

Syk Inhibitor Attenuates Polymicrobial Sepsis in FcγRIIb-Deficient Lupus Mouse Model, the Impact of Lupus Characteristics in Sepsis

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Keywords

FcγRIIb-deficient mice · Systemic lupus erythematosus · Endotoxin · Gut leakage · Spleen tyrosine kinase · Sepsis

Abstract

The impact of spleen tyrosine kinase (Syk) signaling might be prominent in lupus because (i) Syk is a shared downstream signaling molecule among circulating immune complex, LPS, and (1→3)-β-D-glucan (BG), and (ii) all of these factors are detectable in the serum of FcγRIIb-deficient (FcγRIIb^{-/-}) mice with sepsis. As a proof of concept study, we activated macrophages with BG combined with LPS (BG + LPS). We found that BG + LPS predominantly up-regulated Syk expression and proinflammatory cytokines in FcγRIIb^{-/-} macrophages compared with wild-type (WT) macrophages. Syk inhibition downregulated several inflammatory pathways in FcγRIIb^{-/-} macrophages activated with BG + LPS, as determined by RNA sequencing analysis, suggesting the potential anti-inflammatory impact of Syk inhibitors in lupus. Indeed, administration of a Syk inhibitor prior to cecal ligation and puncture (CLP) sepsis in FcγRIIb^{-/-} mice reduced baseline lupus-induced proinflammatory cytokines and attenuated sepsis severity as evaluated by mortality,

organ injury, serum LPS, and post-sepsis serum cytokines. In conclusion, it was easier to induce Syk expression in FcγRIIb^{-/-} macrophages than in WT macrophages. This might be because of the loss of inhibitory signaling, which might be responsible for prominent Syk abundance in the spleens of 40-week-old FcγRIIb^{-/-} mice and the potent effect of Syk inhibitor in lupus mice compared with WT.

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Introduction

Systemic lupus erythematosus is associated with functional defects of FcγRIIb (FcγRIIb), the only inhibitory receptor among the FcγR family [1–3], and FcγRIIb dysfunction polymorphism is high in the Asian population [4]; therefore, FcγRIIb^{-/-} mice have been used as a representative lupus model. Interestingly, FcγRIIb^{-/-} mice demonstrated spontaneous endotoxemia without gastrointestinal symptoms at 40 weeks of age. This might be because of gut permeability defects related to the deposition of circulating immune complex in the

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intestine [5, 6]. Because of the age-dependent development of lupus characteristics, representative models of asymptomatic lupus and symptomatic full-blown lupus can be induced in FcγRIIb^{-/-} mice younger and older than 40 weeks, respectively [6]. LPS, a major cell wall component of Gram-negative bacteria, and (1→3)-β-d-glucan (BG), a fungal cell wall component, are dominant in the gastrointestinal tract [7]. Of note, the induction of systemic inflammation models induced by LPS and BG translocated from the gut was reported [8–10] to involve synergistic activity through TLR-4 and dectin-1, receptors for LPS and BG, respectively [11–13]. In addition, severe bacterial sepsis caused by cecal ligation and puncture (CLP) induced glucanemia through sepsis induced gut leakage [14], which enhanced systemic inflammation and sepsis susceptibility in active lupus FcγRIIb^{-/-} mice [6]. Interestingly, the infectious complications of patients with lupus were more prominent with a higher severity, even before the era of immunosuppressive drugs, when compared with the normal population [15]. Likewise, the severity of sepsis in FcγRIIb^{-/-} mice with symptomatic lupus was higher than that in age-matched WT mice because previous studies reported (i) prominent responses to LPS with or without BG in FcγRIIb^{-/-} mice [6, 16] and (ii) synergistic activity between LPS and BG via TLR-4 and dectin-1, respectively [11–13]. Of note, spleen tyrosine kinase (Syk) is a shared downstream signaling molecule of FcγR, TLR-4, and dectin-1 [17, 18], which is activated by stimulators present in active lupus [6], including circulating immune complex, endotoxemia, and glucanemia. Accordingly, Syk is important for FcγR-mediated signal transduction, inflammatory induction, and organismal responses [17, 18]. In addition, the Syk inhibitor, fostamatinib, previously known as R788, is a US Food and Drug Administration approved drug for the treatment of chronic immune thrombocytopenia [19] and is considered a good candidate for the treatment of other autoimmune diseases. Syk inhibitors attenuate inflammatory processes in several lupus models [20–22] but have never been tested in FcγRIIb^{-/-} mice. Therefore, Syk inhibitors might have significant effects in FcγRIIb^{-/-} mice by rescuing the potent Syk activation caused by FcγR inhibitory signaling defect. Moreover, because there is high prevalence of FcγRIIb dysfunction polymorphisms in the Asian population, further studies to explore Syk inhibitors in FcγRIIb^{-/-} mice as another lupus model might yield promising results for the treatment of lupus patients [4].

Syk inhibitors inhibit FcγR signaling and attenuate TLR-4 activation as demonstrated by the attenuation of LPS-induced sepsis in mice [23]. Despite frequent studies

of the therapeutic effects of Syk inhibitors in autoimmune diseases, their effects on sepsis and sepsis preconditioning with lupus characteristics are unclear. The CLP model is a sepsis model with conditions similar to those of lupus patients. The CLP sepsis model and symptomatic lupus model develop gut permeability defect-induced endotoxemia and glucanemia [14]. Hence, a study of the therapeutic effect of Syk inhibitors on sepsis superimposed on lupus is of interest before clinical translation in patients with lupus. LPS and BG are pathogen-associated molecular patterns that mainly activate innate immunity, especially macrophages [11–13], and induce FcγR receptor expression related to adaptive immunity [1–3]; therefore, the simultaneous impact of Syk inhibitors on innate and adaptive immunity warrants further study. Here, we report *in vitro* and *in vivo* studies to determine the effect of a Syk inhibitor on sepsis using FcγRIIb^{-/-} lupus mice.

Materials and Methods

Animals

This study gained approval from the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, and followed the animal care and use protocol of the National Institutes of Health (NIH), USA. Only female mice were used in experiments. FcγRIIb-deficient mice on a C57BL/6 background (FcγRIIb^{-/-}), a lupus mouse model, were provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA) and female wild-type (WT) mice were purchased from the Nomura Siam International (Pathumwan, Bangkok, Thailand). FcγRIIb^{-/-} mice develop anti-dsDNA antibodies as early as 16–24 weeks and have increased serum creatinine (Scr) levels at 40 weeks of age, indicating lupus nephritis [6, 24, 25]. Therefore, FcγRIIb^{-/-} mice at 24 and 40 weeks of age were used as representative models of asymptomatic and symptomatic lupus, respectively.

Induction of the CLP Sepsis Model and Syk Inhibitor Administration

CLP was induced following a previous publication [6] with some modifications to induce polymicrobial sepsis in asymptomatic and symptomatic lupus mice. In brief, cecal puncture with a 21-gauge needle was performed under isoflurane anesthesia. Tramadol, 20 mg/kg diluted in 0.5 mL normal saline, and antibiotic (imipenem/cilastatin), at 14 mg/kg in 0.5 mL normal saline, were administered subcutaneously after surgery and at 6 h after CLP. Mice were sacrificed at 24 h after CLP under isoflurane anesthesia for tissue sample collection. The collected serum was kept at –80°C until analysis. A Syk inhibitor (R788 disodium; Selleckchem, Houston, TX, USA) in 0.1 M citrate buffer (pH 6.8) at 25 mg/kg/dose was orally administered in 2 separate groups of experiments including (i) daily oral administration for 14 days prior to CLP and at 6 h after CLP surgery and (ii) daily oral administration for 3 days prior to CLP and at 6 h after CLP surgery. Blood collection through tail vein was performed 2 days prior to CLP and at sacrifice for pre-

Table 1. List of primers used in this study

Primers	Forward	Reverse
Arginase-1 (Arg-1)	5'-CTTGGCTTGCTTCGGAAGCTC-3'	5'-GGAGAAGGCGTTTGCTTAGTTC-3'
Transforming growth factor- β (TGF- β)	5'-CAGAGCTGCGCTTGACAGAG-3'	5'-GTCAGCAGCCGGTTACCAAG-3'
Resistin-like molecule- α (FIZZ-1)	5'-GCCAGGTCCTGGAACCTTTC-3'	5'-GGAGCAGGGAGATGCAGATGAG-3'
Interleukin-10 (IL-10)	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
Inducible nitric oxide synthase (iNOS)	5'-ACCCACATCTGGCAGAATGAG-3'	5'-AGCCATGACCTTTCGCATTAG-3'
Tumor necrosis factor- α	5'-CCTCACACTCAGATCATCTTCTC-3'	5'-AGATCCATGCCGTTGGCCAG-3'
Interleukin-1 β	5'-GAAATGCCACCTTTTGACAGTG-3'	5'-TGGATGCTCTCATCAGGACAG-3'
Spleen tyrosine kinase (Syk)	5'-CTACTACAAGGCCAGACCC-3'	5'-TGATGCATTCCGGGGCGTAC-3'
β -Actin	5'-CGGTTCCGATGCCCTGAGGCTCTT-3'	5'-CGTCACACTTCATGATGGAATTGA-3'

and post-CLP parameters, respectively. Then, blood was collected through tail vein or cardiac puncture to explore lupus characteristics including Scr (QuantiChrom Creatinine Assay, DICT-500; BioAssay, Hayward, CA, USA), serum anti-dsDNA by a protocol using coated calf DNA (Invitrogen, Carlsbad, CA, USA) [26], and serum cytokines by ELISA (PeproTech, Oldwick, NJ, USA). Symptomatic lupus was defined as increased serum anti-dsDNA antibodies and high Scr compared with age-matched control WT mice. In addition, endotoxin (LPS) was measured as a parameter for sepsis severity using the Limulus Amebocyte lysate test (Associates of Cape Cod, East Falmouth, MA, USA). Values of LPS <0.01 EU/mL were recorded as 0.

