



รายงานการวิจัย

การศึกษาทางชีววิทยาเชิงระบบของการควบคุมภูมิคุ้มกันเซลล์ระดับโมเลกุล
ในหนูโรคลูปัส

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

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บทคัดย่อภาษาไทย

โรคแพ้ภูมิตนเองเกิดขึ้นเมื่อเซลล์ภูมิคุ้มกันตอบสนองต่อแอนติเจนของตัวเองผิดปกติและนำไปสู่การอักเสบในเนื้อเยื่อ ปฏิสัมพันธ์ระหว่างพันธุกรรมและสิ่งแวดล้อมเป็นส่วนส่งเสริมให้อาการของโรคเป็นมากขึ้นสารไซโตไคน์ที่ทำให้เกิดโรคเอสแอลอีและโรครุมมาตอยด์ที่สำคัญคือ อินเตอร์เฟอรอนแอลฟา โรคเอสแอลอีเกิดจากภูมิต้านทานผิดปกติเรื้อรังซึ่งจะทำให้เกิดการสร้างแอนติบอดีต่อต้วเนื้อเยื่อตนเองและทำให้เกิดไตอักเสบ การส่งสัญญาณผ่านเซ็นเซอร์กรดนิวคลีอิกสามารถกระตุ้นการผลิตอินเตอร์เฟอรอนแอลฟาจากเดนไดรติกเซลล์ และส่งเสริมความรุนแรงของโรคเอสแอลอี Stimulator of interferon genes (Sting) เป็นตัวรับดีเอ็นเอและส่งสัญญาณต่อเพื่อให้เกิดการสร้างอินเตอร์เฟอรอนแอลฟา ความผิดปกติของ Stingสามารถก่อให้เกิดโรคหลอดเลือดอักเสบในเด็กซึ่งมีอาการคล้ายโรคเอสแอลอี อย่างไรก็ตามการศึกษาการทำงานของ 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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SLE, Systemic lupus Erythematosus; Fcgr2b, Fc gamma receptor IIb; Sting; type I interferon,

โครงการวิจัยย่อยที่ 1

“รายงานการวิจัยเรื่อง การศึกษาการควบคุมของวิถีสัญญาณอินเตอร์เฟียร์รอนผ่านเซ็นเซอร์ของไวรัสดีเอ็นเอในเซลล์ภูมิคุ้มกันของหนูโรคลูปัสด้วยวิธีโปรตีโอมิกส์”

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

ส่วนประกอบเนื้อเรื่อง

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

Fcgr2b เป็น Fc receptor ที่ทำหน้าที่ควบคุมและยับยั้งการทำงานของเซลล์ในร่างกาย เพื่อป้องกันไม่ให้เซลล์ที่ถูกกระตุ้นนั้นตอบสนองต่อตัวกระตุ้นและทำงานเพิ่มขึ้นเพียงอย่างเดียวโดยที่ไม่สามารถกลับเข้าสู่ภาวะปกติได้เมื่อหมดตัวกระตุ้นแล้ว ดังนั้นในหนูที่ปราศจาก Fcgr2b (Fcgr2b^{-/-}) จึงมีการทำงานของเซลล์ที่ผิดปกติโดยเฉพาะ 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

การแสดงออกของ TLR9 และ TLR7 มีผลต่อการทำให้เกิดโรคลูปัสและการสร้าง autoantibody (7-9) นอกจากนี้ การเพิ่มมากขึ้นของ RNA-sensing โมเลกุลอย่างเช่น MDA5 ก็สามารถกระตุ้นให้หนูที่เป็นโรคลูปัสอยู่แล้วมีอาการที่รุนแรงขึ้นได้ (10) และความผิดปกติของ DNA metabolism ในหนูที่ขาดยีน Trex-1 (11) และ DNase (12, 13) ก็ทำให้เกิดโรคลูปัสได้เหมือนกัน นอกจากนี้ในผู้ป่วย SLE ก็ตรวจพบว่าการสร้างแอนติบอดีต่อ DNA-sensing โมเลกุล IFI16, เชื้อไวรัส HHV8 และ CMV (14-16) อย่างไรก็ตามเป็นที่ทราบกันว่าอาการของโรค SLE จะเพิ่มความรุนแรงขึ้นอย่างมีนัยสำคัญในระหว่างการติดเชื้อจากไวรัสต่างๆ เช่น CMV และ EBV (14, 17) จะเห็นได้ว่าข้อมูลเหล่านี้บ่งชี้ให้เห็นถึงความ

เชื่อมโยงระหว่างการติดเชื้อไวรัส, เซ็นเซอร์ของกรดนิวคลีอิกในเซลล์และการเกิดโรคลูปัส แต่ถึงกระนั้น ปัจจุบันก็ยังไม่มีการศึกษาที่ทำให้ทราบถึงบทบาทและกลไกของ DNA-sensing โมเลกุลที่อยู่ใน cytoplasm ของเซลล์ว่ามีผลต่อการเกิดโรคลูปัสหรือไม่ และจนถึงปัจจุบันความเข้าใจในกลไกที่ทำให้เกิดปรากฏการณ์นี้ก็ยังไม่ทราบชัดเจน ซึ่งถ้าเราสามารถทำงานวิจัยที่นำมาซึ่งความเข้าใจในกลไกการเกิดโรคนี้ได้ ข้อมูลนี้อาจนำไปสู่ความรู้ขั้นสูงในการพัฒนาวัคซีนรักษาโรคในการรักษาผู้ป่วยโรคลูปัสในอนาคต โดยทั่วไป DNA-sensing โมเลกุลที่ถูกกระตุ้นโดย Interferon (IFNs) จะส่งสัญญาณผ่านทางโปรตีน adaptor ที่เรียกว่า STING (Stimulator of interferon genes) (18-20) นอกจากนี้ STING ยังเป็นโมเลกุลที่จำเป็นสำหรับการรวมตัวของไวรัสและ membrane ของ host ซึ่งขบวนการนี้จะกระตุ้นให้เกิดการหลั่ง Interferon (21) ดังนั้น STING จึงเป็นโมเลกุลตัวแทนสำหรับการศึกษาถึงบทบาทของ DNA-sensing pathway ว่ามีผลต่อการเกิดโรคลูปัสหรือไม่

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).

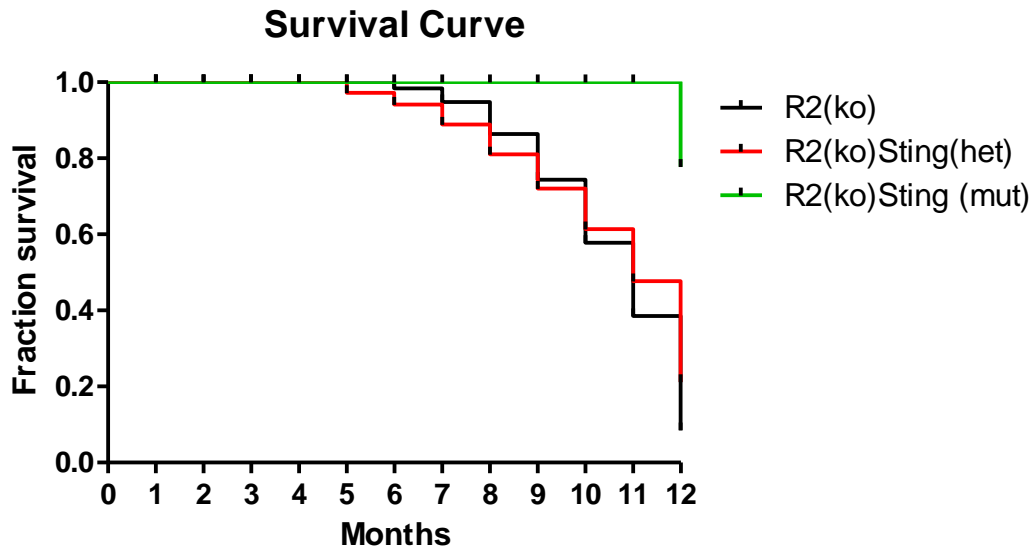


Figure 1 Increase of survival rates of the Fcgr2b-deficient mice in the absence of Sting

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).

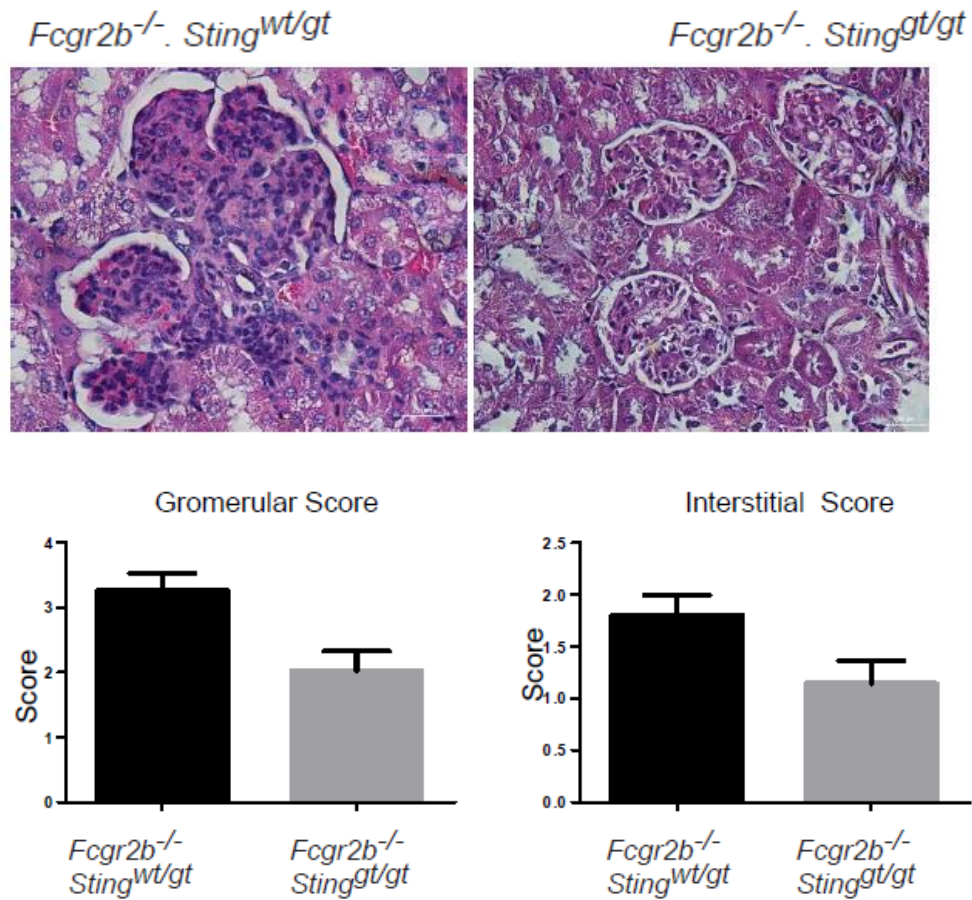


Figure 2 Sting deficiency diminishes fatal glomerulonephritis in the Fcgr2b-deficient mice

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).

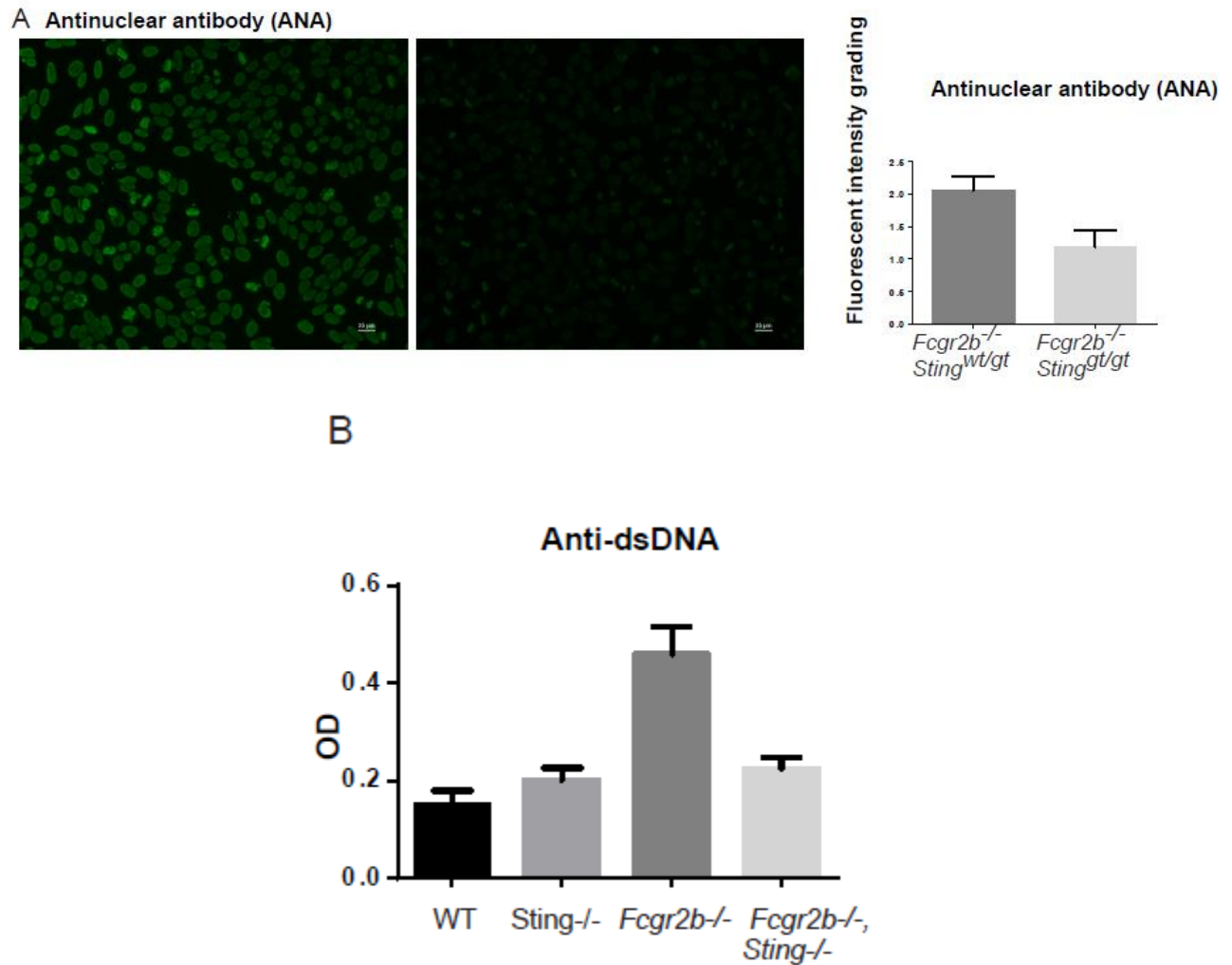


Figure 3 Decrease autoantibody productions in the double deficient mice

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 IgG deposition and leukocyte infiltration in the kidneys of the double-deficient mice

