

A Synergy Between Endotoxin and (1→3)-Beta-D-Glucan Enhanced Neutrophil Extracellular Traps in *Candida* Administered Dextran Sulfate Solution Induced Colitis in FcGRIIB^{-/-} Lupus Mice, an Impact of Intestinal Fungi in Lupus

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Introduction: The translocation of organismal molecules from gut into blood circulation might worsen the disease severity of lupus through the induction of neutrophil extracellular traps (NETs).

Methods: An impact of lipopolysaccharide (LPS) and (1→3)-β-D-glucan (BG), components of gut bacteria and fungi, respectively, on NETs formation, was explored in lupus models, Fc gamma receptor IIB deficiency (FcGRIIB^{-/-}) and Pristane injection, using *Candida*-administered dextran sulfate solution induced colitis (*Candida*-DSS) model.

Results: Severity of *Candida*-DSS in FcGRIIB^{-/-} mice was more prominent than wild-type (WT) and Pristane mice as indicated by (i) colonic NETs using immunofluorescence of Ly6G, myeloperoxidase (MPO) and neutrophil elastase (NE) together with expression of *PAD4* and *IL-1β*, (ii) colonic immunoglobulin (Ig) deposition (immunofluorescence), (iii) gut-leakage by FITC-dextran assay, endotoxemia and serum BG, (iv) systemic inflammation (neutrophilia, serum cytokines, serum dsDNA and anti-dsDNA) and (v) renal injury (proteinuria, glomerular NETs and Ig deposition).

Discussion: The formation of NETs in *Candida*-DSS mice was more severe than non-*Candida*-DSS mice and NETs in *Candida*-DSS were more profound in FcGRIIB^{-/-} mice than Pristane mice. Prominent NETs in *Candida*-DSS FcGRIIB^{-/-} mice might be due to the profound responses against LPS+BG in FcGRIIB^{-/-} neutrophils compared with WT cells. These data implied an impact of the inhibitory FcGRIIB in NETs formation and an influence of gut fungi in lupus exacerbation. Hence, gut fungi in a DSS-induced gut-leakage lupus model enhanced colonic NETs that facilitated gut translocation of organismal molecules and synergistically exacerbated lupus activity.

Keywords: FcGRIIB deficient mice, Pristane mice, systemic lupus erythematosus, neutrophil extracellular traps, *Candida*, gut-leakage

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease caused by genetic predispositions and environmental factors. There is a high prevalence of

dysfunction polymorphism of Fc gamma receptor IIb (*FcGRIIB*) gene, the only inhibitory receptor in the Fc gamma receptor (FcγR) family, in Asian populations.¹ As such, FcGRIIB^{-/-} mice represent a genetic prone lupus model through the loss of inhibitory FcGRIIB signaling,^{2,3} while injection of Pristane, a hydrocarbon oil, in wild-type (WT) mice represents a lupus model from environmental activation via the sustained IFN-γ mediated-inflammation.^{4,5} The gut translocation of endotoxin (a Gram-negative bacterial component) in active lupus, referred to as “gut-leakage”,^{6,7} and the common enterocolitis from infections or adverse drug reactions in lupus⁸ are mentioned. Despite the non-prominent gastrointestinal (GI) symptoms in patients with lupus, peritonitis is found in 60–70% of postmortem autopsy in patients with SLE.⁷ Due to a significant burden of acute diarrhea in developing countries^{9–11} and a high prevalence of both acute diarrhea and lupus among Asians,¹ the co-existence of diarrhea-induced gut leakage and lupus is possible.

Previous reports note that (i) gut-leakage from chronic intestinal inflammation exacerbates lupus disease activity,^{12–14} (ii) gut translocation of pathogen molecules such as lipopolysaccharide (LPS) and (1→3)-β-D-glucan (BG), the main component of Gram-negative bacteria and fungi, respectively, synergistically induced systemic inflammation^{4,15} and (iii) the exposure of auto-antigens from neutrophil extracellular traps (NETs; a web-like structure released by activated neutrophils) enhances auto-antibody production in lupus.¹⁶ Interestingly, NETs are inducible by different pathogen molecules, including LPS and BG, and the NETs extracellular structure is composed of nuclear components (e.g., chromatin, DNA and histone) and cytosolic anti-microbial molecules from the neutrophils.^{17,18} Therefore, gut-leakage possibly accelerates immune recognition of the auto-antigens from NETs that is induced by organismal molecules and gut-leakage might become an exacerbating factor of lupus.