Gut Permeability Determination

Fluorescein isothiocyanate-dextran (FITC-dextran), a gut nonabsorbable molecule, was orally administered to determine gut permeability, as previously published [14]. Briefly, FITC-dextran (molecular weight 4.4 kDa, FD4; Sigma, St. Louis, MO, USA) at 0.5 mL (25 mg/mL) diluted in sterile PBS was administered, and serum FITC-dextran was measured by fluorospectrometry (microplate reader; Thermo Scientific, Wilmington, DE, USA) after 3 h. Spontaneous increases in (1 \rightarrow 3)- β -D-glucan (BG) in serum, without systemic fungal infection, measured by Fungitell assay (Associates of Cape Cod), were used as an indicator of gut leakage. Values of BG <7.8 pg/mL were recorded as 0.

Histology Analysis

Semiquantitative evaluation of renal histology on paraffin-embedded slides was performed after 10% neutral buffered formalin fixation, followed by hematoxylin and eosin (H&E) staining [27, 28]. In brief, sepsis-induced renal injury (defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, cast formation, and desquamation) was performed at $\times 200$ magnification in 10 randomly selected fields for each animal using the following scoring method: 0, area of damage <5%; 1, area of damage 5–10%; 2, area of damage 10–25%; 3, area of damage 25–50%; and 4, area of damage >50%.

Western Blot Analysis

Isolated internal organs were maintained at -80°C until use for Western blot analysis, as previously described [29]. In brief, 20 μg of homogenized tissue, as measured by bicinchoninic acid assay (Thermo Fisher Scientific), was used for SDS-PAGE following the standard procedures before incubation with specific

primary antibodies against Syk (Cell signaling, Beverly, MA, USA) or glyceraldehyde 3-phosphate dehydrogenase (Cell signaling) overnight at 4°C . Then, a secondary antibody linked with horseradish peroxidase enzyme was used and visualized by ImageQuantTM LAS 500 (GE-Healthcare, Little Chalfont, Buckinghamshire, UK).

Bone-Marrow-Derived Macrophages and Supernatant Cytokines

Macrophages were derived from bone marrow following a published protocol [29, 30]. In parallel, heat-killed *Candida albicans*, the representative fungus, was prepared by heating at 65°C for 15 min, followed by sonication with a high intensity ultrasonic processor (VC/VCX 130, 500,750) at 25% amplitude until a clear solution was obtained. Macrophages (1×10^5 cells/well) were incubated with the heat-killed *C. albicans* preparation (HK-fungi) with or without LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) at 100 ng/mL or supplemented with DMEM alone (control) for 6 h before the measurement of supernatant cytokines (PeproTech). In addition, to determine different influences of the Syk inhibitor against the activation of Fc γ R1Ib^{-/-} and WT macrophages by heat-killed *C. albicans* with LPS, macrophages were preconditioned with active metabolites of the Syk inhibitor (R406) (Selleckchem) at 10 μg /mL or DMEM alone (control) for 1 h prior to a 6-h incubation of the fungal preparation with LPS and before supernatant cytokine measurement.

Real-Time PCR for Macrophage Polarization and Syk Expression

Macrophage polarization is associated with pro- or anti-inflammatory effects, termed M1 or M2 polarization, respectively [31], and the proinflammatory properties of Fc γ R1Ib^{-/-} macrophages are prominent [1]. Therefore, the polarization of macrophages from WT and Fc γ R1Ib^{-/-} after induction might be different. Accordingly, macrophages at 2×10^6 cells per well were incubated with whole glucan particle (WGP), representative of BG, purified from *Saccharomyces cerevisiae* (WGP[®] Dispersible; Biothera), at 100 μg /mL with or without LPS (100 ng/mL) for 6 h. Then, total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and the reverse transcription of 0.3 μg total RNA was performed using a high capacity reverse transcription assay (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Bio-

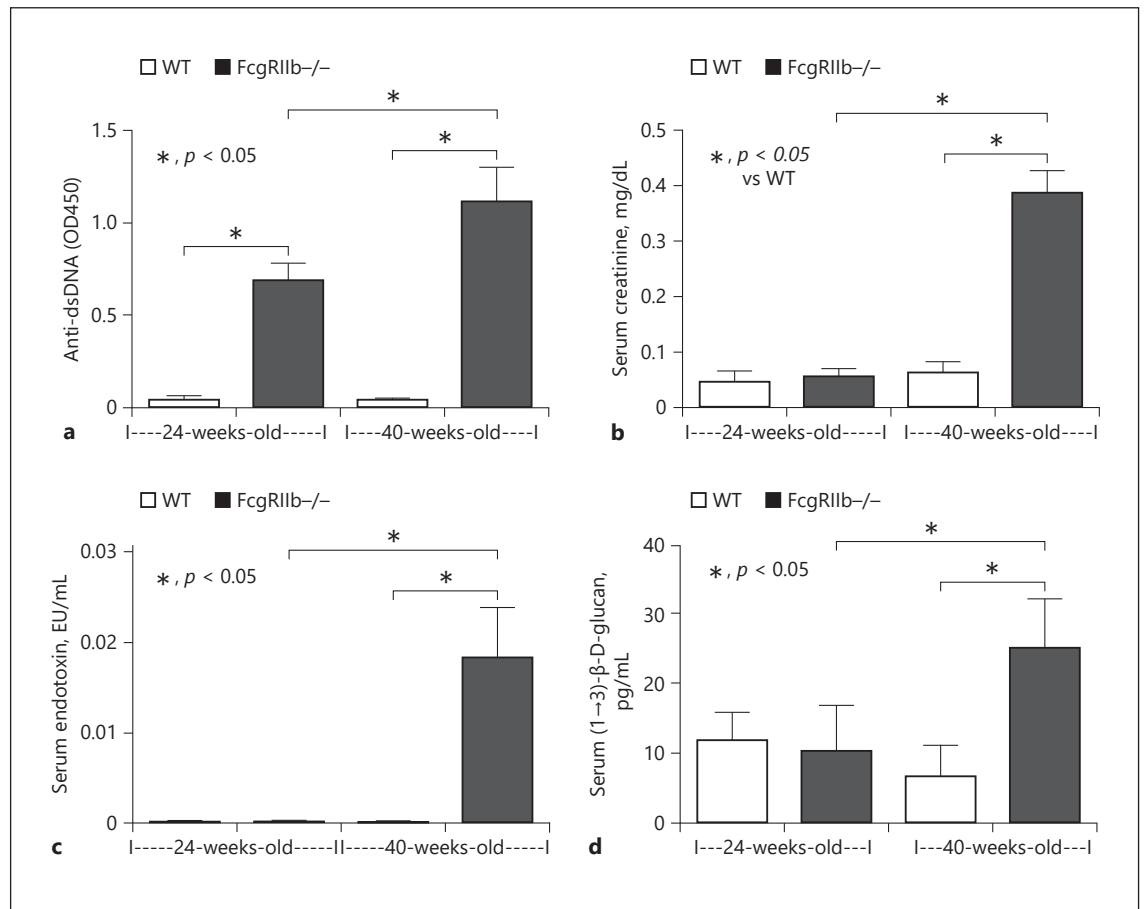


Fig. 1. Lupus characteristics of FcgRIIb^{-/-} mice. Characteristics of FcgRIIb^{-/-} mice at 24 (early onset of lupus) and 40 weeks of age (full-blown lupus) or age-matched wild-type (WT) mice as determined by serum anti-dsDNA (a), renal injury (serum creatinine) (b), endotoxemia (c), and serum (1→3)-β-D-glucan (d) ($n = 5-7$ /group).

systems) with SYBR[®] Green PCR Master Mix (Applied Biosystems). The results were indicated in terms of relative quantitation using the comparative threshold ($\Delta\Delta C_t$) method. The expression of target genes in the sample, normalized to β -actin (an endogenous housekeeping gene), was demonstrated. A list of primers for PCR is shown in Table 1.

RNA Sequencing

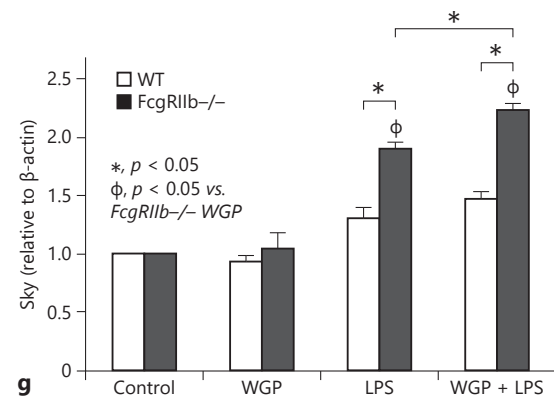
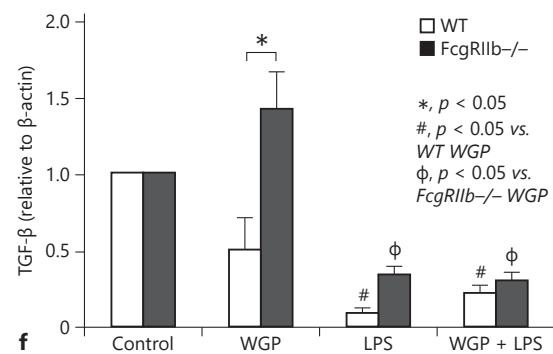
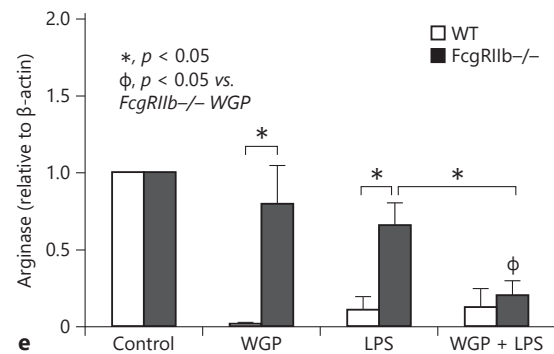
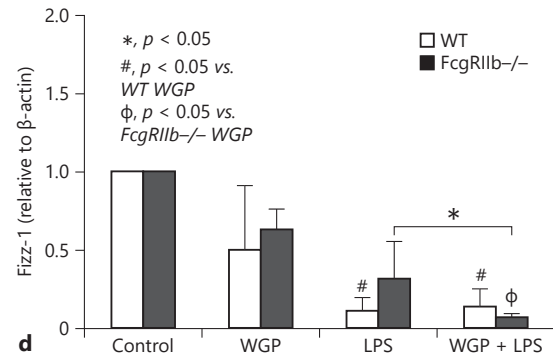
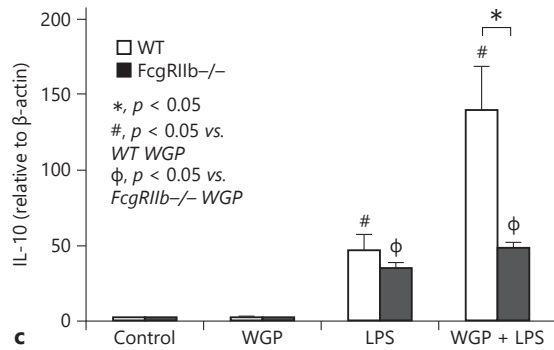
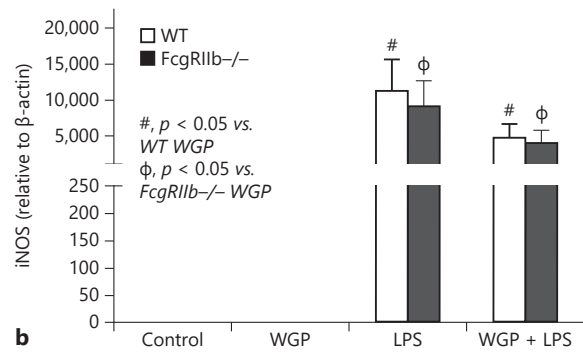
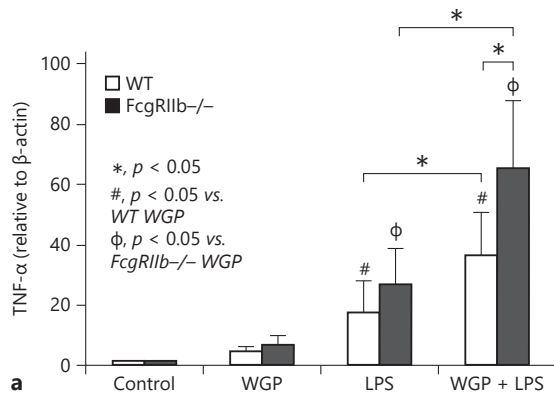
RNA sequencing was performed to determine the influence of a Syk inhibitor on FcgRIIb^{-/-} and WT macrophages after activation by WGP (500 μ g/mL) plus LPS (100 ng/mL). FcgRIIb^{-/-} and WT macrophages were treated with a combination of WGP and LPS, with and without the active form of the Syk inhibitor, R406 (Selleckchem), at 10 μ g/mL for 6 h. Then, the cells were collected for RNA extraction using an RNeasy mini kit (Qiagen). RNA sequencing was performed by the BGI Company. Differential gene expression was determined using R package. Biological process and pathway analysis were performed using GO analysis and gene ontology pathway analysis, respectively.