The kidney section showed that immune complex deposition (IgG) and leukocyte (CD45) infiltration increased in the *Fcgr2b*-deficient mice and reduced in double-deficient mice (Figure 4)

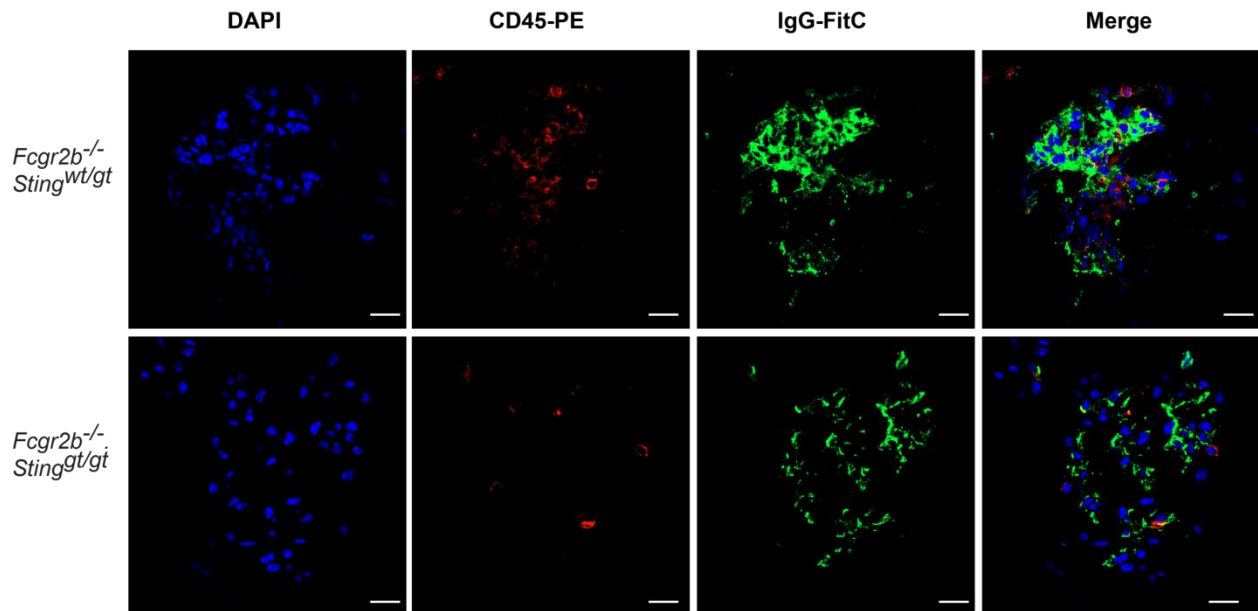


Figure 4 Immunofluorescence staining in the kidney of *Fcgr2b*^{-/-} and double-deficient mice by confocal microscope (DAPI in blue, CD45 in red and IgG in green). Representative of mice (N=3 mice per group, scale bar=10 um).

Decrease interferon inducible gene expression in the kidneys of the double deficient mice

In order to determine the expression of interferon-inducible genes in the kidney of these mice, RNA was performed by microarray. The results found that the higher expression in the *Fcgr2b*^{-/-} mice, especially in the ones with greater severity (mouse number 003, 004) and a significant reduction of interferon-inducible genes in the kidneys of *Fcgr2b*^{-/-}.*Sting*^{gt/gt} mice (Figure 3). However, not all of these interferon-inducible genes decreased in the *Fcgr2b*^{-/-}.*Sting*^{gt/gt} mice.

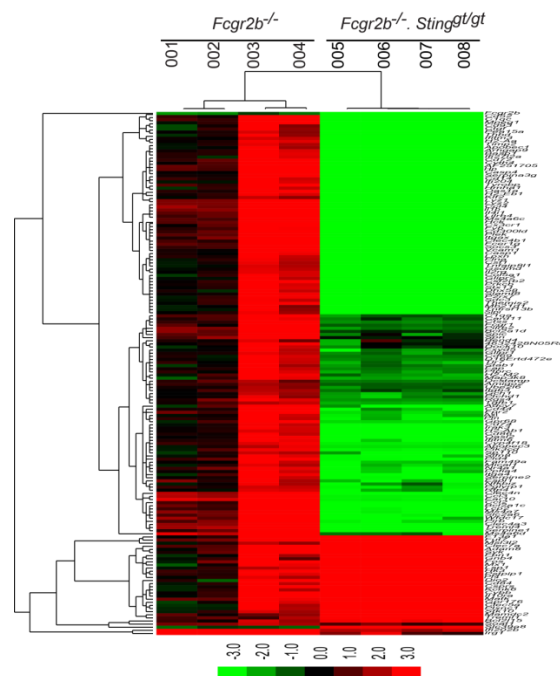


Figure 5 Genes expression profiles of kidneys in the *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-}. *Sting*^{gt/gt} mice. A heat map shows that the interferon signature genes significantly changed in the *Fcgr2b*^{-/-} mice (N= 4 mice per group). Data show in log₂ (sample/wild-type).

Furthermore, the significantly 167 genes of interferon-inducible genes were identified and clustered using the online resource Database for Annotation (DAVID, v 6. 8, <https://david.ncifcrf.gov/>) in the immune system majoring that shown in Table 1.

Table 1. Annotation Cluster of interferon-inducible genes of microarray data from the kidneys of *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-}. *Sting*^{gt/gt} mice

Annotation Cluster	Count	P-Value
Immunity	38	4.20E-31
immune system process	36	5.30E-28
innate immune response	28	1.10E-19
Innate immunity	24	2.00E-19
Adaptive immunity	11	6.70E-10
adaptive immune response	12	7.40E-10
B cell activation	3	1.80E-02

Next, to confirm the expression of interferon-inducible genes by real-time PCR in the kidneys of the *Fcgr2b*^{-/-} mice, the results show that the expressions of *Isg15*, *Mx1*, *Irf3*, and *Irf7* were upregulated in the *Fcgr2b*^{-/-} mice and downregulated in the absence of *Sting* (Figure 6A-6E). Also, the expression of *Irf5*, the lupus susceptibility gene, that upregulated in the kidneys of the *Fcgr2b*^{-/-} mice was *Sting*-dependent (Figure 6D).

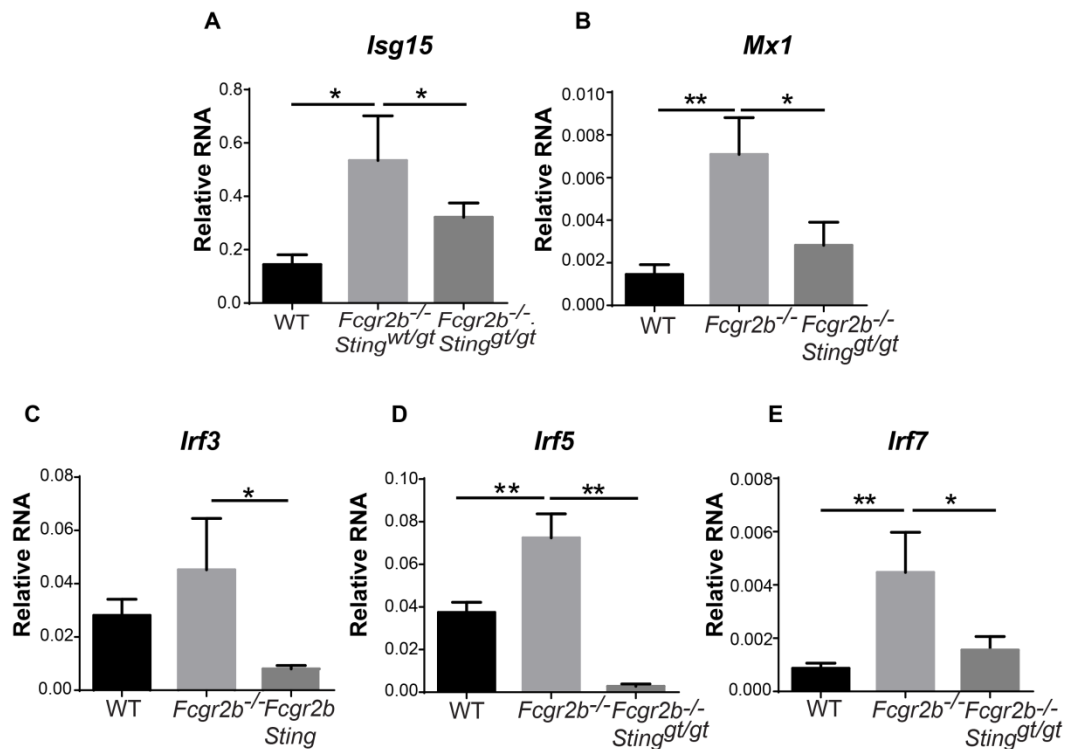


Figure 6. Decrease interferon-inducible gene expressions in the kidneys of the double-deficient mice. (A-E) Gene expression profiles from the kidneys of wild-type, *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-} *Sting*^{gt/gt} mice at the age of 6-7 months were tested by real-time PCR (N=10-17 per group). The mRNA expressions of interferon-inducible genes shown in (A) *Isg15*, (B) *Mx1*, (C) *Irf3*, (D) *Irf5*, and (E) *Irf7*. Data show as mean \pm SEM (*p < 0.05 and **p < 0.01).

Sting signaling is essential for inflammatory phenotypes of the *Fcgr2b*^{-/-} lupus mice

The splenocytes were analyzed from the mice at the age of 6-7 months to characterize the alteration of immune-phenotypes. The expansion of dendritic cells (CD11c⁺) and plasmacytoid dendritic cells (CD11c⁺PDCA⁺) in the *Fcgr2b*^{-/-} mice diminished in the *Fcgr2b*^{-/-} *Sting*^{gt/gt} mice (Figure 7A-7B). Also, these data found that the reduction of T effector memory cells (CD3⁺CD4⁺CD62L^{lo}CD44^{hi}), CD3⁺CD4⁺ICOS⁺ cells, and germinal center B cells (B220⁺GL7⁺) in the

double-deficient mice (Figure 7C-7E). Besides, the mean fluorescence intensity of MHC-II (IA-b) on B cells significantly reduced in the double-deficient mice (Figure 7F). However, the expansion of plasma cells did not show the difference between single and double-deficient mice (Figure 7G).

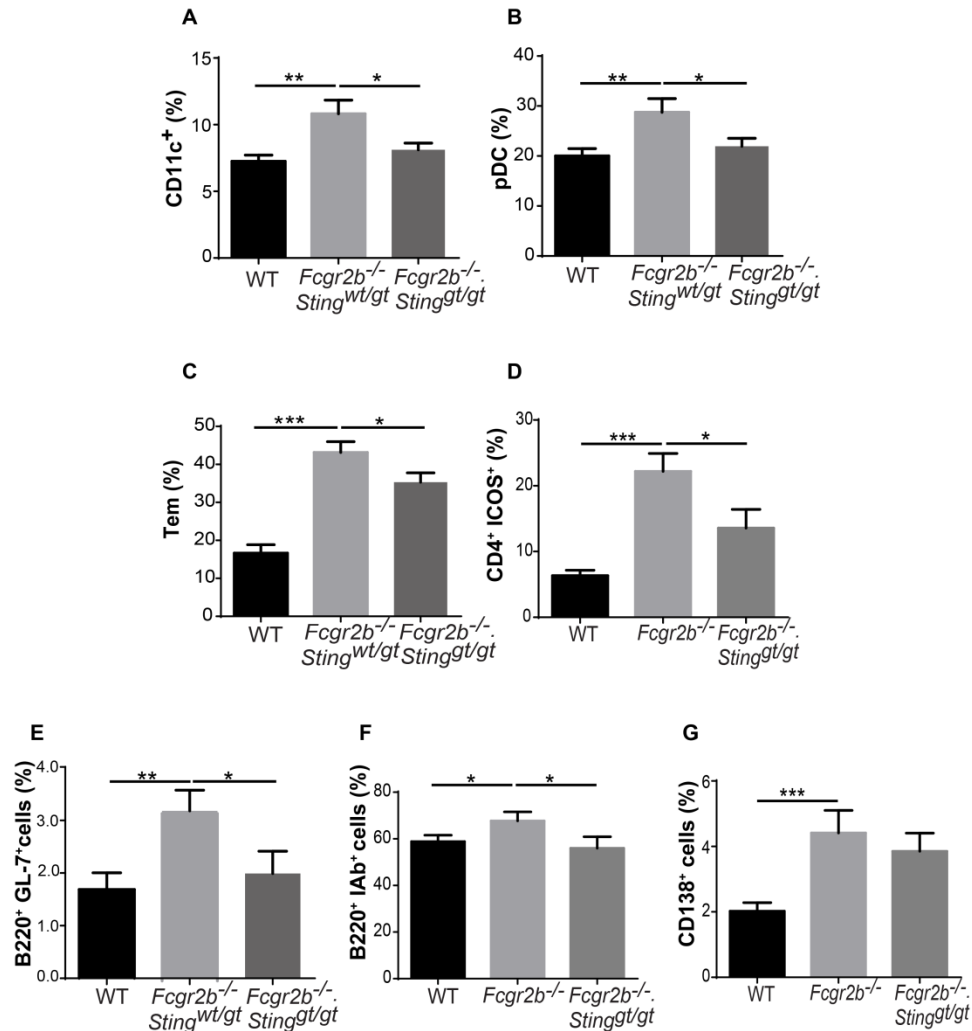


Figure 7. Decrease of dendritic cells, plasmacytoid dendritic cells, effector T cells, and germinal center in the double-deficient mice. (A-G) Flow cytometry analysis of splenocytes isolated from wild-type, *Fcgr2b*^{-/-}, and *Fcgr2b*^{-/-} *Sting*^{gt/gt} mice at the age of 6-7 months (N= 13-14 per group). Data shown in the percentage of (A) CD11c⁺, (B) plasmacytoid dendritic cells (pDC), (C) T effector memory (CD3⁺ CD4⁺ CD44^{hi} CD62L^{lo}), (D) CD3⁺ CD4⁺ ICOS⁺ cells, (E) B220⁺ GL7⁺ cells and (G) CD138⁺ cells. Data show as mean ± SEM (*p < 0.05, **p < 0.01 and ***p < 0.001).