In the gut, Gram-negative bacteria are an important source of LPS, an endogenous toxin, in both human and mice. Meanwhile, in mice, there is less abundance of fungi, particularly *C. albicans*, which is a source of gut BG.^{19,20} Hence, *Candida* administration in mice might, at least in part, make the mouse model resemble more that of human conditions.^{21,22} Indeed, gut translocation of BG enhances systemic inflammation in active lupus^{6,7} and the synergistic activation of LPS with BG through TLR-4 and Dectin-1, respectively, accelerates inflammation.^{23,24} Although a crosstalk between TLR-4 and Dectin-1

receptors in lupus macrophages has been previously explored,^{4,15,25} the effect on neutrophils in lupus remains unclear. Due to the detectable gut-leakage in active lupus,^{6,7} gut-leakage might exacerbate lupus activity partly through the neutrophils cell death, including NETosis (the cell death after NETs formation), that is induced by gut translocation of LPS and BG.

Although infective diarrhea is the most common cause of gut-leakage,²⁶ the administration of a pathogenic organism into the model induces not only gut-leakage but also the immune responses against infection, making it difficult to separate the impact from both factors. Hence, to study only the effect of gut-leakage in acute diarrhea, non-infectious acute diarrheal induction using dextran sulfate solution (DSS), a well-known agent for gut-leakage induction in mice,^{12,27} was performed. Here, DSS administration, with or without *C. albicans*, was conducted in two lupus mouse models from genetic predisposition (FcGRIIB^{-/-}) and chemical induction (Pristane).

Materials and Methods

Animals and Animal Model

The Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, gave approval following the protocol of the National Institutes of Health (NIH), USA. FcGRIIB deficient mice on a C57BL/6 background (FcGRIIB^{-/-}) were provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA) and wild-type (WT) mice were purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand). Only female mice were used in the experiments.^{12,26} Because FcGRIIB^{-/-} mice and Pristane (administered in 4-wk-old-mice) model develop anti-dsDNA (a major auto-antibody of lupus) as early as 24 wks old,^{4–6,15,28} FcGRIIB^{-/-} mice and Pristane mice at 16 wks old were used as a representative model of asymptomatic lupus.

To induce gut-leakage, dextran sulfate solution (DSS) (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dilution into drinking water at the concentration of 3% (v/v) for 1 wk following a previous publication.²⁹ In parallel, *C. albicans* (American Type Culture Collection, ATCC90028, Fisher Scientific, Waltham, MA, USA) at 1×10⁶ colony forming unit (CFU), using a hemocytometer, diluted in 0.3 mL of phosphate buffer solution (PBS) were orally administered with DSS and at days 1, 3 and 5 of the experiment as previously published.²⁹ Mice were sacrificed at day 7 post-DSS by cardiac puncture under isoflurane anesthesia with

blood and organ collection. Urine was collected for 24 h before sacrifice by placing mice into a metabolic cage (Hatteras Instruments, NC, USA) for the determination of urine protein by Bradford protein assay (Sigma-Aldrich). The stool consistency was semi-quantitatively evaluated using the following score; 0, normal; 1, soft; 2, loose and 3, diarrhea, as previously published.³⁰

Serum creatinine and cytokines (IL-6, TNF- α and IL-10) were measured by QuantiChrom Creatinine Assay (BioAssay Systems, Hayward, CA, USA) and ELISA (Invitrogen, Vienna, Austria), respectively. Serum anti-dsDNA was analyzed by a protocol using coated Calf-DNA (Invitrogen, Carlsbad, CA, USA).³¹ The percentage of peripheral blood neutrophils was determined by Wright staining following a previous publication.^{32,33} Serum dsDNA, a biomarker of NETs in peripheral blood,¹⁷ was determined using Quanti PicoGreen assay. Additionally, expression of several genes in colon was determined using real-time polymerase chain reaction (PCR)³⁴ and a high-capacity reverse

transcription assay (Applied Biosystems, Warrington, UK), respectively. Real-time PCR was performed using an Applied Biosystems QuantStudio 6 Flex Real-Time PCR system with SYBR[®] Green PCR Master Mix (Applied Biosystems) and the results were demonstrated in terms of relative quantitation using the comparative threshold ($\Delta\Delta Ct$) method ($2^{-\Delta\Delta Ct}$) as normalized by β -actin (an endogenous housekeeping gene). Primer sequences are presented in Table 1. Mouse organs (colon and kidney) were kept in 10% formalin or in Tissue-Tek optimum cutting temperature (O.C.T.) compound (Sakura Finetek, CA, USA) or snap frozen and kept in -80°C before further use.