Statistical Analysis

Statistical differences among groups were examined using the unpaired Student's t test or one-way ANOVA with Tukey's comparison test for the analysis of experiments with 2 groups or more than 2 groups, respectively, and are presented as the mean \pm SE. Statistical comparisons of data before and after treatment were conducted by paired Student's t test. SPSS 11.5 software (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Fig. 2. Expression of activated genes in activated macrophages. Gene expression of macrophage after 6 h activation by whole glucan particle (WGP), representative of (1→3)-β-D-glucan, with or without LPS in FcgRIIb^{-/-} and wild-type (WT) macrophages, as determined by proinflammatory genes ($TNF-\alpha$ and $iNOS$) (a, b), anti-inflammatory genes ($IL-10$, $Fizz-1$, $Arginase-1$, and $TGF-\beta$) (c-f), and spleen tyrosine kinase (Syk) (g). Independent triplicate experiments were performed.

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Results

Asymptomatic and Symptomatic Lupus Characteristics in 24- and 40-Week-Old FcγRIIb^{-/-} Mice

Lupus characteristics including anti-dsDNA antibodies and Scr were evaluated. Anti-dsDNA antibodies were elevated as early as 24 weeks of age in FcγRIIb^{-/-} mice, whereas Scr was increased at 40 weeks of age, indicating renal injury (Fig. 1a, b). Spontaneous gut permeability defects as determined by the elevation of endotoxin (LPS) and BG in serum were demonstrated in 40-week-old FcγRIIb^{-/-} mice (Fig. 1c, d). Levels of anti-dsDNA antibody, but not of other parameters, were increased in 24-week-old FcγRIIb^{-/-} mice (Fig. 1). These characteristics confirmed symptomatic and asymptomatic lupus were induced in FcγRIIb^{-/-} mice, as previously published [6].

Prominent Responses against Bacterial and Fungal Molecules in FcγRIIb^{-/-} Macrophages Compared with WT Macrophages

Because LPS and BG in the serum of symptomatic lupus mice might activate macrophages, gene expression related to macrophage polarization was investigated. LPS, a potent proinflammatory inducer, enhanced the expression of the proinflammatory genes, *TNF-α* and *iNOS*, and increased the expression of the anti-inflammatory gene, *IL-10*, to similar levels in WT and FcγRIIb^{-/-} macrophages (Fig. 2a–c). The addition of WGP, a representative BG, enhanced LPS-induced *TNF-α* expression, but not *iNOS*, and decreased *IL-10* expression in FcγRIIb^{-/-} macrophages (Fig. 2a–c), suggesting proinflammatory synergy between BG and LPS. In parallel, activation with LPS, with and without WGP, had a minor effect on the expressions of other anti-inflammatory genes including *Fizz-1*, *Arginase-1*, and *TGF-β*, which were lower than in the control group (Fig. 2c–f), except for activation by WGP alone in FcγRIIb^{-/-} cells (Fig. 2f). These data suggest that WGP plus LPS enhanced the proinflammatory characteristics of FcγRIIb^{-/-} macrophages compared with WT macrophages. Furthermore, the expression of *Syk*, the shared downstream signaling factor of WGP and LPS [32], was higher in FcγRIIb^{-/-} macrophages after activation by LPS alone or LPS with WGP (LPS + WGP) compared with WT macrophages (Fig. 2g). *Syk* expression in FcγRIIb^{-/-}, but not WT, macrophages after activation with LPS + WGP was higher than when activated with LPS alone (Fig. 2g).

To explore further influence of combined bacterial and fungal molecules on macrophages, levels of cytokines

including *TNF-α*, *IL-6*, and *IL-10* were measured in the supernatant after the cells were activated by LPS with or without heat-killed *C. albicans* (HK-fungi). All groups showed elevated supernatant cytokines; however, activation by LPS alone was more potent than HK-fungi alone in both strains of macrophages (Fig. 3a–c). Stimulation by HK-fungi (alone) similarly elevated supernatant levels of cytokines from both strains of macrophages, whereas LPS (alone) induced higher levels of cytokines in FcγRIIb^{-/-} macrophages compared with WT macrophages (Fig. 3a–c). Compared with stimulation by LPS alone, LPS plus HK-fungi (LPS + HK-fungi) enhanced *TNF-α* and *IL-6* levels, but not *IL-10*, in FcγRIIb^{-/-} macrophages and increased *IL-10*, but not other cytokines, in WT macrophages (Fig. 3a–c). This suggests that FcγRIIb^{-/-} macrophages were more proinflammatory compared with WT macrophages. In addition, after activation by LPS + HK-fungi, the *Syk* inhibitor attenuated all supernatant cytokines in FcγRIIb^{-/-} macrophages but only *IL-6* in WT macrophages (Fig. 3d–f).

Prominent Effect of the Syk Inhibitor on FcγRIIb^{-/-} Macrophages Compared with WT Macrophages: RNA Sequencing Analysis

We observed a prominent anti-inflammatory effect of the *Syk* inhibitor on FcγRIIb^{-/-} macrophages compared with WT macrophages. Therefore, RNA sequencing was performed to investigate the potential pathways involved. The numbers of genes expressed in FcγRIIb^{-/-} macrophages after activation by WGP plus LPS (WGP + LPS) with or without a *Syk* inhibitor or in the negative control (culture media without pathogenic molecules) were similar (Fig. 4a). However, the *Syk* inhibitor suppressed most of the expressed genes in WGP + LPS activated FcγRIIb^{-/-} macrophages (Fig. 4b). In addition, the comparison of differentially expressed genes in FcγRIIb^{-/-} macrophages with negative control FcγRIIb^{-/-} macrophages after stimulation with WGP + LPS demonstrated the upregulation of proinflammatory genes (*TNF-α*, *NF-κB*, and *MAPK*) and the downregulation of genes in metabolic pathways (Fig. 4c). In groups treated with the *Syk* inhibitor, differentially expressed genes in most pathways including the signaling pathways of *TNF-α*, Toll-like receptor, and *NF-κB* were downregulated (Fig. 4d).

Interestingly, the direction of gene expression (up- or downregulation) in WGP + LPS activated FcγRIIb^{-/-} macrophages, compared with the negative control, was similar to genes associated with high mortality rate in patients with sepsis [33]. Most upregulated genes in pa-

