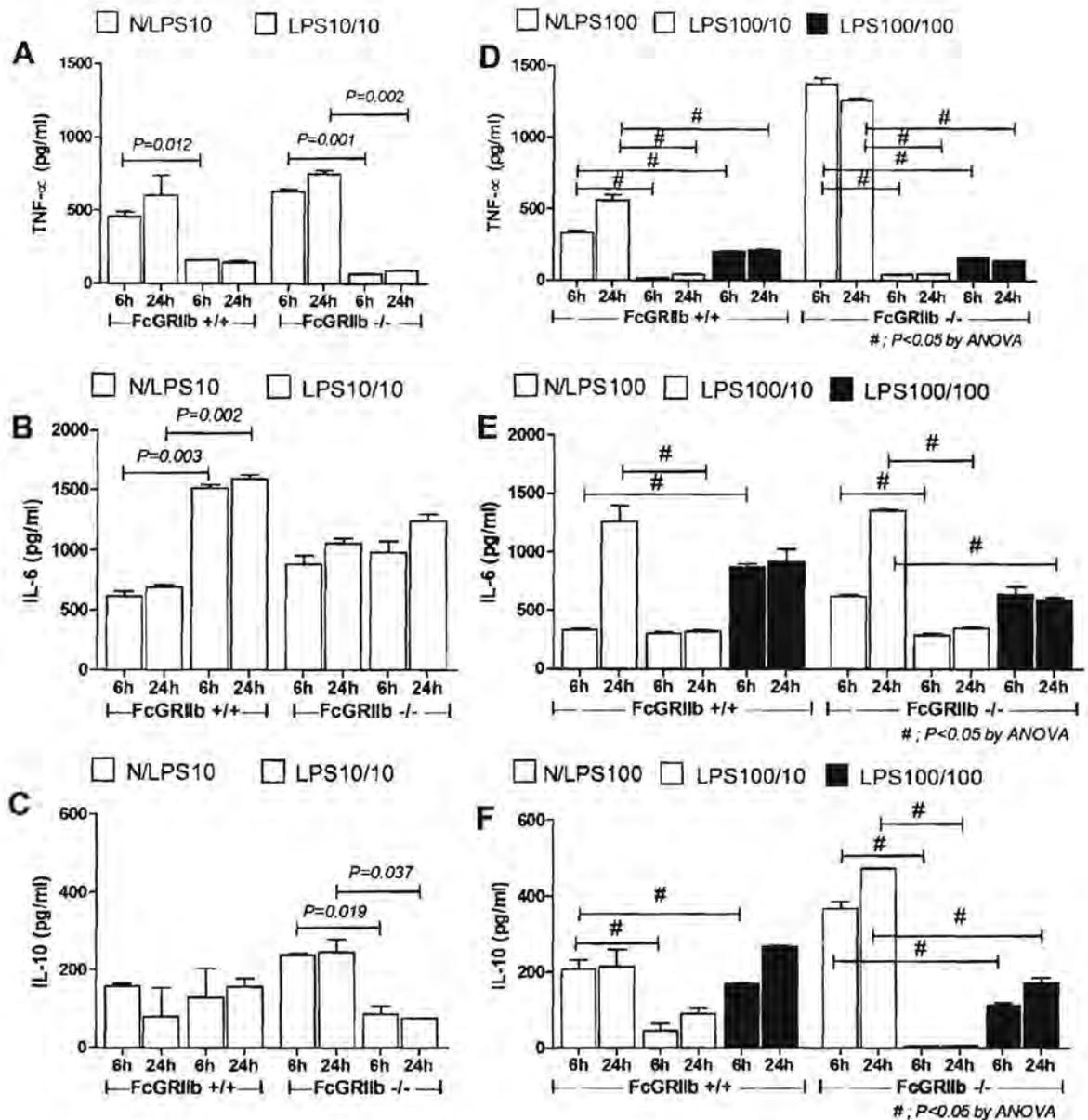


Figure 8. The macrophage endotoxin tolerance emphasized by the difference in cytokine responses (TNF- α , IL-6, IL-10) in supernatant media from macrophages of FcGR11b $^{-/-}$ or wild type (FcGR11b $^{+/+}$) after activated with only once low dose (N/LPS10) versus twice low dose of LPS (LPS10/10) (A-C) and the difference among only once high dose (N/LPS100) versus twice high and then low dose of LPS (LPS100/10) versus twice high dose of LPS (LPS100/100) (D-F) were demonstrated. (Separated experiments were done in triplicate)

Macrophage exhaustion could not be demonstrated by IL-6 responses both in wild type and FcGR11b^{-/-} cells with double low dose LPS stimulation. In parallel, with the comparison among single high dose LPS (N/LPS100) with double LPS dose with low and high 2nd LPS dose (LPS100/10 and LPS100/100, respectively), macrophage exhaustion could be shown by all cytokines despite a less prominent in IL-6 response of the wild type cell (Fig 8D-F). It seems the macrophage exhaustion occur in both knock-out and wild type cells but more prominent in FcGR11b^{-/-} cells.

In contrast, the phagocytosis and killing activity of FcGR11b^{-/-} macrophages were better than wild type cells and there was a non-significant exhaustion in phagocytosis and killing activity after LPS stimulation (Fig 9). Although, the killing activity of FcGR11b^{-/-} macrophage should be able to control sepsis severity, perhaps the more severe bacterial sepsis severity in FcGR11b^{-/-} mice might due to the lower number of mononuclear cell after sepsis (Fig 5).

3. อภิปราย / วิจารณ์ (Discussion) ผลการทดลอง / ผลการวิจัยที่ได้ทั้งหมด (ทั้งที่เป็นและไม่เป็นไปตามสมมติฐานที่ตั้งไว้)