Furthermore, the sera levels of MCP-1 and TNF- α from the *Fcgr2b*^{-/-} mice were significantly increased compared to WT mice (Figure 8A-8B) whereas IL-1 β and IL-23 did not show the significant changes (Figure 8C-8D). However, the levels of TNF- α , IL-1 β , and IL-23 from the *Fcgr2b*^{-/-} mice were significantly decreased in the absence of Sting (Figure 8). These data suggested that Sting participates in the inflammatory mediated pathway in the *Fcgr2b*-deficient lupus mice.

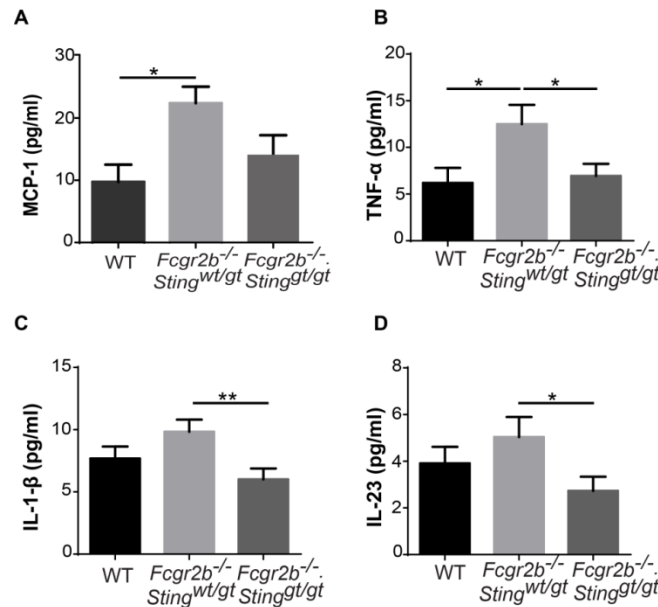


Figure 8. Sting mediated signaling decrease the production of inflammatory cytokines in double-deficient mice. (A-D) The sera cytokines of wild-type, *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-} *Sting*^{gt/gt} mice at the age of 6-7 months were analyzed by cytometric bead array. Serum cytokines of (A) MCP-1, (B) TNF- α , (C) IL-1 β , and (D) IL-23 (N=10-15 per group). Data show as mean \pm SEM (* p < 0.05 and ** p < 0.01).

Sting activated dendritic cells induce the proliferation of naïve CD4⁺ T cells

The splenocytes of *Fcgr2b*^{-/-} mice showed an increase of effector memory T cells (T_{em}) and *Ifn- γ* expression (Figure 15C and 6B). The hypothesis of this study is the high proportion of T_{em} in the *Fcgr2b*^{-/-} mice might contribute to the increase of *Ifn- γ* expression. To test if this assumption is correct, the intercellular staining of IFN- γ from lymph nodes of the affected mice were performed.

The results show that the numbers and percentage of IFN- γ producing CD4⁺ T cells from the *Fcgr2b*^{-/-} mice were higher than wild-type and double-deficient mice (Figure 9).

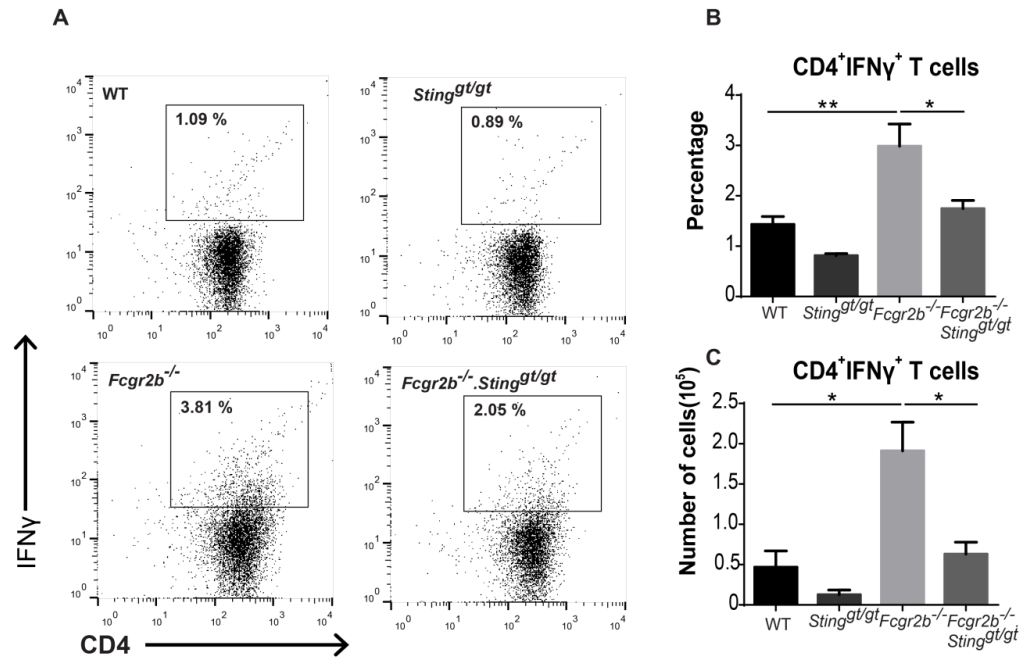


Figure 9. Sting mediated pathway induces IFN- γ producing CD4⁺ T cells from the *Fcgr2b*^{-/-} mice. (A-C) Flow cytometry analysis of (A-B) intracellular staining of IFN- γ -producing CD4⁺ T cells isolated from lymph nodes of wild-type, *Sting*^{gt/gt}, *Fcgr2b*^{-/-}, and *Fcgr2b*^{-/-}. *Sting*^{gt/gt} mice at the age of 6-7 months. (A) Data are representative of 3-4 mice per group. (B) The percentage of IFN- γ ⁺ CD4⁺ T cells and (C) the number of IFN- γ ⁺ CD4⁺ T cells (N=3-4 per group). Data show as mean \pm SEM (*p < 0.05, and **p<0.01).

To determine if the Sting-expressing DC could directly influence the T cell phenotypes, T cells were co-cultured with activated BMDC. The short-interval (6 hours) co-cultured between Sting-activated BMDC derived from *Fcgr2b*^{-/-} and double-deficient mice and CD4⁺ T cells from lymph nodes showed comparable numbers of IFN- γ producing CD4⁺ T cells independent of Sting expression on BMDC (Figure 9A and 9B). However, the CD4⁺ T cells derived from the *Fcgr2b*^{-/-} mice expressed the intracellular staining of IFN- γ higher than the double-knockout mice when co-cultured with *Sting*-sufficient BMDC (Figure 10A and 10B).

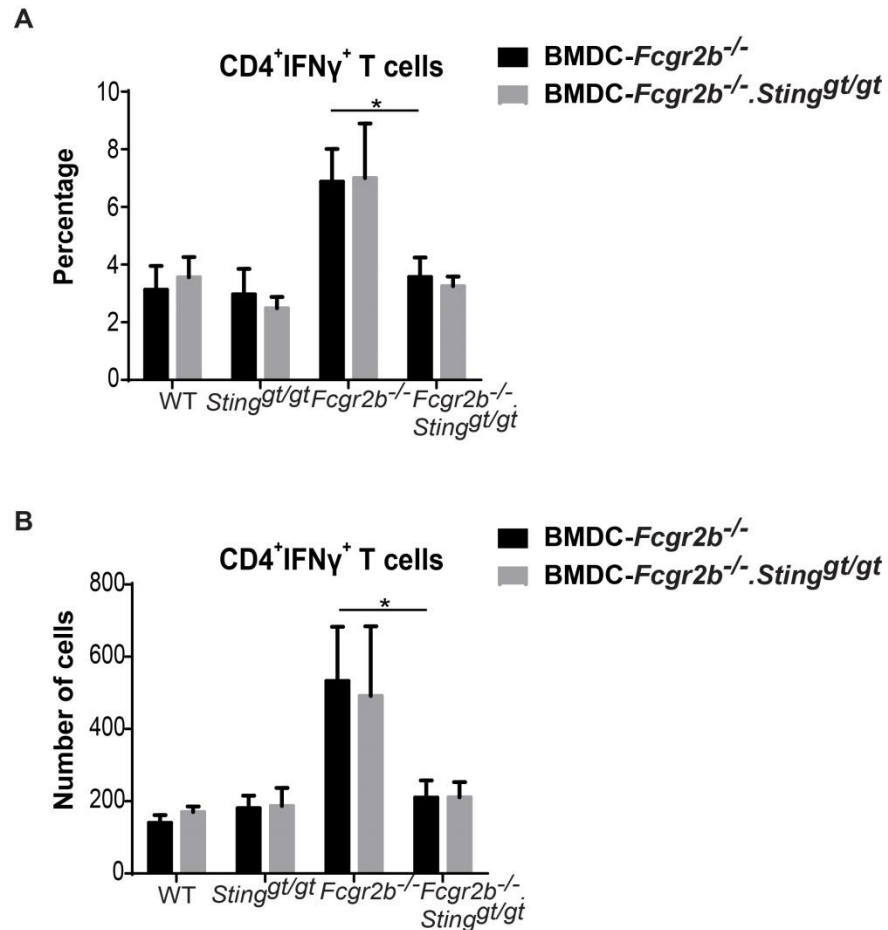


Figure 10 Sting express on BMDC independent IFN- γ producing CD4⁺ T cells for a short time. (A) The percentage and (B) the number of intracellular IFN- γ producing CD4⁺ cells after co-cultured with DMXAA activated BMDC from *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-}. *Sting*^{gt/gt} (6–7 months old) for six hours (N=3-4). Data show as mean \pm SEM (*p < 0.05).

Next, to examine if Sting activated BMDC can promote T cell proliferation, purified naïve T cells from spleen were co-cultured with Sting-activated BMDC for 72 hours and assessed with CFSE dilution assay (Figure 11A and 11B). The proliferation ability of naïve T cells was independent on Sting expression on T cells but depended on Sting-expression on BMDC (Figure 11A and 11B). The naïve T cells that primed with Sting expressing BMDC became more proliferative, and these cells were capable of producing higher IFN- γ compared to the cells that were co-cultured with Sting deficient BMDC (Figure 11C). These data suggest that Sting activation promoted the DC

differentiation, which subsequently the activated DC primed the naïve CD4⁺ T cells to proliferate and become the IFN- γ producing T cells.

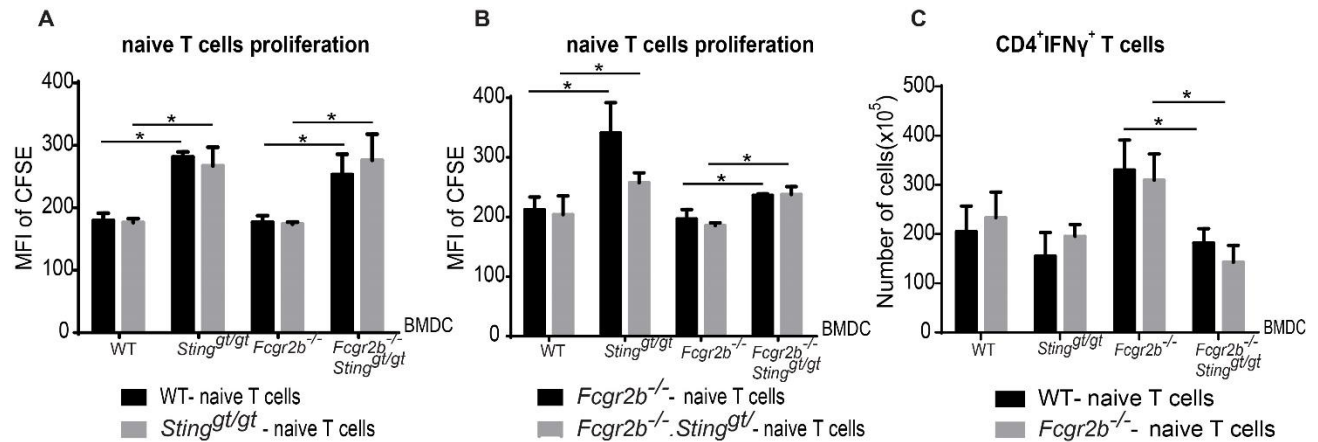


Figure 11 Sting activated dendritic cells induce the proliferation of naïve CD4⁺ T cells. (A-C) Co-culture of naïve T cells with DMXAA activated BMDC from wild-type, *Sting*^{gt/gt}, *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-}.*Sting*^{gt/gt} mice (labeled at x-axis) for 72 hours. (A and B) CFSE dilution of isolated naïve T cells showed in mean fluorescence intensity (MFI), and (C) the total numbers of IFN- γ ⁺ CD4⁺ T cells (N=4 per group). Data show as mean \pm SEM (*p < 0.05).