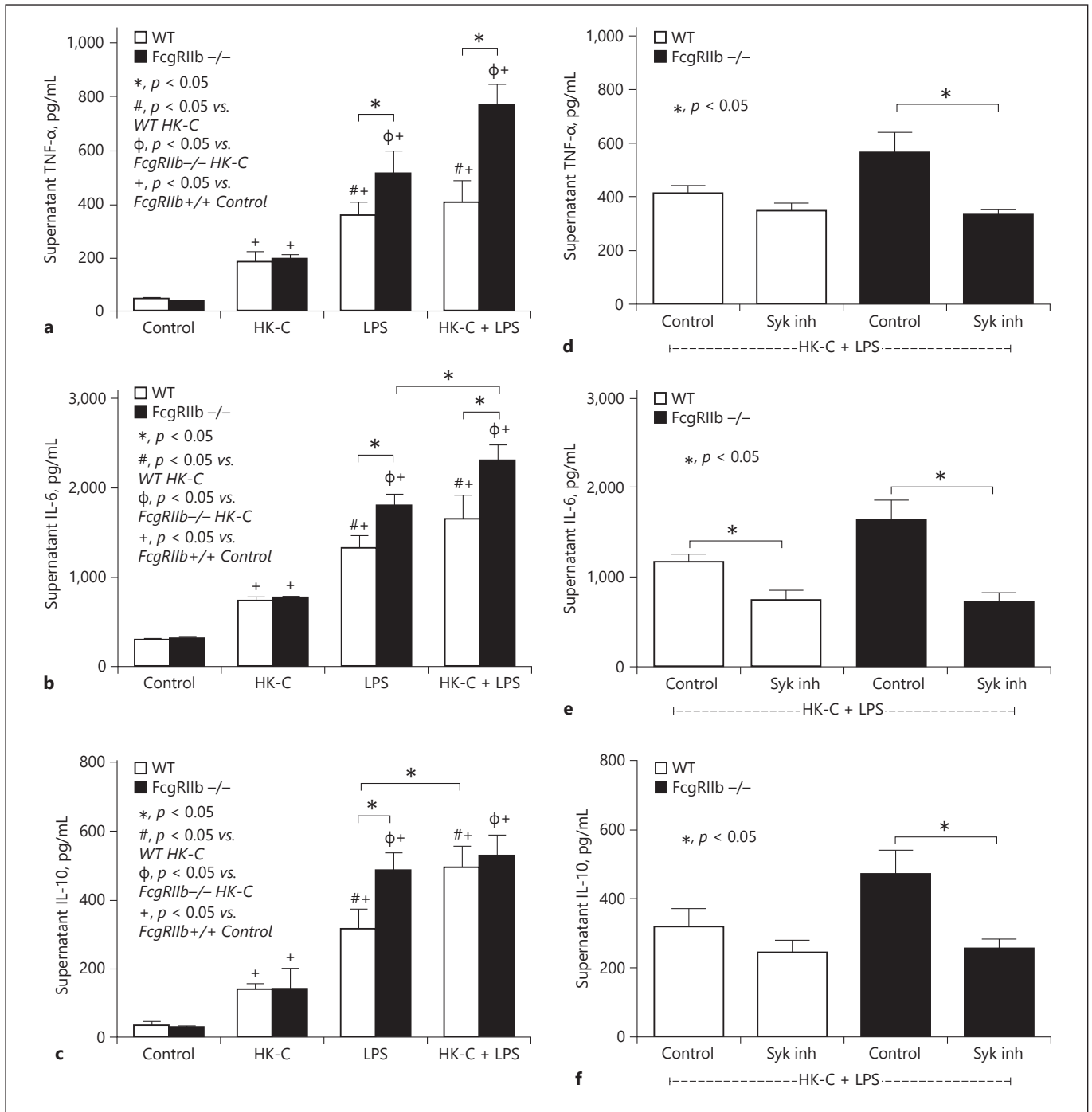
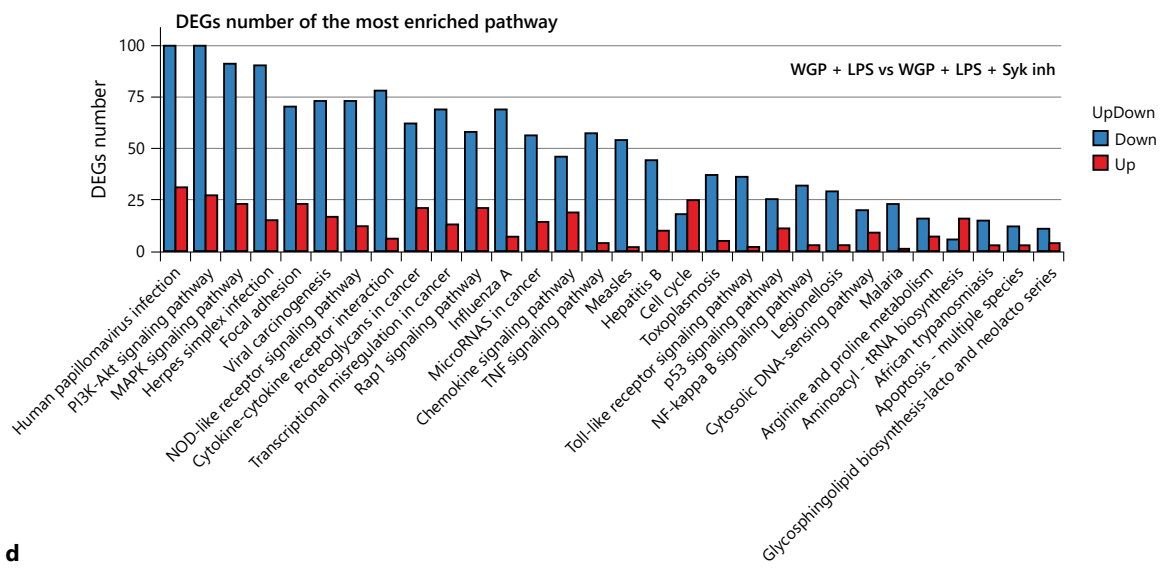
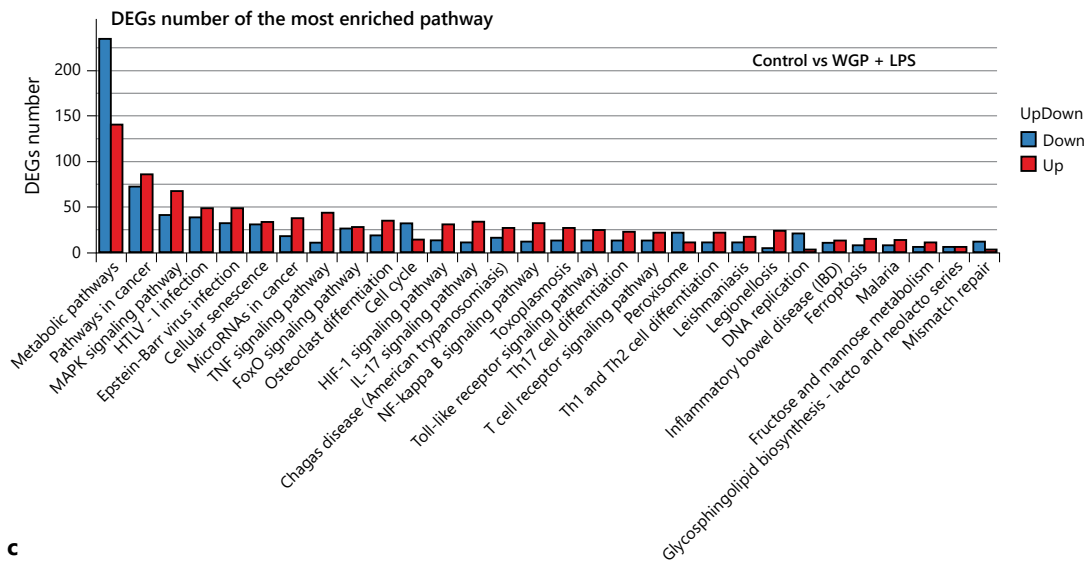
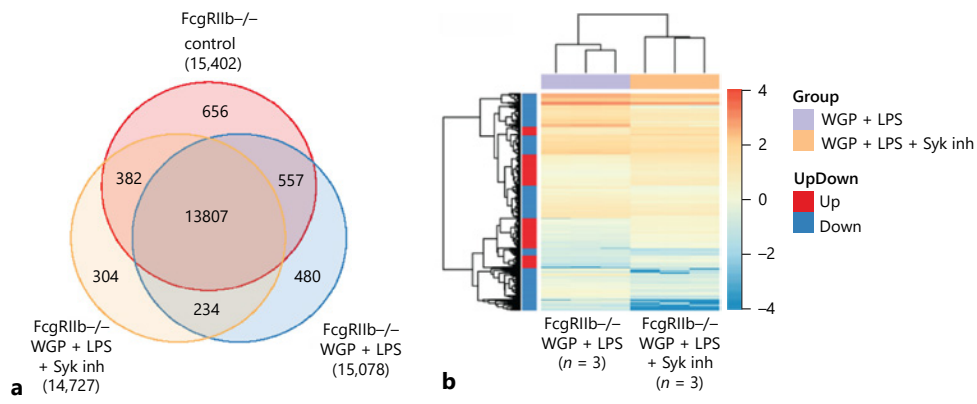


Fig. 3. Supernatant cytokine levels of activated macrophages. Supernatant cytokines secreted by macrophages from FcgRIIb $^{-/-}$ and wild-type (WT) mice after 6 h incubation with heat-killed *Candida albicans* (HK-C) with or without LPS (**a-c**). The effect of a Syk inhibitor against supernatant cytokine secretion by HK-C with LPS in FcgRIIb $^{-/-}$ and WT mice (**d-f**). Independent triplicate experiments were performed.



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tients with severe sepsis, except *PLK1* and *CD-163*, were also upregulated in WGP + LPS activated FcγRIIb^{-/-} macrophages (Fig. 5; control vs. WGP + LPS). Likewise, most downregulated genes in patients with severe sepsis, except *AIM2*, *GSTM1*, and *VNN3*, were downregulated in WGP + LPS activated FcγRIIb^{-/-} macrophages (Fig. 5; control vs. WGP + LPS). These data indicated the similarity between genes expressed in patients with severe sepsis and those expressed in WGP + LPS activated FcγRIIb^{-/-} macrophages. Of note, the Syk inhibitor reversed the direction of the expressed genes in WGP + LPS activated FcγRIIb^{-/-} macrophages (Fig. 5; WGP + LPS vs. WGP + LPS + Syk inhibitor), indicating Syk inhibitors might be a potential candidate for sepsis treatment in lupus.

High Levels of Syk in FcγRIIb^{-/-} Mice and Sepsis Attenuation by Syk Inhibition

Because Syk is a shared downstream signaling factor of FcγR, TLR-4, and dectin-1 [34, 35] and was activated by anti-dsDNA antibodies, LPS, and BG in symptomatic FcγRIIb^{-/-} mice (Fig. 1), we investigated the levels of Syk in FcγRIIb^{-/-} and WT mice. Syk levels were higher in the spleen than in other organs in FcγRIIb^{-/-} mice at 24 and 40 weeks of age, and in WT mice at 40 weeks, but not 24 weeks, of age (Fig. 6a). Syk levels in the spleens of 40-week-old WT mice were similar to those in 24-week-old FcγRIIb^{-/-} mice, and Syk levels in 40-week-old FcγRIIb^{-/-} mice were highest among all groups (Fig. 6a). In addition, Syk levels in spleens were enhanced by CLP sepsis in both mouse strains, but levels were highest in FcγRIIb^{-/-} mice with sepsis (Fig. 6b). Because Syk levels were increased in the spleens of CLP sepsis mice, we investigated the effect of the Syk inhibitor *in vivo*.