FcGR11b deficiency is one of the genetic defects of SLE and FcGR11b polymorphism with a lower gene activity commonly reported in patients with SLE in Asia (3-5, 8). Additionally, sepsis is the leading cause of death in patients with SLE (9). We showed a high susceptibility to CLP in symptomatic SLE mice but not in asymptomatic group in comparison with age-match wild type control. In asymptomatic genetic prone mice, the high susceptibility to CLP was showed only after with repeated endotoxin induced immunoparalysis. The more severe macrophage paralysis in FcGR11b^{-/-} macrophages might responsible for the more immunoparalysis in mice leading to higher sepsis severity after CLP surgery.

The susceptibility to bacterial infection in patients with SLE is associated with several factors; immunosuppressive drugs, activity of disease, organs involvement, etc (9). Studies on FcGR11b^{-/-} mice allow for exploring SLE without several confounding factors, especially

immunosuppressive drugs. As expected, the higher mortality rate of sepsis was showed in 24-wk-old FcGR11b-/- mice, positive proteinuria but normal Scr classified into symptomatic SLE group, in comparison with age-matched wild type mice. These results supported the association between SLE disease activity and bacterial infection susceptibility reported previously (9). Interestingly, the mortality rate of sepsis without LPS preconditioning in asymptomatic 8-wk-old FcGR11b-/- mice, positive anti-dsDNA without proteinuria, did not different to wild type mice supported the effective immune responses to bacterial infection previously published (18).

More prominent endotoxin-induced immunoparalysis in FcGR11b-/- mice demonstrated by the high mortality rate of CLP sepsis

The immunoparalysis was induced by the double separate LPS administration and the severity of immunoparalysis, the condition with a more susceptible to infection, was determined by the severity of CLP sepsis (20). With this model, there was a higher mortality rate of CLP after LPS preconditioning compare with NSS placebo control within either 8-wk-old FcGR11b-/- or wild type mice demonstrated immunoparalysis occur in both groups. Nevertheless, FcGR11b-/- mice showed a higher mortality rate than wild type implied a more severe immunoparalysis.