Sting activation increases the maturation of dendritic cells and cytokine production

In order to understand the immunological importance of Sting in lupus *Fcgr2b*-deficient mice, the flow cytometry was characterize the subsets of splenocytes from affected mice and their controls. The activated immune cells showed that the expansion of the DC in the spleen of the *Fcgr2b*^{-/-} mice was Sting-dependent, and the cGAS/Sting pathway is important for DC activation. To investigate if the expansion of DC in the *Fcgr2b*^{-/-} mice is directly mediated by Sting signaling, the bone-marrow derived dendritic cells (BMDC) were differentiated into immature DC and subsequently stimulated with Sting ligands (DMXAA), DMSO, and LPS (as a control) to assess if Sting played a role in DC maturation. The LPS control induced the immature DC to increase the expression of MHC-II (IA-b) and CD80, which suggested the phenotypes of mature DC, from both *Sting*-sufficient and *Sting*-deficient mice (Figure 12A and 12C). While the immature DC from wild-

type and *Fcgr2b*^{-/-} mice also showed the increasing percentage of IA-b⁺ and CD80⁺ DC cells after DMXAA stimulation, but the *Sting*-deficient mice did not develop these mature phenotypes (Figure 12B and 12D).

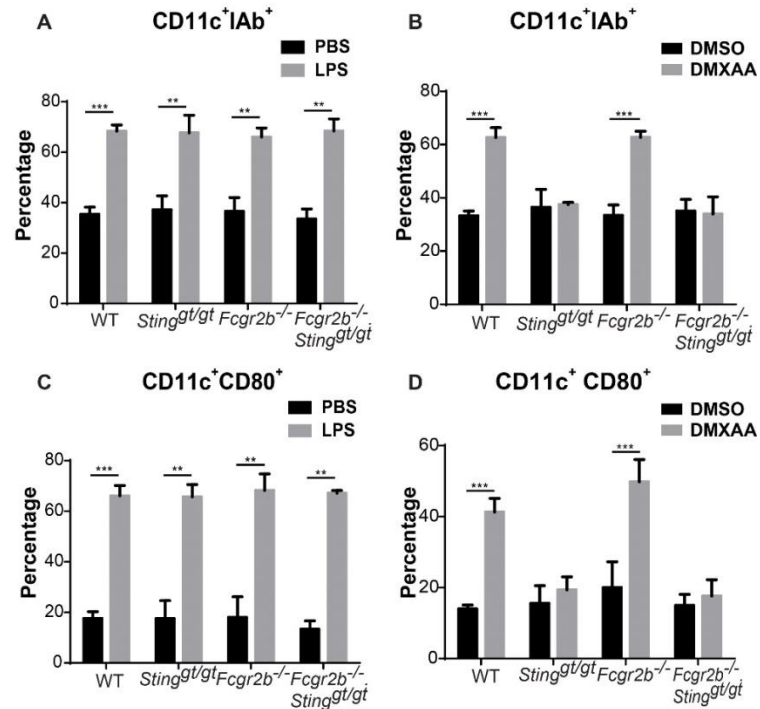


Figure 12 *Sting* activation increases the maturation of dendritic cells. Bone marrows were isolated from wild-type, *Sting*^{gt/gt}, *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-} *Sting*^{gt/gt} mice at the age of 6-7 months. (A-D) IL-4 and GM-CSF differentiated bone marrow-derived dendritic cells (BMDC) for five days then immature BMDC were stimulated with LPS or DMXAA for 24 hours. Flow cytometry analysis shows the percentage of (A-B) CD11c⁺ IAb⁺ cells and (C-D) CD11c⁺ CD80⁺ cells. Data show as mean ± SEM (N=3-5; *p < 0.05, **p<0.01 and ***p<0.001).

Sting signaling promotes the differentiation of plasmacytoid dendritic cells (pDC)

To better understand the function of Sting in DC, the quantitative proteomic analysis of Sting activated BMDC in the *Fcgr2b*^{-/-} mice compared to the double-deficient mice were performed using a dimethyl labeling method. The Volcano plot showed the protein that highly expressed were interferon-regulated proteins (Figure 13) . This finding may result from the increase of IFN-I production in the culture medium, which could upregulate the interferon-regulated proteins.

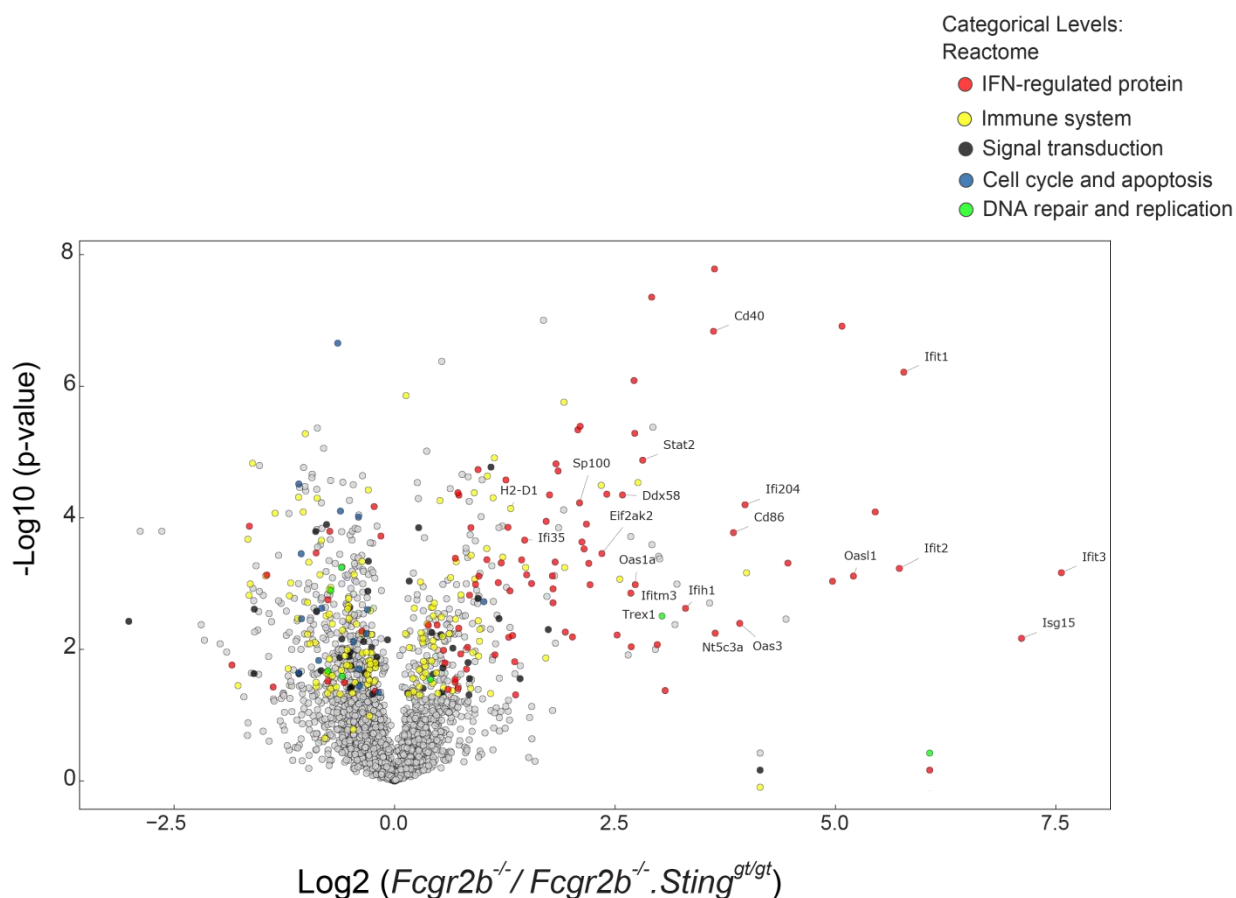


Figure 13 *Fcgr2b*^{-/-} increase the expression of interferon-regulated proteins. Volcano plot of protein expressions from proteomic analysis of DMXAA activated BMDC of *Fcgr2b*^{-/-} and *Fcgr2b*^{-/-} . *Sting*^{gt/gt} mice at the age of 6-7 months (N=4 per group).

The hypothesis of this study is Sting might promote the differentiation of pDC (the major producer of IFN-I). To confirm this hypothesis, the in vitro culture of BMDC with DMXAA and LPS (as a control) showed a significant increase in pDC and IFN- β production with DMXAA but not with LPS stimulation (Figure 14A-14D). Also, the results show the morphology of these cells by the imaging flow cytometry and found the pDC expressed CD80 and IA-b as well (Figure 14E -14F).

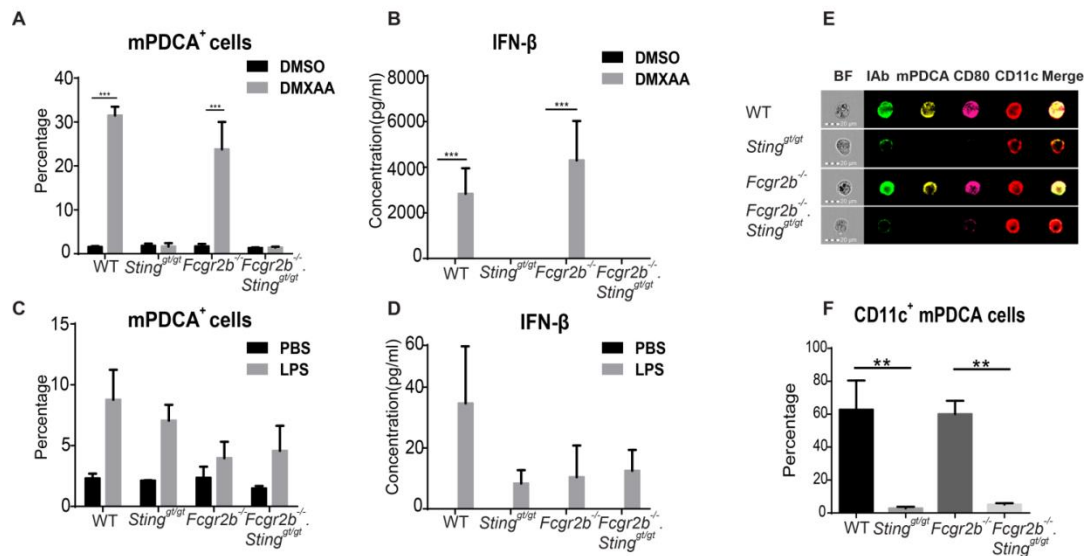


Figure 14 Sting activation promotes differentiation of plasmacytoid dendritic cells (pDC). The percentage of pDC (PDCA⁺ cells) after (A) DMXAA activation and (C) LPS activation for 24 hours (N = 3–4 per group). (B and D) The level of IFN- β from the culture supernatant of activated BMDC with (B) DMXAA and (C) LPS (N = 5 per group). (E and F) Imaging flow cytometry of DMXAA activated BMDC shows (E) the representative staining of IA-b (green), mPDCA (yellow), CD80 (pink), and CD11c (red) and (F) the percentage of CD11c⁺ mPDCA⁺ cells (N = 3 mice per group). A representative of 3 experiments. Data show as mean \pm SEM (**p < 0.01 and ***p < 0.001).

Adoptive transfer of Sting expressing BMDC from *Fcgr2b*^{-/-} mice increases autoantibodies in the wild-type mice.

To determine if Sting contributes lupus pathogenesis in wild-type mice, the adoptive transfer of Sting-activated BMDC from *Fcgr2b*^{-/-} mice to wild-type mice were performed. The results show that the anti-dsDNA significantly increased in the wild-type mice who received *Sting*-sufficient

BMDC compared to the wild-type (non-recipient) controls (Figure 15). The results suggest that the BMDC derived from the *Fcgr2b*^{-/-} mice induced the recipients to produce a higher level of anti-dsDNA than normal mice.

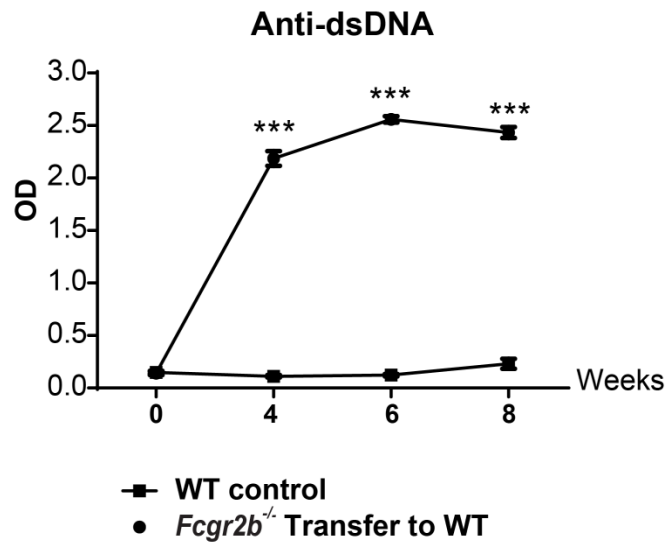


Figure 15 Adoptive transfer of Sting expressing BMDC increase anti-dsDNA in wild-type mice. DMXAA activated BMDC from *Fcgr2b*^{-/-} were transferred into the recipient mice (wild-type). (A) The level of anti-dsDNA from the sera (1:100) measured by ELISA (N=4-5 per group).

Additionally, to investigate the immune-phenotypes in wild type that received the Sting-activated BMDC compared to the control, the flow cytometry analysis of spleens from all WT-recipient mice were performed. The results showed that the increased percentage of T effector memory (CD4⁺CD44^{hi}CD62L^{lo}), CD4⁺ICOS⁺ cells, plasma cells (CD138⁺ cells) and germinal center B cells (B220⁺GL7⁺ cells) compared with the wild-type (non-recipient) controls (Figure 16).