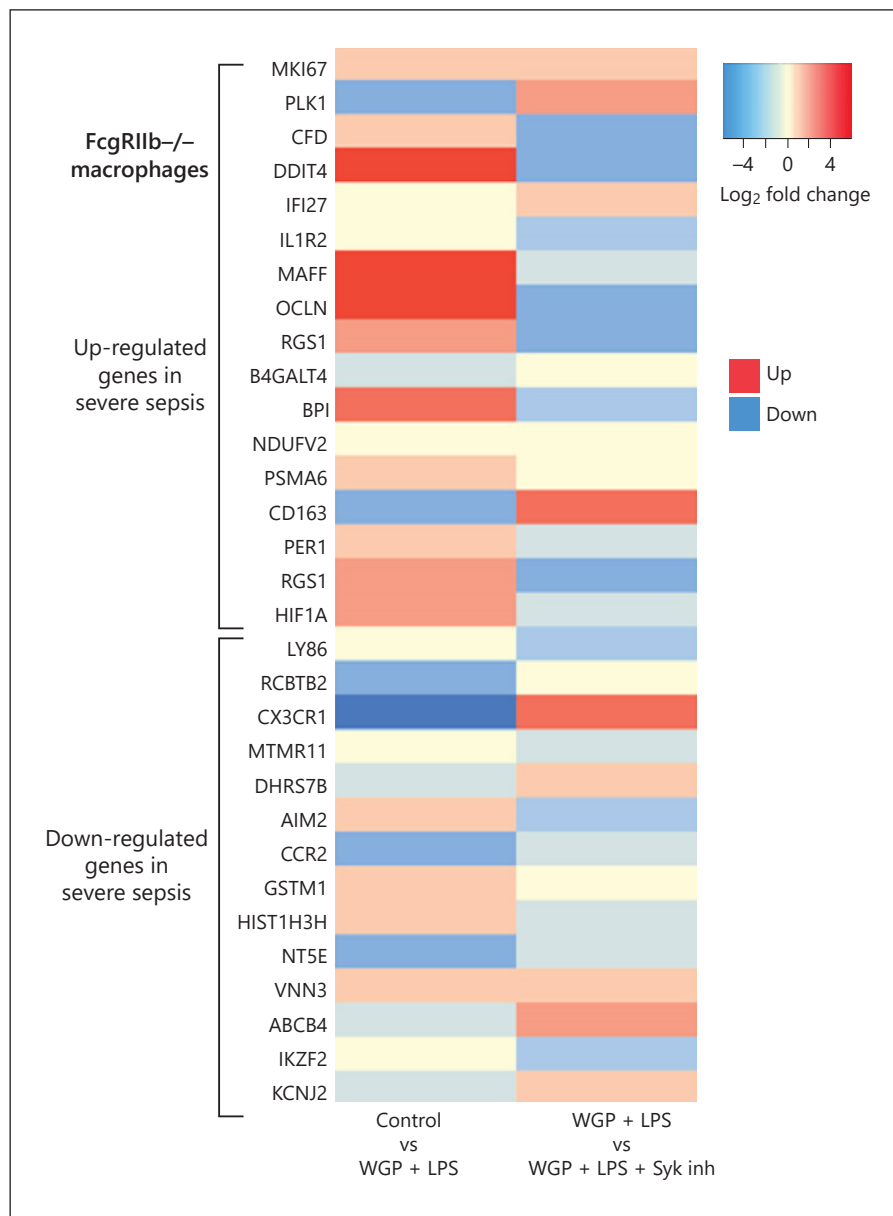
The mortality rate of CLP sepsis in 40-week-old FcγRIIb^{-/-} mice was higher than that in age-matched WT

sepsis mice, but a similar mortality rate was observed for 24-week-old mice of either strains (Fig. 7a, b). Furthermore, the mortality rate of sepsis in FcγRIIb^{-/-} mice aged 40 weeks was higher than that in 24-week-old mice (Fig. 7c). Organ injury determined by Scr, renal histology, liver enzyme (ALT) (Figs. 7d–i, 8), gut leakage (FITC-dextran, serum LPS, and serum BG) (Fig. 9), and inflammatory cytokines (Fig. 10) at 24 h post-CLP was more severe in 40-week-old FcγRIIb^{-/-} mice than in 24-week-old FcγRIIb^{-/-} mice or WT mice. Levels of anti-dsDNA antibodies were higher in 40-week-old FcγRIIb^{-/-} mice than in 24-week-old FcγRIIb^{-/-} mice, and sepsis did not alter anti-dsDNA antibody levels (Fig. 7j–l). In addition, there was preconditioning damage in 40-week-old FcγRIIb^{-/-} mice (pre-CLP), as indicated by the higher baseline levels of Scr (Fig. 7f), gut leakage (Fig. 9c, f, i), and inflammatory cytokines (Fig. 10c, f, i), but not ALT or anti-dsDNA antibodies (Fig. 7i, l), compared with 24-week-old FcγRIIb^{-/-} mice, which might explain the higher sepsis mortality rate in 40-week-old FcγRIIb^{-/-} mice compared with 24-week-old FcγRIIb^{-/-} mice (Fig. 7c). In parallel, there was no preconditioning injury in 24-week-old FcγRIIb^{-/-} mice compared with age-matched WT mice by these pre-CLP parameters (Figs. 7–10), except for anti-dsDNA antibodies (Fig. 7j), resulting in a similar mortality rate of CLP sepsis between 24-week-old FcγRIIb^{-/-} mice and age-matched WT mice (Fig. 7a).

Compared with the PBS control group, after 14-day administration of a Syk inhibitor prior to CLP surgery, the severity of sepsis was lower in 40-week-old FcγRIIb^{-/-} mice, but not 24-week-old FcγRIIb^{-/-} mice and WT mice, as determined by mortality rate and several post-CLP parameters including Scr, renal histology, liver damage (ALT) (Figs. 7, 8), and inflammatory cytokines (Fig. 10). Furthermore, the Syk inhibitor did not decrease the severity of sepsis-induced gut leakage, as indicated by FITC-dextran and serum BG (Fig. 9b, h), but did attenuate serum LPS levels (Fig. 9e), which might explain the attenuation of sepsis severity. However, 14-day treatment with the Syk inhibitor reduced baseline proinflammation, as indicated by reduced levels of pre-CLP serum cytokines (Fig. 10b, e, h) despite no effect on Scr, anti-dsDNA antibodies (Fig. 7e, k), or gut leakage (Fig. 9b, e, f). These data indicate the importance of preconditioning injury upon sepsis severity [28, 36]. Indeed, the 3-day administration of a Syk inhibitor prior to CLP did not decrease pre-CLP serum cytokine levels or sepsis severity in lupus mice of both ages (Fig. 11).

Fig. 4. RNA sequencing analysis of activated macrophages. Venn diagram of the RNA sequencing analysis demonstrating gene expression of FcγRIIb^{-/-} macrophages after 6 h activation with whole glucan particle (WGP) and LPS (WGP + LPS) with or without Syk inhibitor (Syk inh) or control culture media (Control) (a). A heat map of differentially expressed genes in activated FcγRIIb^{-/-} macrophages by WGP + LPS with or without a Syk inhibitor (b). Comparison of differentially expressed genes (DEGs) from the most enriched pathways between activated FcγRIIb^{-/-} macrophages and control culture media (Control vs. WGP + LPS) (c) and between activated FcγRIIb^{-/-} macrophages with or without a Syk inhibitor (WGP + LPS vs. WGP + LPS + Syk inhibitor) (d) (*n* = 3/group).

Fig. 5. Comparison of gene expression between activated macrophages and patients with severe sepsis. A heat map comparison of the up- and downregulated genes in macrophages associated with genes expressed in patients with high mortality rate sepsis. Left column: fold change in gene expression after activation of FcγRIIb^{-/-} macrophages by whole glucan particle (WGP), a representative of (1→3)-β-D-glucan, and LPS (WGP + LPS) versus Control. Right column: fold change in gene expression of FcγRIIb^{-/-} macrophages activated by WGP + LPS with Syk inhibition (WGP + LPS + Syk inh) versus no Syk inhibition (WGP + LPS).



Discussion

Serum inflammatory cytokines and Syk signaling in FcγRIIb^{-/-} mice at baseline before CLP operation were higher than in WT mice. This might be related to Syk-mediated preconditioning injury caused by immune complex, endotoxemia, and glucanemia in active lupus. Marked Syk activation in FcγRIIb^{-/-} mice with sepsis compared with WT mice might explain the therapeutic effect of Syk inhibitor in lupus with sepsis.

More Severe Sepsis in Full-Blown Lupus in FcγRIIb^{-/-} Mice Compared with WT Mice: Impact of Preconditioning Inflammation through Gut Permeability Defects

Spontaneously developed lupus characteristics including anti-dsDNA antibodies and renal injuries (Scr and renal pathology) caused by hyper-immune responsiveness related to a defect in negative signaling [1] were demonstrated in 40-week-old FcγRIIb^{-/-} mice, a full-blown lupus model. In parallel, it was shown that prominent intestinal deposition of immune complex with gut perme-