Despite immunoparalysis could be demonstrated by several biomarkers (42), cytokines responses are frequently used. Then we tested cytokines responses in panels of pro- and anti-inflammatory cytokines, TNF- α , IL-6, IL-1 β , IFN- γ and IL-2, IL-4, IL-5, IL-10, respectively, with luminex-based measurement in mice with double doses of LPS administration. Most of the cytokines selected to measure were mainly produced by macrophage except for IFN- γ and IL-5 which produced prominently by NK cell or T cell and mast cell, respectively. With double dose of LPS administration by the 2nd dose of the 5 times higher than the 1st dose, all of these cytokines response after the 2nd LPS stimulation were not higher than the responses after the 1st LPS dose. These results suggested immunoparalysis in both FcGR11b-/- and wild type mice. Interestingly, in comparison with wild type mice, most of the pro- and anti-inflammatory cytokines, except for IL-5, were higher in FcGR11b-/- mice after the 1st LPS stimulation, implied the vigorous cytokines responses in FcGR11b-/- mice. Then after 2nd LPS administration, all cytokine except for IL-1 β and IL-5 were lower than the 1st responses in both wild type and but with a more prominent difference,

demonstrated by the cytokine difference between 1st and 2nd LPS administration, in FcGR11b^{-/-} mice. These results demonstrated a more severe immunoparalysis in FcGR11b^{-/-} group. Of note, most of these cytokines produced from macrophages except for IL-5 and IFN- γ which produced mainly from mast cell and NK cell, respectively. Although functional FcGR11b expression on mast cell (43) and murine NK cell (44) were reported, IFN- γ , but not IL-5, response differently between FcGR11b^{-/-} and wild type mice. These implied the difference of FcGR11b function between these cells. More experiments needed but out of the scope of this article.

Nevertheless, the double LPS preconditioning seems to affect CLP severity. At the initial time-point of CLP surgery, 12h after 2nd dose of LPS, there was non-difference in TNF- α , IL-6 and IL-10 between LPS preconditioning and NSS control (data not showed) supported by the base-line value before CLP surgery (Figure 5). Interestingly, blood bacterial burdens were higher with the lower of these cytokines in FcGR11b^{-/-} mice compare with wild type at 3h and 6h after CLP surgery. However, at 18h after CLP the sepsis severity was more severe in FcGR11b^{-/-} mice as demonstrated by bacterial burdens, cytokines and organs injury. Perhaps, the initial cytokine responses were needed for the initial innate immune responses to control the infection and the loss of the initial control in FcGR11b^{-/-} mice due to LPS preconditioning induced a more severe sepsis. These results support the importance of the initial bacterial control, especially in patients with SLE and /or FcGR11b polymorphism.

More prominent immunoparalysis in FcGR11b^{-/-} macrophage demonstrated by cytokine responses after LPS stimulation

The LPS induction *in vitro* alters macrophage characteristics from classical pro-inflammatory macrophage responses into a less pro-inflammatory stage of the macrophage (38) implied the importance of macrophage in immunoparalysis. With the double low doses of LPS (LPS10/10), macrophage exhaustion could be demonstrated in both FcGR11b^{-/-} and wild type but cytokines production after 2nd dose of LPS was lower in FcGR11b^{-/-} macrophages. Moreover, the exhaustion seems to be more apparent with the higher 1st dose of LPS which needed the higher 2nd dose of LPS to re-stimulate. With the initial high dose of LPS followed by a low dose (LPS100/10), only subtle cytokines responses were demonstrated. But with the larger 2nd LPS dose (LPS100/100),

the difference between FcGR11b-/- and wild type appeared again. However, FcGR11b-/- macrophages produced less cytokines levels after 2nd dose of LPS either with high or low LPS doses. Then LPS could induce a more apparent macrophage paralysis in FcGR11b-/- cells resulted in immunoparalysis state in mice which demonstrated by the higher CLP sepsis severity.