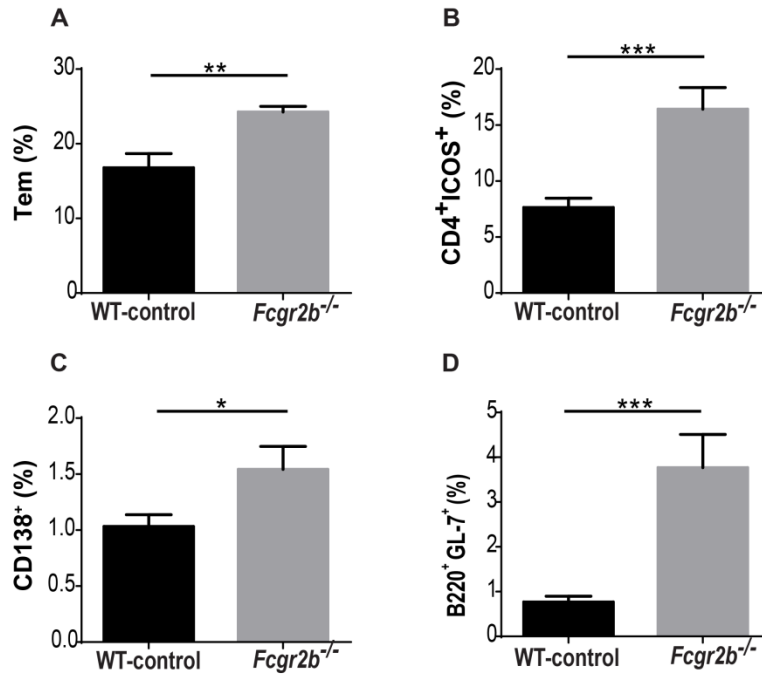
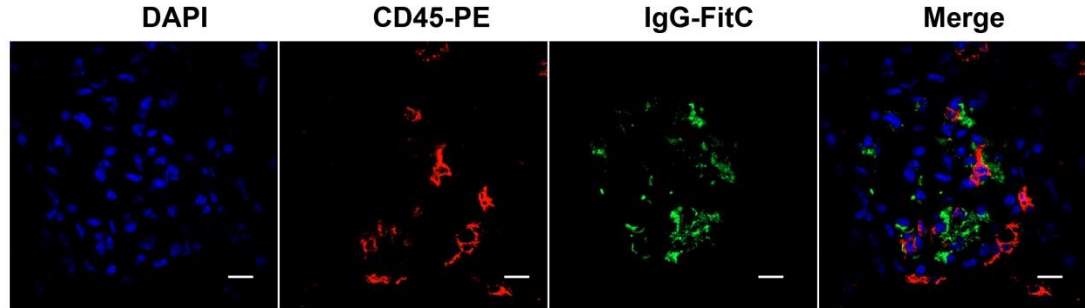


Figure 16 Sting-activated BMDC increase the activated immune cells in recipient wild type mice. Flow cytometry analysis of recipient splenocytes after BMDC transferred every 2 weeks for 4 times show the percentage of (A) effector T cells (CD4⁺ CD44^{hi} CD62L^{lo}), (B) CD4⁺ ICOS⁺ cells, (C) CD138⁺ cells and (D) B220⁺ GL7⁺ cells (N=4-7 per group). Data show as mean \pm SEM (*p < 0.05, **p < 0.01 and ***p < 0.001).

Also, the immunofluorescences staining at the kidney of the wild type recipient mice were determined. These results found that increase of IgG deposition and CD45⁺ cell infiltration in wild type that received Sting-sufficient BMDC while did not show in the wild type control (Figure 17).

A BMDC from *Fcgr2b*^{-/-} transfer to WT



B WT-control

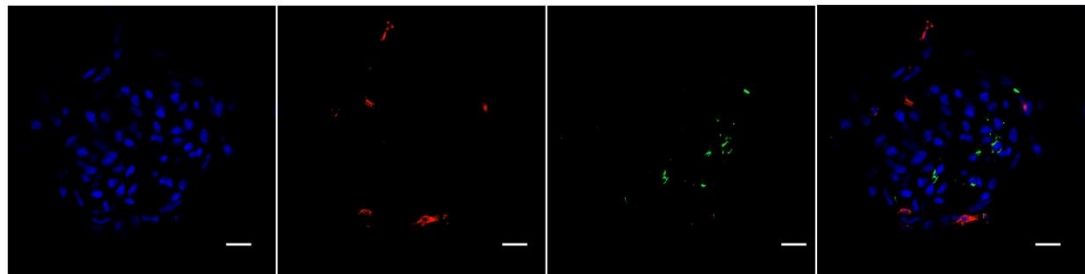


Figure 17 Sting-activated BMDC induce immune complex deposition in the kidney of WT-recipient mice. (A and B) Immunofluorescence staining of the kidneys from recipient wild-type mice show in green (IgG), red (CD45), and blue (DAPI). Data are representative of 4 mice per group (scale bar=10 μ m).

Adoptive transfer of Sting expressing BMDC induce lupus development in the *Fcgr2b*^{-/-}.*Sting*^{gt/gt} mice

The Sting signaling pathway activated the immature BMDC to differentiate into the mature DC and pDC which are capable of promoting T cell proliferation and producing the inflammatory cytokines. The proposed of this study is that Sting may induce the lupus disease by initially acting through the DC activation. To confirm this hypothesis, the adoptive transfers of Sting-activated BMDC into the double-deficient mice were performed. The anti-dsDNA significantly increased in the recipient mice who received *Sting*-sufficient BMDC compared to the non-recipient controls (Figure 18). The BMDC derived from Sting-sufficient mice (both WT and *Fcgr2b*^{-/-}) induced the recipients to produce a higher titer of anti-dsDNA than the ones from the double-deficient mice (Figure 18).

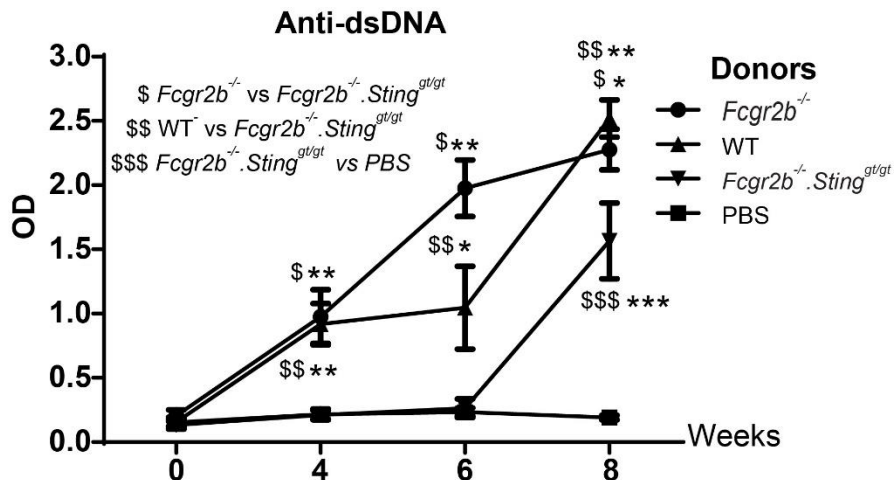


Figure 18 Increase of anti-dsDNA in the recipient mice. DMXAA activated BMDC from *Fcgr2b*^{-/-}, WT, and *Fcgr2b*^{-/-}.*Sting*^{gt/gt} were transferred into the recipient mice (*Fcgr2b*^{-/-}.*Sting*^{gt/gt}). (A) The level of anti-dsDNA from the sera (1:100) measured by ELISA (N=5-10 per group). Data show as mean ± SEM (*p < 0.05, **p < 0.01, and ***p < 0.001).

In addition, the flow cytometry analysis of spleens from all groups of recipient mice showed the increase in the percentage of T effector memory (T_{em}), CD4⁺ICOS⁺, and germinal center B cells when compared with PBS injection group (Figure 35A-35C). However, the transfer of Sting-activated BMDC from the *Fcgr2b*^{-/-} mice significantly induced the T effector memory (T_{em}), CD4⁺ICOS⁺, and germinal center B cells, but did not increase CD138⁺ cells, B220⁺IAb⁺ cells (Figure 19).

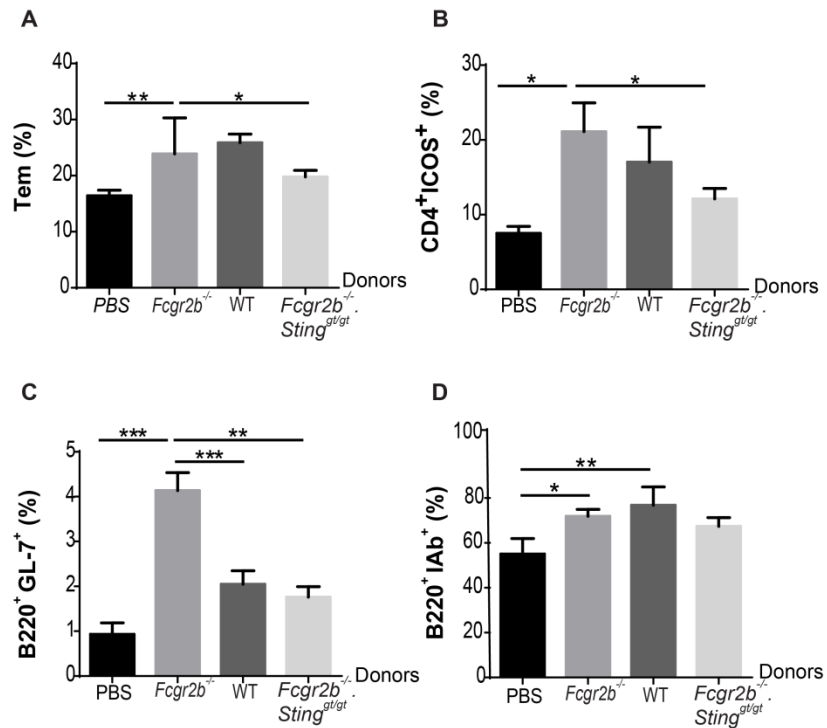


Figure 19 Increase of activated immune cells in recipient mice. (A-E) Flow cytometry analysis of recipient splenocytes after BMDC transferred every 2 weeks for 4 times show the percentage of (A) effector T cells (CD4⁺CD44^{hi}CD62L^{lo}), (B) CD4⁺ICOS⁺ cells, (C) B220⁺GL7⁺ cells and (D) B220⁺IAb⁺ cells (N=5-10 per group). Data show as mean ± SEM (*p < 0.05, **p<0.01, and ***p<0.001).

Next, the immunofluorescences staining at the kidney of the recipient mice were performed. The recipient of *Sting*-sufficient BMDC showed the increase of IgG deposition and CD45⁺ cell infiltration while *Sting*-deficient BMDC did not (Figure 20A-D). Nevertheless, the *Fcgr2b*^{-/-} BMDC induced more immune complex and CD45⁺ cells in the kidneys (Figure 20B and 20C). The results suggested that the restoration of *Sting* signaling pathway in dendritic cells is essential for lupus development in the *Fcgr2b*^{-/-}.*Sting*^{g/gt} mice.

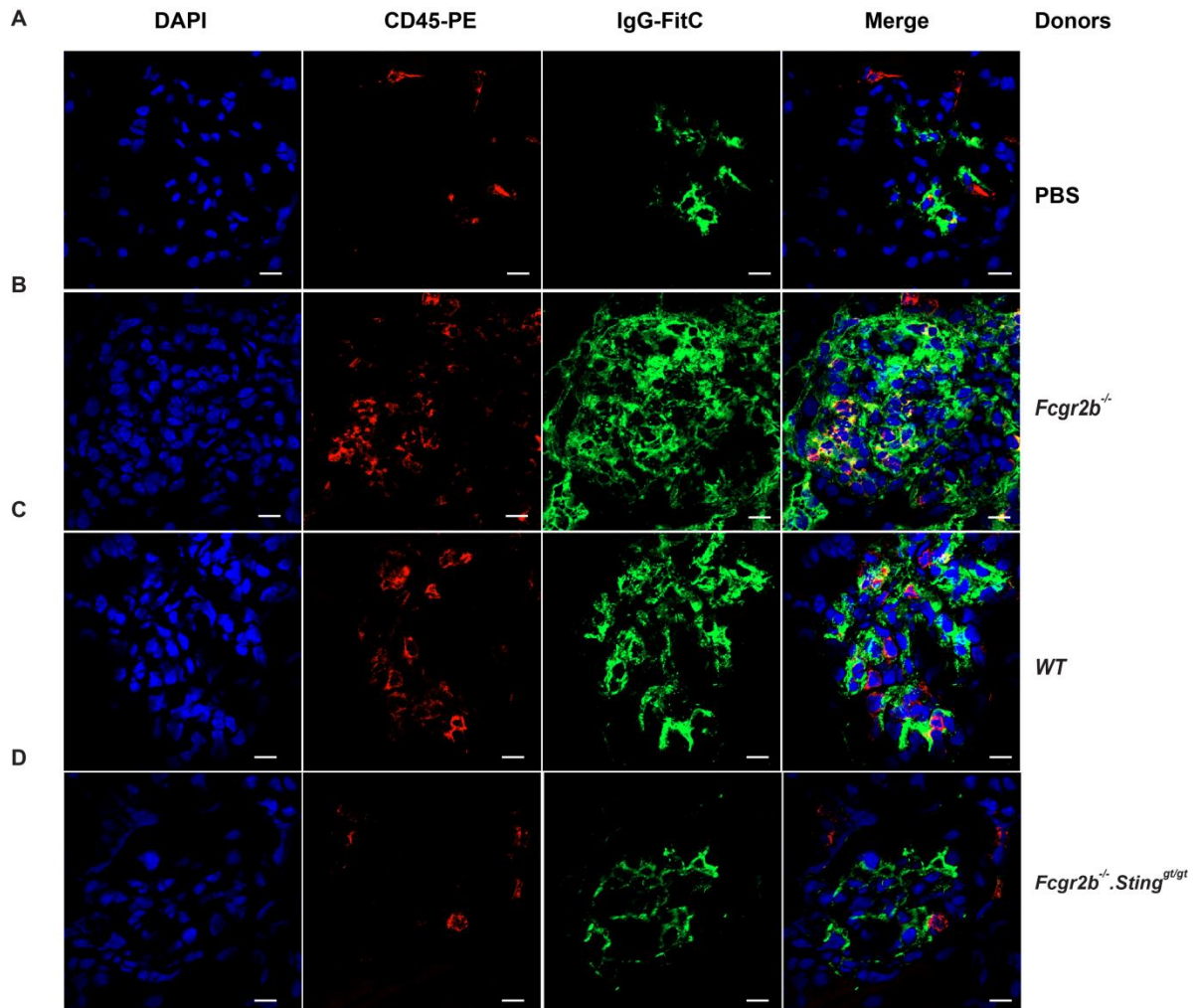


Figure 20 Sting-activated BMDC induce immune complex deposition in the kidney. (A-D) Immunofluorescence staining of the kidney from the *Fcgr2b^{-/-}.Sting^{gt/gt}* recipient mice after the transfer with (F) PBS control, DMXAA activated BMDC from (G) *Fcgr2b^{-/-}*, (H) WT and (I) *Fcgr2b^{-/-}.Sting^{gt/gt}*. The confocal microscope shows DAPI (blue), CD45 (red) and IgG (green). The representative of 3 experiments (scale bar=10 μ m).