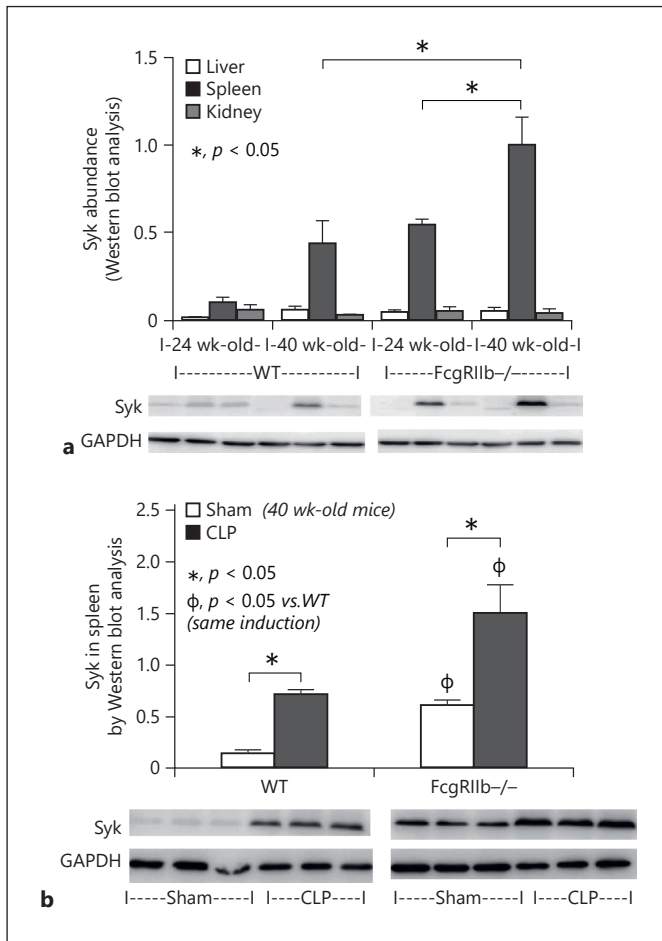


Fig. 6. Syk abundance in mouse organs. Syk levels in organs from wild-type (WT) and *FcgRIIb*^{-/-} mice aged 24 and 40 weeks. **a** Western blot analysis without cecal ligation and puncture (CLP) ($n = 5/\text{group}$). **b** Syk levels in spleens of 40-week-old WT and *FcgRIIb*^{-/-} mice 24 h after CLP or sham surgery (Sham) ($n = 5/\text{group}$).

ability defects induced endotoxemia/glucanemia in 40-week-old *FcgRIIb*^{-/-} mice, leading to more severe sepsis than in WT mice [6]. Although endotoxemia in the CLP model cannot be used as an indicator of gut leakage because of sepsis-induced Gram-negative bacteremia, glucanemia without fungemia did indicate sepsis-induced leaky gut [14]. Nevertheless, LPS and BG are pathogenic molecules foreign to the host, which synergistically activate innate immune responses in the host through TLR-4 and dectin-1, respectively [32], resulting in systemic inflammatory responses [6, 8, 9, 14, 37]. Indeed, gut leakage and levels of systemic cytokines before sepsis induction and sepsis severity in 40-week-old *FcgRIIb*^{-/-}

mice (full-blown lupus) were higher than those in 24-week-old *FcgRIIb*^{-/-} mice (asymptomatic lupus). Accordingly, enhanced sepsis severity after preconditioning inflammation in 40-week-old *FcgRIIb*^{-/-} mice supports the impact of chronic inflammation at baseline (before sepsis induction) on sepsis severity [28, 36, 38–40], similar to other 2-hit sepsis models [41–43]. Therefore, the attenuation of baseline systemic inflammation in full-blown lupus mice, before CLP induction, might be a good strategy to reduce sepsis severity.

*High Syk Signaling in *FcgRIIb*^{-/-} Lupus Mice and Marked Anti-Inflammatory Effects of a Syk Inhibitor in Lupus: Potential for Clinical Translation*

Syk is a shared downstream signaling factor of FcγR, TLR-4, and dectin-1 [34]. Interestingly, the stimulatory molecules of these receptors, including anti-dsDNA antibodies, LPS, and BG, were presented in the serum of 40-week-old *FcgRIIb*^{-/-} mice, but not WT mice, because of lupus-induced gut permeability defects [6]. Indeed, levels of Syk in the spleens of fully developed lupus *FcgRIIb*^{-/-} mice at 40 weeks of age were higher than in age-matched WT mice. In addition, activation of *FcgRIIb*^{-/-} macrophages by LPS alone, or LPS + BG, was more potent than the activation of WT macrophages, as indicated by higher cytokine production and enhanced Syk expression supporting the hyper-responsiveness of *FcgRIIb*^{-/-} macrophages [1]. In WT macrophages, activation by LPS + BG increased the expression of *TNF-α*, a proinflammatory cytokine, and increased the expression of *IL-10* and levels of supernatant IL-10, which are anti-inflammatory biomarkers, compared with activation by LPS alone. There appeared to be a balance between pro- and anti-inflammatory responses in WT macrophages after LPS + BG activation.

However, LPS + BG activation of *FcgRIIb*^{-/-} macrophages increased the expressions of *TNF-α* and Syk, but reduced *IL-10* expression, together with increased TNF-α and IL-6, but not IL-10 levels, in the supernatant, supporting the activation of LPS + BG through Syk signaling and the synergistic proinflammatory effect of LPS + BG [32]. The activation of *FcgRIIb*^{-/-} macrophages by LPS + BG shifted toward a proinflammatory response, which might be related to the loss of inhibitory signaling through FcγRIIb. Furthermore, the greater anti-inflammatory effect of the Syk inhibitor in *FcgRIIb*^{-/-} macrophages compared with WT macrophages might indicate the greater impact of Syk signaling in lupus.

Although it was previously demonstrated that Syk inhibitor attenuated sepsis in LPS model (a model induced

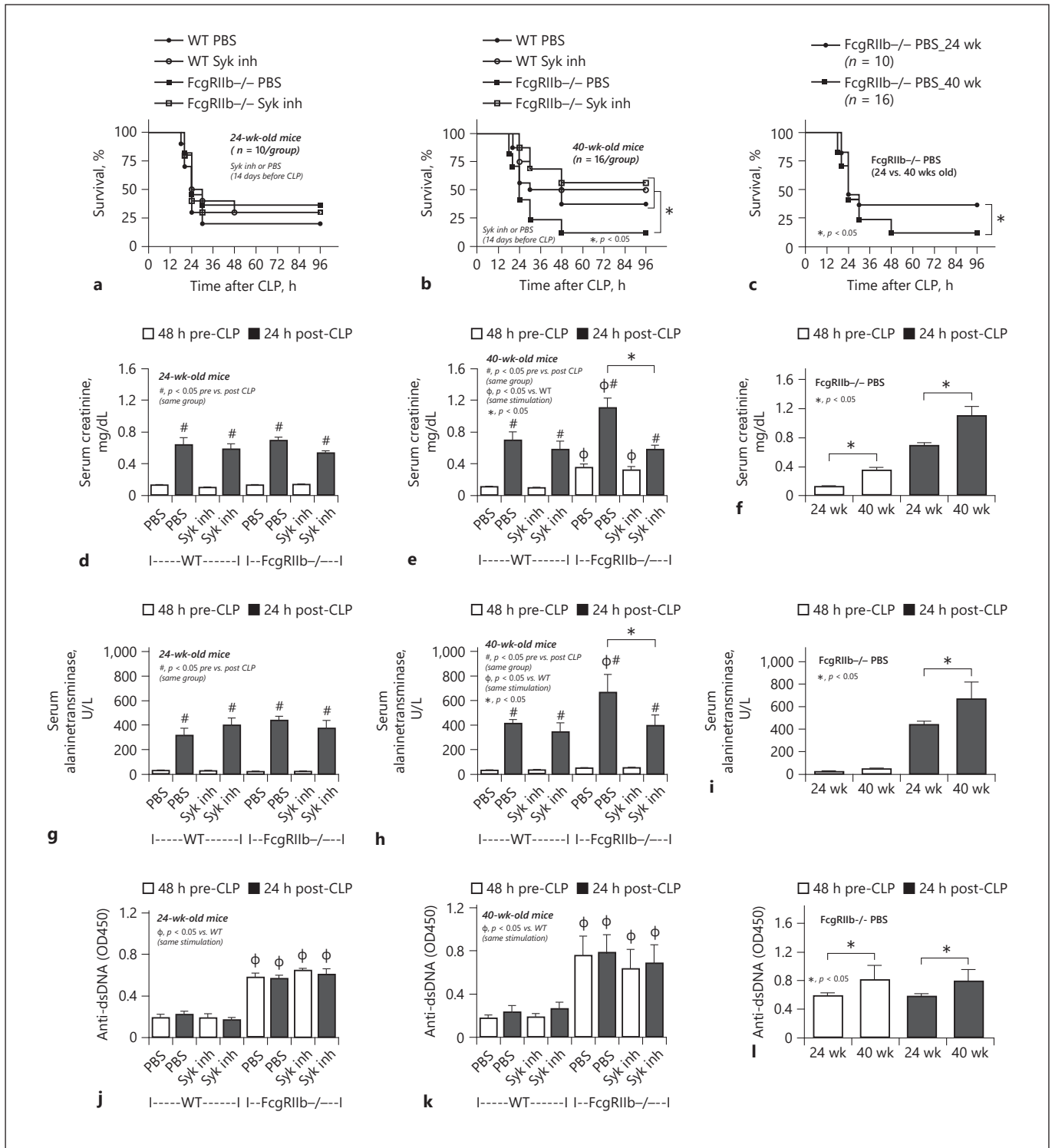


Fig. 7. Sepsis severity after 14 days of Syk inhibitor treatment in sepsis mice. Characteristics of *FcγRIIb*^{-/-} or wild-type (WT) mice aged 24 and 40 weeks before and after cecal ligation and puncture (CLP) surgery and 14 days after Syk inhibitor administration (Syk inh) or PBS control by survival analysis (**a–c**) (*n* = 10 and 16/group for **a** and **b**, respectively), serum creatinine (**d–f**), serum alanine transaminase (**g–i**), and serum anti-dsDNA antibodies (**j–l**) (*n* = 5–7/group for **d–l**). **c, f, i, l** show better visualization of the difference in sepsis between *FcγRIIb*^{-/-} mice aged 24 and 40 weeks.