In conclusion, we demonstrated the impact of the repeated infection in patients with SLE through the CLP preconditioning with LPS in FcGR11b-/- mice. Despite a good response to the only once bacterial infection in FcGR11b-/- mice, the responses to the repeated exposure might be impaired lead to a more severe bacterial burdens and infection. The repeated infection in patients with SLE could be more severe due not only to hyperimmunoglobulin induced hyper-immuneresponse and sepsis (18), but also from immunoparalysis with the higher bacterial burdens as currently demonstrated. In the translational aspect, we suggested that the repeated infection in patients with SLE should be vigorously concerned and the FcGR11b polymorphism screening in Asian patients with SLE might be a useful clinical practice.

4. สรุปและเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

The next final part of the project is the experiments to see if macrophage of FcGR11b knock-out which demonstrated exhaustion but intact killing activity show shorter half-life in comparison with wild type cell. If the last experiment results go along with the hypothesis then FcGR11b knock-out mice will be susceptible to sepsis due to macrophage exhaustion and shorten half life of macrophage which will be stronger support our in vivo results.

Regarding to the translation, the exploration of FcGR polymorphisms in patients with SLE might be beneficial for the prediction of sepsis and might require a more aggressive antibacterial drug at the beginning of the simple infectious diseases. More studies will be needed.

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2. ภาคผนวก (Appendix) ถ้ามี

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สัญญาเลขที่ GRB_BSS_๕๕_๕๘_๓๐_๑๓

โครงการวิจัย เรื่อง โครงการวิจัยการศึกษาเชิงระบบของการส่งสัญญาณผิดปกติในเซลล์
ของระบบภูมิคุ้มกันในหนูโรคลูปัส

รายงานการรับ-จ่ายเงิน *

	ประมาณการ	งบที่เกิดขึ้นจริง
<u>รายได้</u>		
เงินอุดหนุนงบประมาณแผ่นดิน	290,000.00	00.00
รวมรายได้	00.00	00.00
<u>รายจ่าย</u>		
<u>หมวดค่าจ้างชั่วคราว</u>		
ค่าจ้างนักวิจัยเต็มเวลาปริญญาตรี		140,000.00
<u>หมวดค่าตอบแทน</u>		
-	00.00	00.00
<u>หมวดค่าใช้สอย</u>		
-	00.00	00.00
<u>หมวดค่าวัสดุ</u>		
- CALF THYMUS DNA SOLUTION, 5 ML		17,441.00
- Collagenase D, 500 mg		8,560.00
- Human activating transcription factor 3(ATF3) ELISA Kit-96 well		51,843.11
- QUANTICHRON CREATININE ASSAY KIT		27,830.70
- Tube 15 ml, 50 ml, Dish 90 mm., Triple vent, 500 pairs/pkg		14,102.60
- Mycoplasma-off, 1 litre		9,630.00

- Creatinine assay		20,865.00
<u>หมวดค่าครีเอตินีน (ถ้ามี)</u>		
	00.00	00.00
รวมรายจ่าย		<u>290,272.41</u>

รายรับสูงกว่ารายจ่าย นำส่งส่วนการคลังจุฬาลงกรณ์มหาวิทยาลัย (เอกสารแนบ)

0

ดอกเบี้ย (ถ้ามี)

00.00

รวมจำนวนเงินที่นำส่งส่วนการคลังทั้งสิ้น

0

ขอรับรองว่ารายงานการรับ-จ่ายเงินข้างต้นเป็นความจริงทุกประการ



 (.....
 (อานุสรณ์ สรีนอชใจ)
 (หัวหน้าโครงการวิจัย)

7 / 25 59

หมายเหตุ: รายงานตลอดโครงการเมื่อสิ้นสุดโครงการ/ปิดโครงการ