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

The 129/B6.*Fcgr2b*^{-/-} mice carrying Nba2 region, a locus on a spontaneous lupus mouse model that important for SLE pathogenesis, expressed constitutively the IFN-activated gene 202 (*Ifi202*) . The *Ifi202* is a lupus-susceptibility gene that increased the progression of glomerulonephritis in lupus mouse models. The CD19⁺ cells from B6.Nba2 show the increase of *Ifi202* and the decreases of *Sting* expression. However, the overexpression of *Ifi202* can activate *Sting*-dependent IFN-I response and the 129/B6.*Fcgr2b*^{-/-} mice increase the expression of IFN- β . Here, these studies show the high expression of *Ifi202*, *Sting*, IFN- β , and interferon-inducible genes including *Irf7*, *Ifn- γ* , *Mx1*, and *Cxcl10* from the spleen of the 129/B6.*Fcgr2b*^{-/-} mice. Although *Sting* functions as a negative regulator in the *Mrl/lpr* lupus mice, this data shows that *Sting* is required for the lupus development in the 129/B6.*Fcgr2b*^{-/-} mice. It is possible that *Sting* will also play a crucial role in other lupus mouse models which contained Nba2 region.

The survival of the 129/B6.*Fcgr2b*^{-/-} mice depends on the autoantibody production and glomerulonephritis and the data from the previous study indicated that 6 months old of *Fcgr2b*^{-/-} generated in 129 background mice showed the full lupus phenotypes. Moreover, it is found that *Sting* is required for the antibody production induced by cyclic-di-GMP in vitro. This study suggested that *Sting* facilitated the autoantibody production, inflammatory cell infiltration, and glomerulonephritis in the 129/B6.*Fcgr2b*^{-/-} mice. Therefore, in the absent of *Sting*, resulting in improved the survival rate of 129/B6.*Fcgr2b*^{-/-} mice.

Investigations by several studies conclude that the expression of interferon-inducible genes associated with SLE disease activity. This study detected the very high expression of a hundred of IFN inducible genes in the kidneys of 129/B6.*Fcgr2b*^{-/-} mice by microarray that showed more severe pathology. The absence of *Sting* signaling in the *Fcgr2b*^{-/-} mice partly decreased the expression of interferon-inducible genes in the kidney. This data suggested that other nucleic acid sensors may promote the type I interferon production or signaling leads to destructive of kidneys in the *Fcgr2b*^{-/-} mice as well. The *Sting*-dependent lupus phenotypes do not mediate only through type-I interferon pathway.

Sting expresses and functions differentially depended on the cell types. The previous study reported that STING was a low expression in B cell from SLE patients and MRL/lpr mice. These finding may contribute to the pathogenesis of SLE by increasing the activation of the JAK1-STAT1 signaling indirectly by STING. Whereas, Sting signals coordinately with B cell receptor (BCR) signaling to promote antibody response. The results showed that the spontaneous germinal center B cells and MHC-II expression in the *Fcgr2b*^{-/-} mice were *Sting*-dependent. However, plasma cell expansion was *Sting*-independent. This data suggested Sting may contribute to the autoantibody production through memory B cells.

Sting also activates T cells by treatment with Sting ligand (DMXAA) induced not only *Sting*-dependent expression of ISGs and type I IFN production but also mediated cell stress and death. Nevertheless, this study found that the increase of T effector memory (T_{em}) in the *Fcgr2b*^{-/-} mice was *Sting*-dependent. The expansion of T_{em} may directly mediate through the interaction with antigen presenting cells, not directly via Sting signaling in T cells.

Sting agonist (DMXAA) treated mice show the increased expression of CD80, CD86, and MHC-II on DC and IFN- β production suggesting promotes the mature phenotypes of DC as the antigen-presenting cells (APC) which increase the expansion of T cells. This observation found the reduction of DC expansion in the *Fcgr2b*^{-/-} mice, which depended on Sting signaling.

To confirm if Sting was required for DC maturation and cytokine production, co-culture of activated DC with T cells were performed. These DC became professional APC and could promote T cell differentiation. The IFN- γ producing CD4⁺ cells in the spleen and lymph nodes of the *Fcgr2b*^{-/-} mice were reduced in the absence of Sting. The Sting-expressing DC derived from WT and *Fcgr2b*^{-/-} mice stimulated naïve T cells to proliferate, but the ability of T cell to differentiate and produce IFN- γ did not depend on intrinsic Sting expression on T cells. Interestingly, only DC from the *Fcgr2b*^{-/-} mice can increase the IFN- γ production in CD4⁺ T cells. Furthermore, the previous data found that Sting-deficient mice fail to prime CD8⁺ T cell leading to nonproductive T cell activation *in vivo*. Thus, this data suggested the DC from the *Fcgr2b*^{-/-} mice have the intrinsic property that promotes the generation of Tem or autoreactive T cells.

The cGAS-STING signaling can activate human pDCs to produce IFN-I and knockdown of Sting using siRNA in CAL-1 cells can cause the reduction of IFN response. The proteomic data

showed the upregulation of interferon-regulated protein after Sting-activation with DMXAA, which implied that the culture environment should enrich with type-I IFN. Sting activation with DMXAA led to phosphorylation of Ser357 of mouse Sting (homolog Ser358 in human Sting), and this site is phosphorylated by TBK1 which subsequently promoting type I IFN production. The identification of pDC, a major producer of type-I IFN, after Sting activation, uncovered the role of Sting in the differentiation of pDC. These data revealed that Sting was essential for the generation of pDCs. Besides, this study identified several Sting-interacting proteins by mass spectrometry. Lyn kinase has been shown the role in the differentiation of pDC.

The recruitment of Lyn kinase to Sting after DMXAA stimulation suggested Sting mediated signaling through Lyn kinase. Also, the proteomics data of Sting-activated BMDC showed a significant increase of phosphoinositide 3-kinase adapter protein 1 (Pik3ap1) and receptor of activated protein C kinase 1 (Rack1). Pik3ap1 is an adaptor that signals to the phosphoinositide 3-kinase (PI3K). Lyn and RACK1 are co-immunoprecipitated in membrane complexes. RACK1 silencing effected on the phosphorylation of AKT. This data suggest that the downstream of Sting-Lyn signaling may mediate through the PI3K-AKT pathway. The inhibition of LYN kinase during Sting activation abolished DC maturation and pDC differentiation. The data suggested Sting mediated differentiation of BMDC through the LYN signaling pathway.

Depletion of pDC ameliorates the autoimmune phenotypes in BXSB lupus-prone mice and B6.Nba2 mice. These data strongly suggested Sting involving in DC function both DC maturation and pDC differentiation. The adoptive transfer of *Sting* sufficient BMDC can induce autoantibody production regardless of *Fcgr2b* status. However, the absence of *Fcgr2b* in the BMDC can accelerate the autoimmune phenotypes, including the immune complex deposition and inflammatory cell infiltration in the double-deficient recipient mice. Additionally, the adoptive transfers of *Sting* sufficient BMDC derived from *Fcgr2*^{-/-} mice increase the antibody production and activated immune cells but did not change the kidney pathology in wild type recipients.

Nevertheless, wild type recipient mice do not develop glomerulonephritis. The data implied that these wild type mice require lupus-susceptibility gene to progress of the disease. In summary, these data elaborated the vital function of Sting in the autoimmune *Fcgr2b*^{-/-} lupus mouse model. The inhibition of STING signaling is a promising therapeutic target for SLE patients.

4. สรุปและเสนอแนะเกี่ยวกับการวิจัยในขั้นต่อไป ตลอดจนประโยชน์ในทางประยุกต์ของผลงานวิจัยที่ได้

129/B6.*Fcgr2b*^{-/-} mice present the strong lupus phenotype.

First, the results show the increase of *Irf202* and *Sting* expression in the 129/B6.*Fcgr2b*^{-/-} mice. Moreover, it is found that the interferon-inducible genes, including *Ifn-β*, *Irf3*, *Irf5*, *Ifn-γ*, *Cxcl10*, and *Mx1*, were also increased in the spleen of 129/B6.*Fcgr2b*^{-/-} mice.

The *Fcgr2b*-deficient mice start to die at the age of 6 months, and the survival rates drop to 22.2 % by 12 months old, while the survival rates of double-deficient mice are 77.7 %. The effect of one allele of *Sting* to survival rates of *Fcgr2b*-deficient mice does not show a significant difference. This finding concludes that *Sting* increases survival rates and improves the lupus phenotypes in 129/B6.*Fcgr2b*^{-/-} lupus mice model.

The activation of *Sting* pathway involved in the pathogenesis of SLE in lupus mice

In the absence of *Sting*, the lupus phenotypes of 129/B6.*Fcgr2b*-deficient mice were improved, including:

1. The kidney staining of *Fcgr2b*-deficient mice shows inflammatory cell infiltrations, enlarged glomeruli, and crescentic glomeruli, but double-deficient mice do not develop glomerulonephritis.

2. Antinuclear antibody (ANA) and anti-dsDNA production in the serum were decreases in the double-deficient mice. The results suggest that the high levels of autoantibodies are from the *Sting*-dependent.

3. *Sting*-mediated signaling induces type I interferon production and leads to the increase of interferon-inducible gene expression while the interferon signature genes in the kidneys were diminished in the double-deficient mice.

4. In order to understand the immunological importance of *Sting* in lupus *Fcgr2b*-deficient mice, the flow cytometry were characterized by the subsets of splenocytes from affected mice and their controls. The activated immune cells decrease in the percentage and numbers in the double-deficient mice, especially CD11c⁺ cells, plasmacytoid dendritic cells, and effector T cells. Also, B cells in the germinal center reduced in the double-deficient mice but not plasma cells.

The Sting-mediated pathway promotes maturation of dendritic cells and plasmacytoid dendritic cells differentiation via Lyn kinase pathway.

1. The in vitro culture of bone-marrow derived dendritic cells with Sting ligands were performed and tested for the differentiation, activation stage, and cytokine production. Sting activation using DMXAA promoted the differentiation of immature DC to become pDC in the wild type and *Fcgr2b*^{-/-} mice. In the absence of Sting, the immature DC cannot differentiate into pDC or get activated to express MHC class II (IAb⁺) and become professional antigen-presenting.

2. The proteomic analysis of activated BMDCs was performed and found the interferon-regulated proteins increase in *Sting-sufficient* BMDC.

3. To discover the new unbiased Sting-signaling pathway by immunoprecipitation assay, After Sting activation, Sting was phosphorylated, and Lyn was recruited to interact with Sting.

4. Regarding the observation of PI3K adaptor proteins associate with Sting after agonist treatment. The association of Sting and Lyn-PI3K-Akt pathway suggested Sting mediated maturation and differentiation of BMDC through the Lyn signaling pathway

The Sting-mediated pathway contribute to SLE via DNA sensor-mediated signaling in antigen presenting cells

The adoptive transfer of Sting-activated bone marrow-derived dendritic cells (BMDC) into the *Sting*-deficiency 129/B6.*Fcgr2b*^{-/-} mice restored the lupus phenotypes. These data suggested that Sting signaling expressed in the dendritic cells induced the autoimmune development in the 129/B6.*Fcgr2b*^{-/-} mice.

This study concludes that the signaling through stimulator of interferon genes (Sting) leads to type I interferon production and lupus pathogenesis. These findings provide the proof of concept that inhibition of STING signaling is a promising therapeutic target for SLE patients. Sting is a new molecule which promising therapeutic target for lupus disease.

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2. ภาคผนวก (Appendix) ถ้ามี

3. ประวัตินักวิจัยและคณะ พร้อมหน่วยงานสังกัด

ส่วน ค : ประวัติคณะผู้วิจัย

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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

2. ผู้ร่วมโครงการวิจัย

1. ชื่อ - นามสกุล (ภาษาไทย) ประภาพร พิสิษฐกุล
ชื่อ - นามสกุล (ภาษาอังกฤษ) Prapaporn Pisitkun
2. เลขหมายบัตรประจำตัวประชาชน 3100100484089
ตำแหน่งปัจจุบัน อาจารย์
เวลาที่ใช้ทำวิจัย 40 (ชั่วโมง : สัปดาห์)
3. ตำแหน่ง อาจารย์
4. ภาควิชาภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

โทรศัพท์ 092-484-4550

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5. ประวัติการศึกษา

2002 Board of Clinical Rheumatology, Thailand

2000 Board of Internal Medicine, Thailand

1995 M.D., Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

1993 Bachelor of Science, Mahidol University, Bangkok, Thailand

6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

อิมมูโนวิทยา โรคอโตอิมมูน การวิจัยในสัตว์ทดลอง

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำ
การวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น

7.1 ผู้อำนวยการแผนงานวิจัย :-

7.2 หัวหน้าโครงการวิจัย: -

7.3 ผู้ร่วมงานวิจัย: -

7.4 โครงการที่ทำเสร็จแล้ว :

1. Benjachat T, Tongyoo P, Tantivitayakul P, Somparn P, Hirankarn N, Prom-On S, Pisitkun P, Leelahavanichkul A, Avihingsanon Y*, Townamchai N. Biomarkers for Refractory Lupus Nephritis: A microarrays study of kidney tissue. *Int. J. Mol. Sci.* 2015 (in press).
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10. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B Cell Responses to RNA-Related Antigens Due to TLR7 Gene Duplication. *Science*. 2006 Jun; 312(5780):1669-72.
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โครงการวิจัยย่อยที่ 2

“รายงานการวิจัย” เรื่อง “หนูทดลองโมเดลโรคเอสแอลอีโดยการขาดตัวรับ FcGR11b เกิดความรุนแรงของการติดเชื้อในกระแสเลือดด้วยโมเดลการผูกและเจาะลำไส้ในหนูทดลองสูงขึ้นเนื่องจากภาวะ Macrophage exhaustion”

“Fc gamma receptor 11b 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”

บทคัดย่อภาษาไทย

การติดเชื้อแทรกซ้อนและซ้ำซ้อนพบได้บ่อยในผู้ป่วย เอสแอลอี ซึ่งเป็นสาเหตุการตายที่สำคัญในผู้ป่วย ผู้วิจัยเลียนแบบภาวะการติดเชื้อซ้ำซ้อนโดยการฉีด endotoxin 2 ครั้ง ห่างกัน 5 วัน แล้วจึงทำการผ่าตัดเจาะและผูกลำไส้ใหญ่เพื่อกระตุ้นการติดเชื้อในกระแสเลือดที่ 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 แมคโครเฟสของหนูเอสแอลอีเมื่อเปรียบเทียบกับหนูปกติตอบสนองต่อ endotoxin ในครั้งแรกโดยการสร้างไซโตคายต์ในระดับที่สูงมาก แต่มีระดับต่ำมากคล้ายกับที่พบในสัตว์ทดลองหลังจากการกระตุ้นครั้งที่สอง เข้าได้กับภาวะ macrophage paralysis

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

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 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 FcGR11b^{-/-} 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 FcGR11b^{-/-} 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 FcGR11b^{-/-} cells in parallel to the immune paralysis, highly susceptible to CLP, in FcGR11b^{-/-} mice compare with wild type group. These implied the importance of the repeated infections in patients with SLE, especially with FcGR11b polymorphisms.

6. คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย (List of Abbreviations)

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

ส่วนประกอบเนื้อเรื่อง

1. บทนำ (Introduction) ซึ่งกล่าวถึงเนื้อหาของเรื่องที่เคยมีผู้ทำการวิจัยมาก่อน ความสำคัญและที่มาของปัญหา วัตถุประสงค์และขอบเขตการวิจัย วิธีดำเนินการวิจัยโดยสรุปทฤษฎีและ/หรือแนวทางการความคิดที่นำมาใช้ในการวิจัย 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 IIb (FcGR IIb), the only inhibitory signaling receptors in the FcGR family, is one of the genetic susceptibility to SLE (2, 6). Interestingly, FcGR IIb 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 FcGR IIb polymorphisms in patients with SLE in Asia Pacific region is also common (3-5, 8). Perhaps FcGR IIb 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 FcGR IIb^{-/-} mice, one of the established SLE mouse model (6), could be resemble to untreated patients with SLE, especially with FcGR IIb 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 (FcGR I, FcGR III, FcGR IV) and only one inhibitory receptor (FcGR IIb) (16). The deficiency of all classes of FcGR in mice (FcGR^{-/-}) protected from sepsis (17) and FcGR IIb^{-/-} 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 FcGR IIb^{-/-} 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, FcGR IIb^{-/-} 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 FcGR11b^{-/-} mice might result in a prominent response but is easier exhausted after repeated stimulation. Then we test immunoparalysis of FcGR11b^{-/-} 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

FcγRIIb^{-/-} 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 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 μl 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 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, InvitrogenTM, 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 FcGR11b-/- 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).

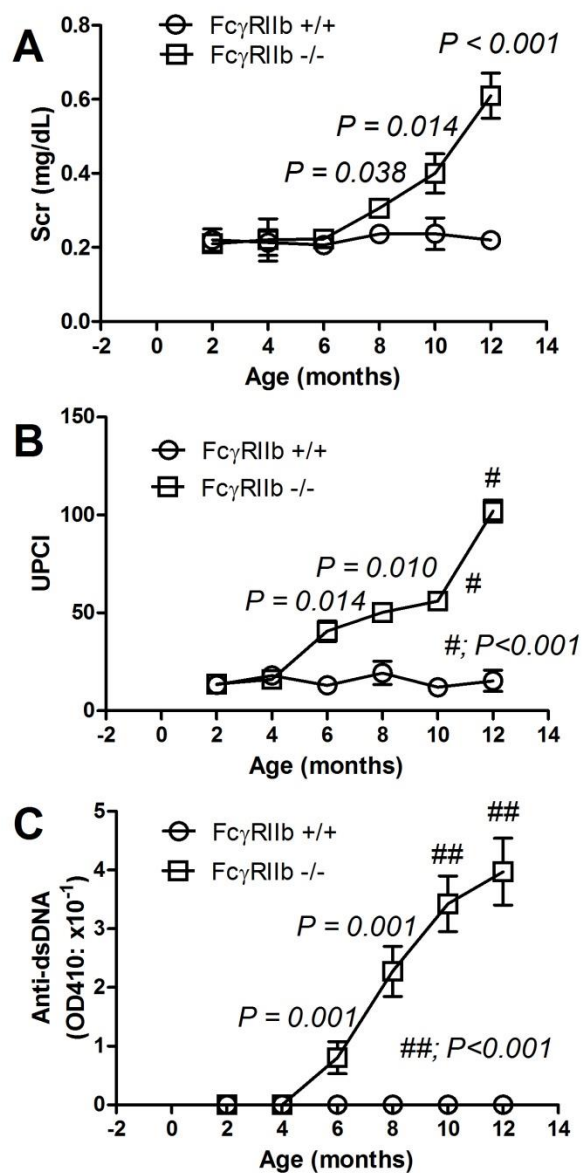


Figure 1. The natural history of Fc γ RIIb $^{-/-}$ and wild type (Fc γ RIIb $^{+/+}$) mice as determined by serum creatinine (Scr), urine protein creatinine index (UPCI) and anti-dsDNA (n=4-5/groups).

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).

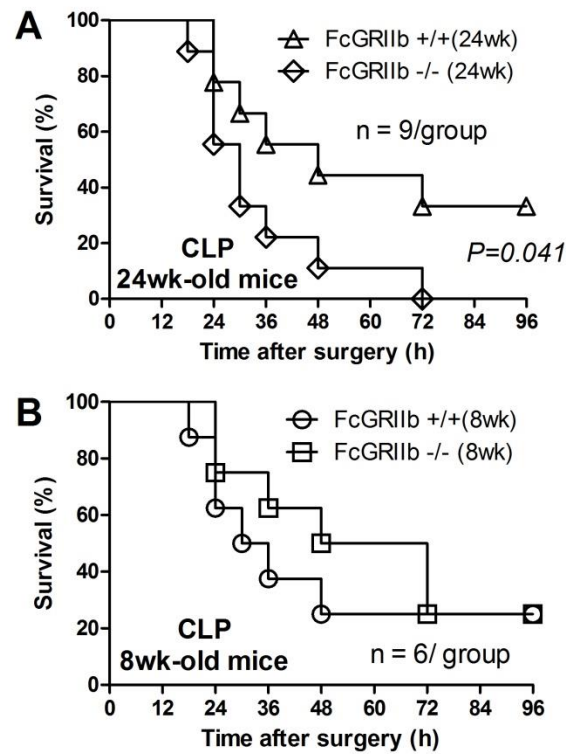


Figure 2. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery in asymptomatic *FcGR11b*^{-/-} mice (8-wk-old) (A) and symptomatic, proteinuria positive but normal serum creatinine, *FcGR11b*^{-/-} mice (24-wk-old) (B) in comparison with age-matched wild type mice (*FcGR11b*^{+/+}).

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).

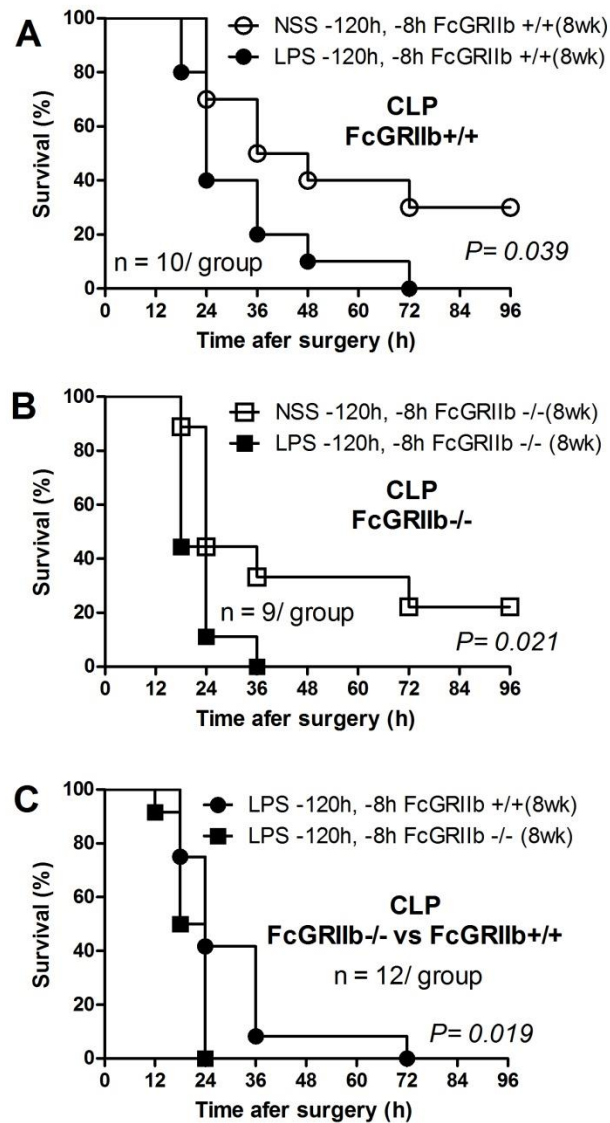


Figure 3. Survival analysis of cecal ligation and puncture (CLP) sepsis surgery preconditioning with 2 separated doses of LPS at 120h and 12h prior to CLP, CLP in endotoxin preconditioning model, in comparison with normal saline (NSS) placebo injection within wild type mice (*FcGR11b*^{+/+}) (A) and *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).

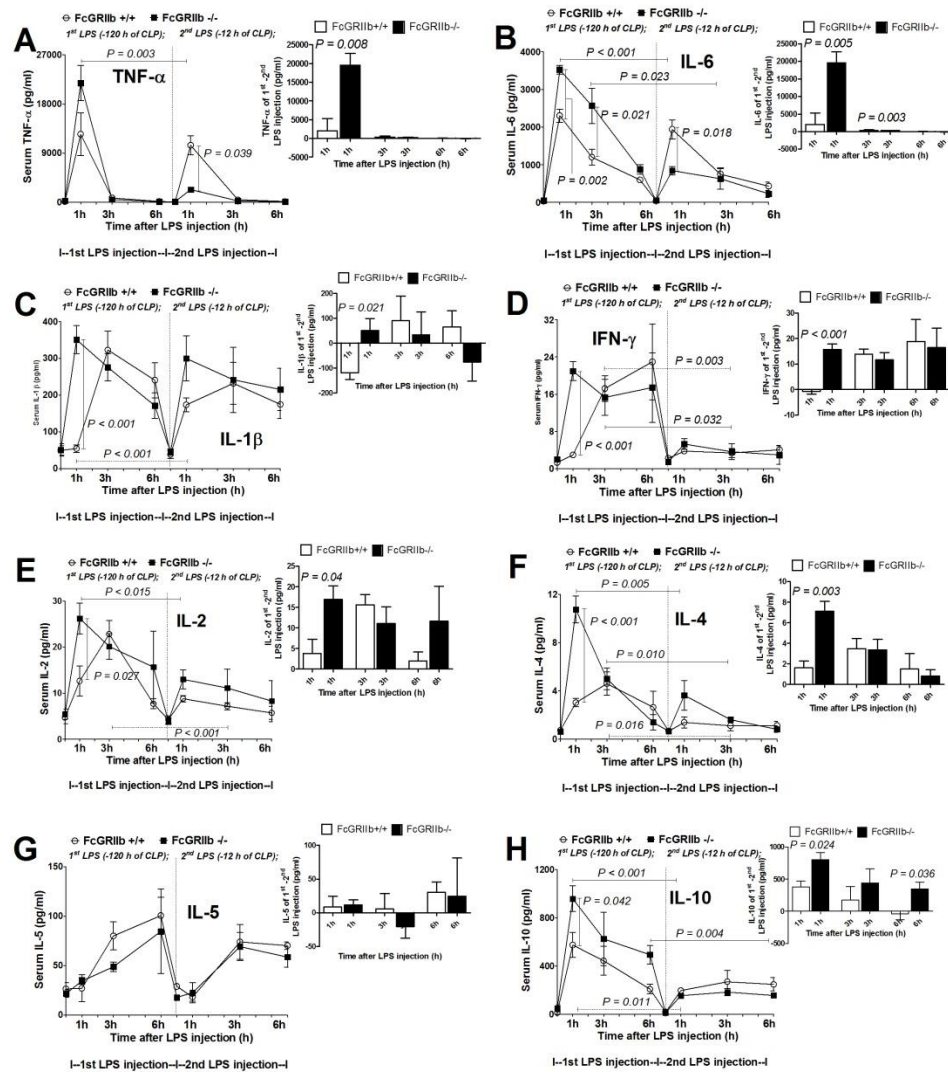


Figure 4. Serum cytokines in wild type (FcGR11b+/+) or FcGR11b-/- mice after at 1, 3 and 6h after 1st LPS injection (0.8 g/kg) and 2nd LPS injection (4 g/kg) as measured by TNF- α (A), IL-6 (B), IL-1 β (C), IFN- γ (D), IL-2 (E), IL-4 (F), IL-5 (G) and IL-10 (H) was demonstrated. To emphasize the difference 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*.