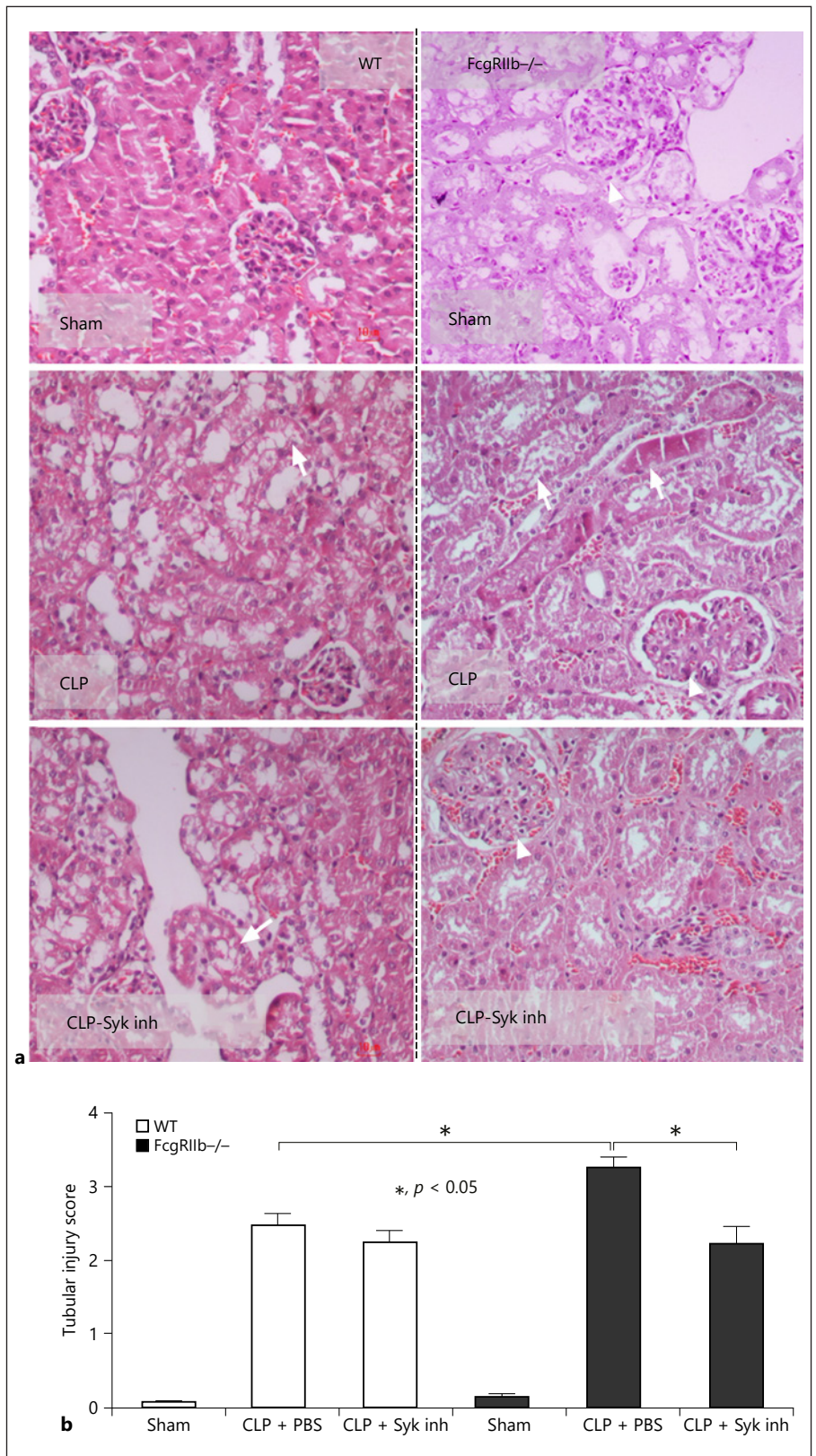


Fig. 8. Kidney histology after 14 days of Syk inhibitor treatment in sepsis mice. Representative figures of kidney histology (hematoxylin and eosin staining) of 40-week-old FcgRIIb^{-/-} and wild-type (WT) mice with sham operation or CLP with and without Syk inhibitor (Syk inh) (**a**) (original magnification $\times 200$) and tubular injury score (**b**) ($n = 4-6$ /group). Data of 24-week-old mice are not shown because there were no differences between Syk-treated and control CLP mice. Arrow head, lupus-induced mesangial glomerulonephritis; arrow, sepsis-induced tubular injury demonstrated by cast formation and tubular cell vacuolization.

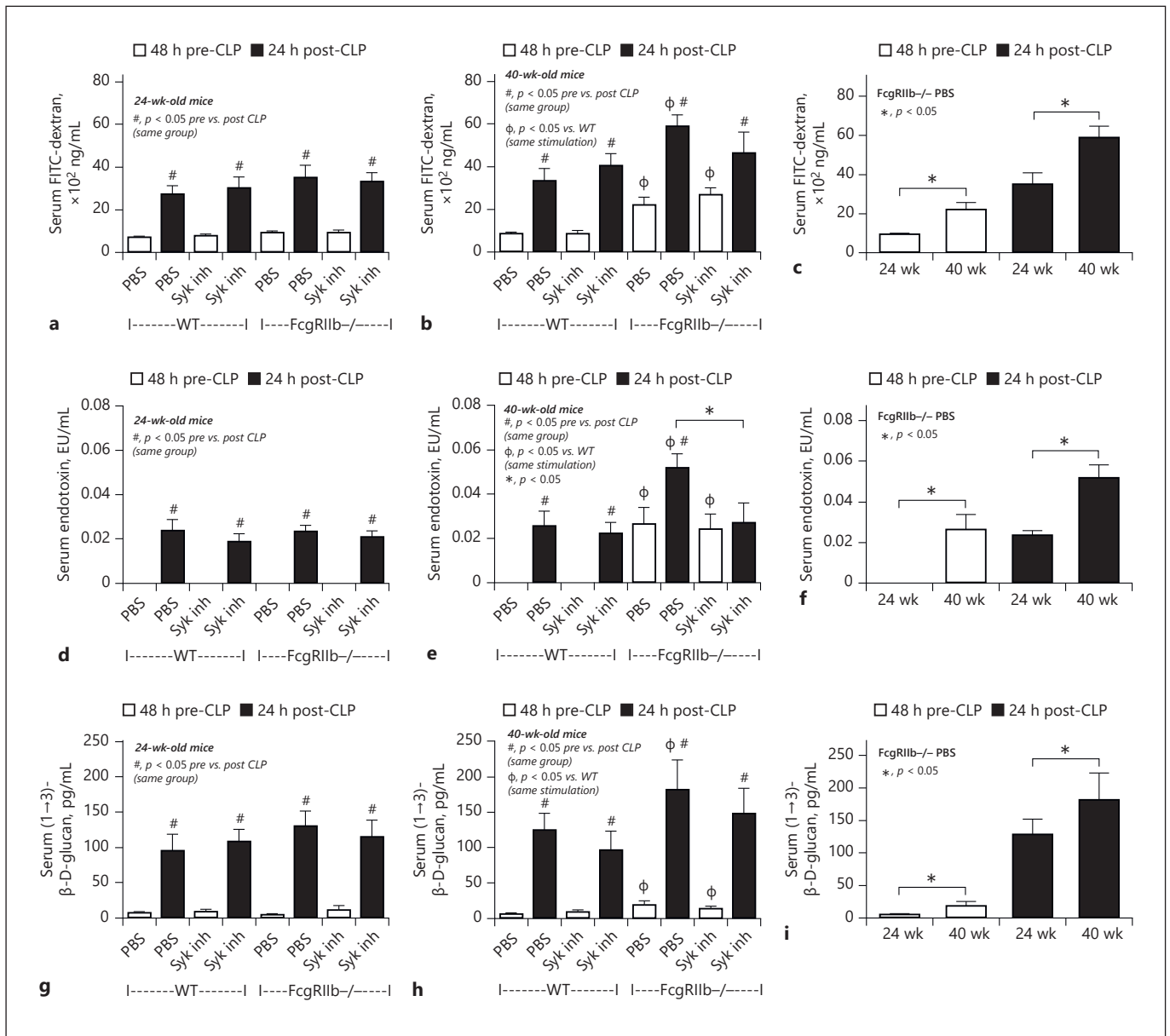


Fig. 9. Gut leakage after 14 days of Syk inhibitor treatment in sepsis mice. Characteristics of FcγRIIb^{-/-} or wild-type (WT) mice aged 24 and 40 weeks before and after cecal ligation and puncture (CLP) surgery 14 days after Syk inhibitor administration (Syk inh) or PBS control. Serum FITC-dextran (**a-c**), serum endotoxin (**d-f**), and serum (1→3)-β-D-glucan (BG) (**g-i**) levels ($n = 5-7$ /group). **c, f, i** show a better visualization of the difference in sepsis between FcγRIIb^{-/-} mice aged 24 and 40 weeks.

by exogenous endotoxin injection without bacteremia) [23], it has not been studied in CLP sepsis model (a model with both bacteremia and endotoxemia) [44]. Interestingly, the 14-day administration but not the 3-day gavage of a Syk inhibitor decreased lupus-induced systemic inflammation at baseline (pre-CLP surgery) and attenuated

sepsis severity in 40-week-old FcγRIIb^{-/-} mice. In contrast, the Syk inhibitor had a reduced anti-inflammatory effect on sepsis in mice at 24-week-old of both FcγRIIb^{-/-} and WT groups. This might be explained by the lack of gut leakage and/or systemic inflammation at baseline in 24-week-old FcγRIIb^{-/-} mice and WT mice (both 24- and