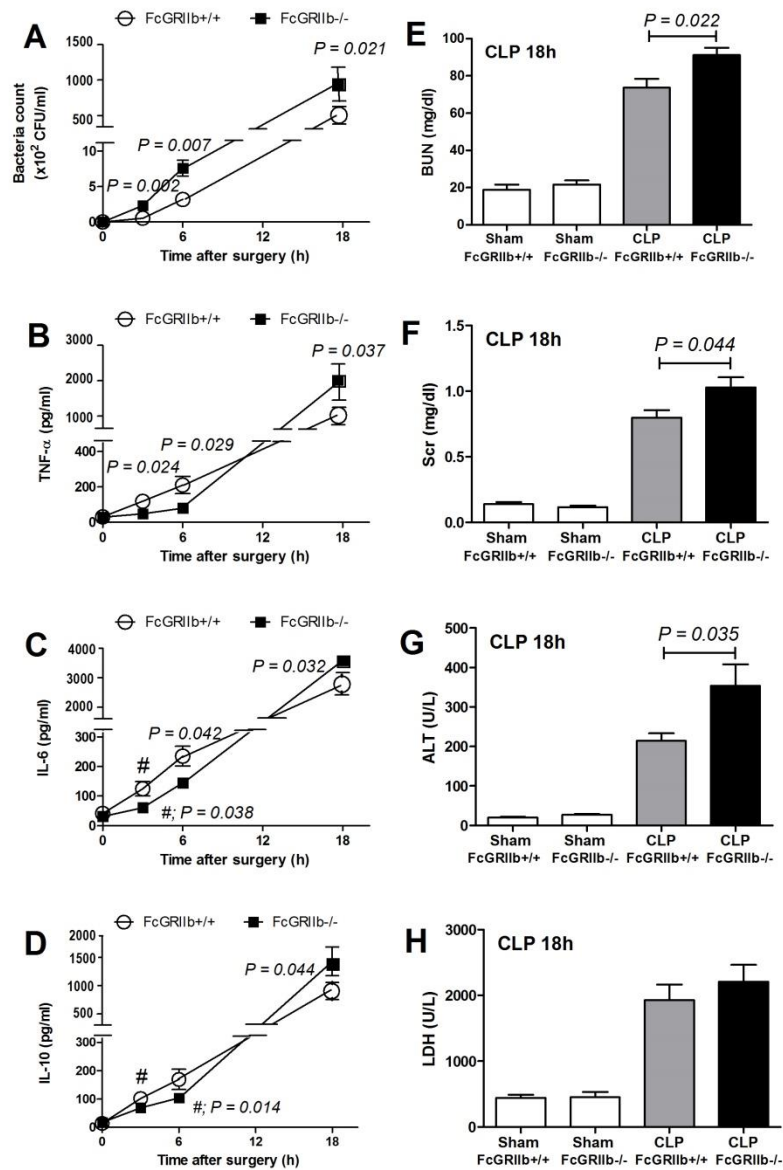


Figure 5. The time-course of bacterial burdens after cecal ligation and puncture (CLP) in blood bacterial burdens (A), mononuclear cell (B), PMN (C), TNF- α (D), IL-6 (E), IL-10 (F) and the severity of organs injury after 18h of wild type (FcGRIIb+/+) and FcGRIIb-/- as demonstrated by serum creatinine (Scr) (G) and alanine transaminase (ALT) (H) was showed. (n= 5-7/ time-point and group)

Interestingly, bacterial burdens in 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 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 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.

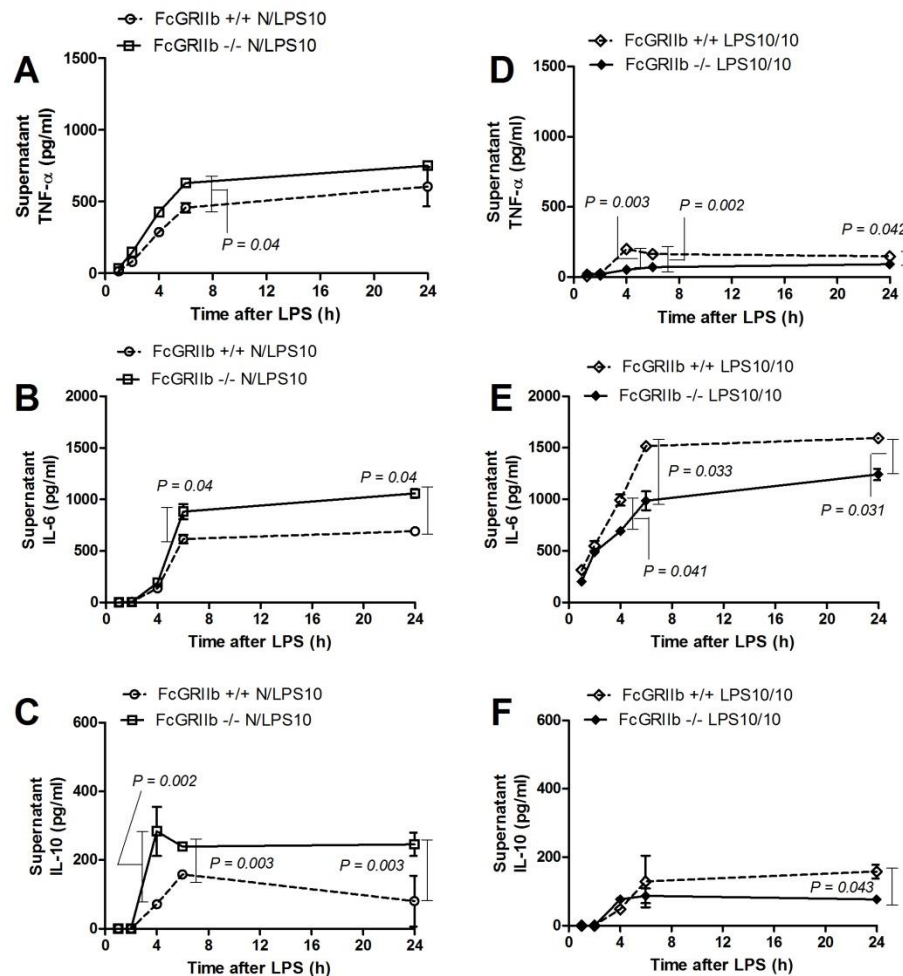


Figure 6. The cytokine responses in supernatant media from macrophages of FcGR11b^{-/-} or wild type (FcGR11b^{+/+}) after activated with only once low dose LPS (N/LPS10) as measured by TNF- α (A), IL-6 (B), IL-10 (C) and after activated with twice low dose LPS (LPS10/10) as measured by TNF- α (D), IL-6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

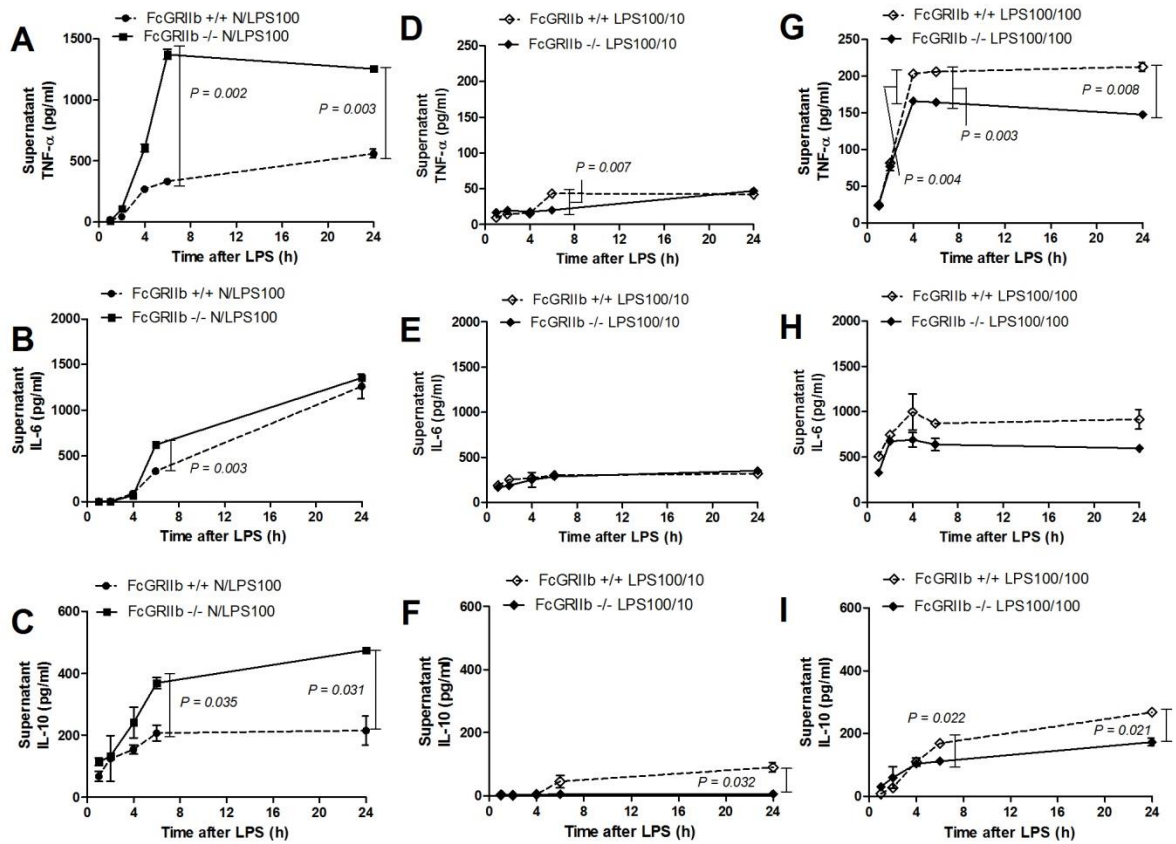


Figure 7. The cytokine responses in supernatant media from macrophages of *FcGR11b*^{-/-} or wild type (*FcGR11b*^{+/+}) after activated with only once high dose LPS (N/LPS100) as measured by TNF- α , IL-6, IL-10 (A-C) and double LPS doses, low and high dose LPS (LPS100/10 and LPS100/100, respectively),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

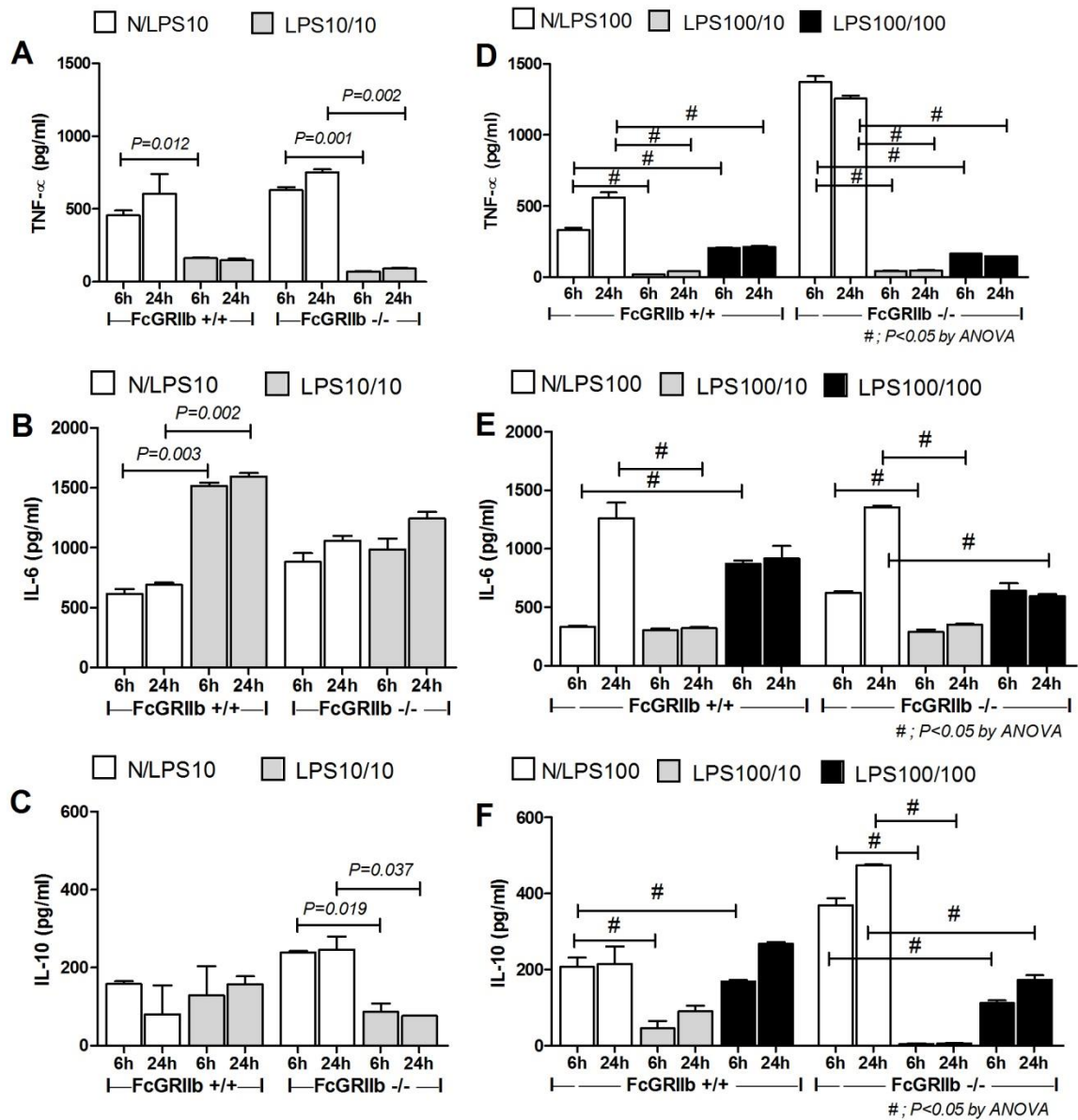


Figure 8. The macrophage endotoxin tolerance emphasized by the difference in cytokine responses (TNF- α , IL-6, IL-10) in supernatant media from macrophages of FcGR11b $^{-/-}$ or wild type (FcGR11b $^{+/+}$) after activated with only once low dose (N/LPS10) versus twice low dose of LPS (LPS10/10) (A-C) 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 differ 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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