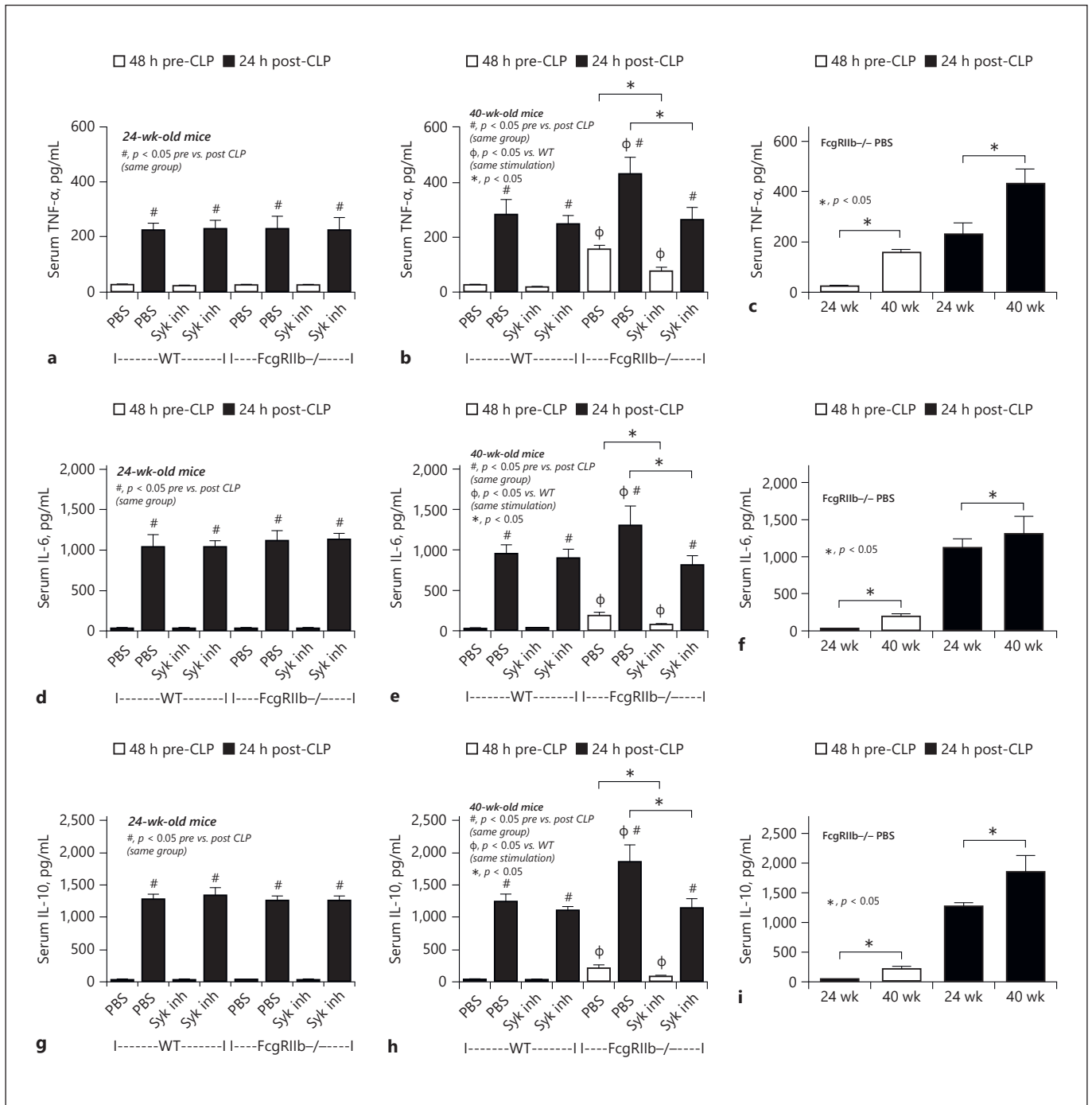


Fig. 10. Systemic inflammation after 14 days of Syk inhibitor treatment in sepsis mice. Levels of systemic cytokines in FcγRIIb^{-/-} or wild-type (WT) mice aged 24 and 40 weeks before and after cecal ligation and puncture (CLP) surgery 14 days after Syk inhibitor administration (Syk inh) or PBS control. Serum TNF-α (a-c), IL-6 (d-f), and IL-10 (g-i) levels (*n* = 5-7/group). c, f, i show a better visualization of the difference in sepsis between FcγRIIb^{-/-} mice aged 24 and 40 weeks.

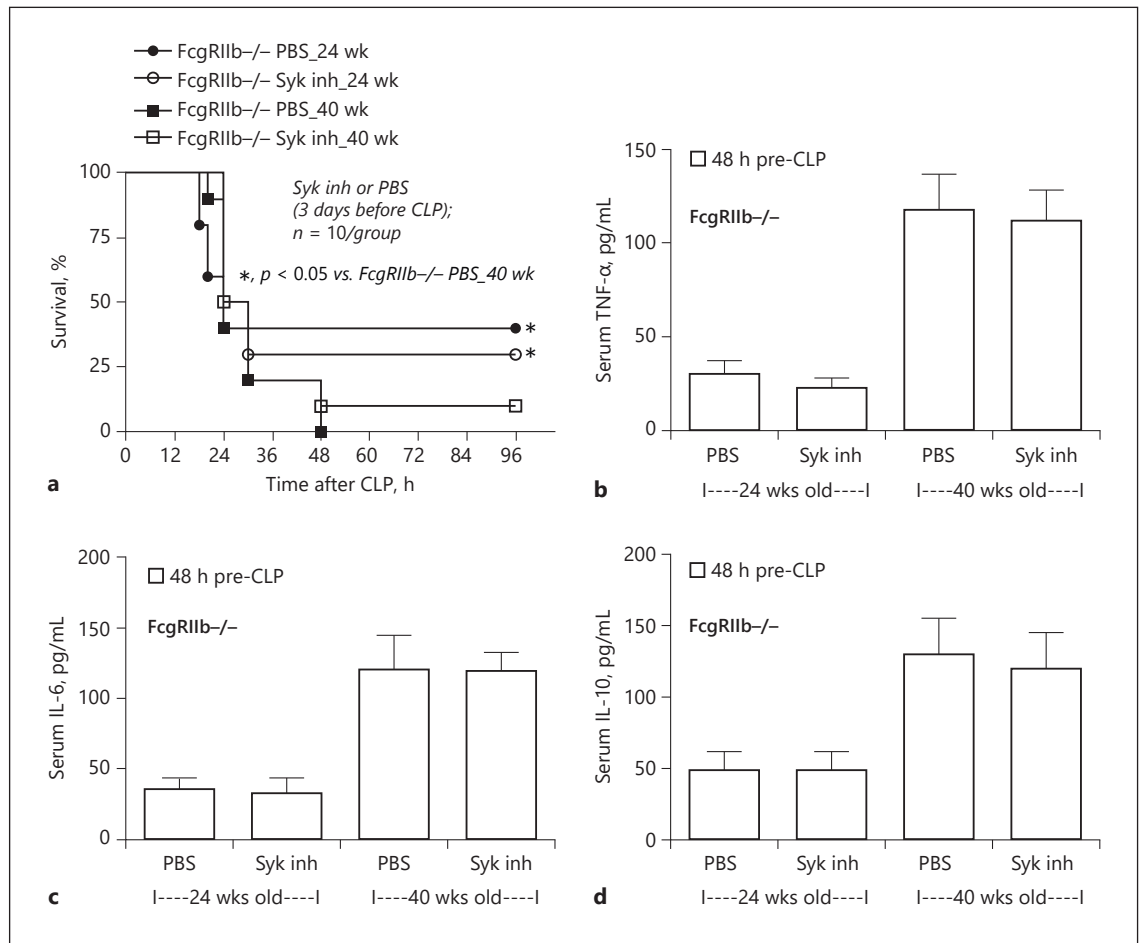


Fig. 11. Sepsis severity after 3 days of Syk inhibitor treatment in sepsis mice. **a** Survival analysis of FcgRIIb^{-/-} mice aged 24 and 40 weeks after 3 days of Syk inhibitor administration (Syk inh) or PBS control ($n = 10/\text{group}$). **b-d** Systemic inflammation determined by serum cytokines before cecal ligation and puncture (CLP) surgery ($n = 10/\text{group}$).

40-week-old age groups). Despite increased levels of anti-dsDNA antibodies in 24-week-old FcgRIIb^{-/-} mice, the 14-day administration of the Syk inhibitor did not reduce baseline anti-DNA antibody titers (pre-CLP surgery) or sepsis severity, implying a lesser impact of Syk induction through FcgR, by the activation from immune complex of anti-dsDNA antibodies, upon sepsis severity.

The anti-inflammatory effects of Syk inhibitors might also be related to the blockade of FcgR, dectin-1, and TLR-4, as well as interfering with other proinflammatory pathways. RNA sequencing of WGP + LPS activated macrophages treated with the Syk inhibitor demonstrated reduced gene expressions of proinflammatory pathways, including *Akt*, *MAPK*, *NOD*, and cytosolic DNA. Interestingly, the gene expression in LPS + BG activated

FcgRIIb^{-/-} macrophages was similar to that in a large number of patients with severe sepsis [33], suggesting the potential influence of immune activation by LPS + BG, the representatives of pathogenic molecules, in patients with sepsis. Accordingly, increased serum BG levels were observed in patients with severe bacterial sepsis, even without fungal infection, via sepsis-induced gut leakage with gut translocation of BG [14]. Some of the genes co-expressed by LPS + BG activated macrophages and patients with severe bacterial sepsis are of note. Activation by LPS + BG upregulated DNA damage inducible transcript 4 (*DDIT4*) and occludin (*OCLN*), genes responsible for hypoxic responses and epithelial morphological stability, respectively [45, 46], and downregulated CX3C chemokine receptor 1 (*CXC3CR1*), a gene responsible for

leukocyte migration and monocyte survival [47]. Nevertheless, the Syk inhibitor downregulated *DDIT4* and *OCN* and upregulated *CXC3CR1* in LPS + BG activated macrophages, whereas sepsis was attenuated in 40-week-old FcγRIIb^{-/-} mice, indicating the benefit of a Syk inhibitor for sepsis treatment.

Regarding potential clinical translation, Syk inhibitors are available for the treatment of several autoimmune diseases including lupus and rheumatoid arthritis [48–51]. Although the therapeutic effect of Syk inhibitors against polymicrobial CLP sepsis was low with the short-term administration, our data suggest that susceptibility against bacterial infection might be lower with a longer duration of treatment with a Syk inhibitor, possibly during the treatment of lupus disease activity. Of note, all current lupus treatments, such as steroids and mycophenolate mofetil, are based upon immunosuppression, which can cause several infectious complications [52]. Considering the effect of Syk inhibitors on lupus progression [48–50] together with our data on the reduction of sepsis severity, Syk inhibitors might be an interesting candidate for the treatment of active lupus in patients with FcγRIIb dysfunction polymorphisms.

In conclusion, marked Syk activation was observed in 40-week-old FcγRIIb^{-/-} mice after sepsis induction by proinflammatory activation that related to endotoxemia and glucanemia. A Syk inhibitor attenuated sepsis severity possibly through the reduction of lupus-induced systemic inflammation in full-blown lupus in 40-week-old FcγRIIb^{-/-} mice. Regarding clinical translation, Syk inhibitors might be an interesting drug for lupus treatment with a counter effect on sepsis. Further studies are warranted.

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Statement of Ethics

This study gained approval from the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, and followed the animal care and use protocol of the National Institutes of Health (NIH), USA.

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

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Author Contributions

Conceptualization: A.L.; methodology: J.I.-A, A.L., W.C., and P.V.; investigation: A.L. and W.C.; writing, original draft preparation: A.L. and J.I.-A.; writing, review and editing: A.L., J.I.-A., and W.C.; supervision: A.L.; and funding acquisition: A.L.

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