Gut Permeability Determination

Fluorescein isothiocyanate-dextran (FITC-dextran, a gut non-absorbable molecule at molecular weight 4.4 kDa; Sigma-Aldrich), was orally administered at the concentration of 25 mg/mL in 0.25 mL PBS at 3 h before sacrifice to determine gut permeability.³⁵ Serum FITC-dextran was

Table 1 List of Primers

Target Genes	Primer Sequences
<i>PAD-4</i> forward <i>PAD4</i> reverse	5'-ACAGGTGAAAGCAGCCAGC-3' 5'-AGTGATGTAGATCAGGGCTTGG-3'
<i>IL-6</i> forward <i>IL-6</i> reverse	5'-TACCACTTCACAAGTCGGAGGC-3' 5'-CTGCAAGTGCATCATCGTTGTTC-3'
<i>IL-1β</i> forward <i>IL-1β</i> reverse	5'-GAAATGCCACCTTTTGACAGTG-3' 5'-TGGATGCTCTCATCAGGACAG-3'
<i>TNF-α</i> forward <i>TNF-α</i> reverse	5'-CCTCACACTCAGATCATCTTCTC-3' 5'-AGATCCATGCCGTTGGCCAG-3'
<i>Syk</i> forward <i>Syk</i> reverse	5'-CTACTACAAGGCCAGACCC-3' 5'-TGATGCATTCGGGGCGTAC-3'
<i>NFκB</i> forward <i>NFκB</i> reverse	5'-CTTCCTCAGCCATGGTACCTCT-3' 5'-CAAGTCTTCATCAGCATCAAAGT-3'
<i>FcGR1</i> forward <i>FcGR1</i> reverse	5'-CACAAATGCCCTTAGACCAC-3' 5'-ACCCTAGAGTTCCAGGGATG-3'
<i>FcGR1B</i> forward <i>FcGR1B</i> reverse	5'-CAGAATGCACACTCTGGAAGC-3' 5'-GGGTCCCTTCGCACATCAG-3'
<i>FcGR1I</i> forward <i>FcGR1I</i> reverse	5'-AGGGCCTCCATCTGGACTG-3' 5'-GTGGTTCTGGTAATCATGCTCTG-3'
<i>FcGR1V</i> forward <i>FcGR1V</i> reverse	5'-AACGGCAAAGCAAGAAGTA-3' 5'-CCGCACAGAGAAATACAGCA-3'
<i>β-actin</i> forward <i>β-actin</i> reverse	5'-CGGTTCCGATGCCCTGAGGCTCTT-3' 5'-CGTCACACTTCATGATGGAATTGA-3'

measured by fluorospectrometry (microplate reader; Thermo Scientific, Wilmington, DE, USA). Other gut-leakage parameters, serum endotoxin (lipopolysaccharide; LPS) and (1→3)- β -D-glucan (BG) were measured using HEK-Blue LPS detection (InvivoGen, San Diego, CA, USA) and Fungitell (Associates of Cape Cod, Falmouth, MA, USA). The values of LPS < 0.01 EU/mL and BG < 7.8 pg/mL were recorded as 0 due to the limitation of the standard curves.

Histology Analysis

The semi-quantitative evaluation of intestinal histology on hematoxylin and eosin (H&E) staining at 200x magnification was determined²⁶ based on mononuclear cell infiltration (in mucosa and sub-mucosa), epithelial hyperplasia (epithelial cell in longitudinal crypts), reduction of goblet cell and epithelial cell vacuolization in comparison with control groups using the following scores; 0: leukocyte < 5% and no epithelial hyperplasia (<10% of control); 1: leukocyte infiltration 5–10% or hyperplasia 10–25%; 2: leukocyte infiltration 10–25% or hyperplasia 25–50% or reduced goblet cells (> 25% of control); 3: leukocyte infiltration 25–50% or hyperplasia >50% or intestinal vacuolization; 4: leukocyte infiltration >50% or ulceration. In parallel, renal tissue with H&E staining was determined only by glomerular injury score due to the very subtle injury in other parts of kidney (tubule and interstitial area) (data not shown). All glomeruli in the slides were examined and the percentage of glomeruli with mesangial expansion (>25% or normal) at 400x magnification represented the glomerular injury score.

In parallel, NETs formation in colon and in glomeruli was visualized by immunofluorescence prepared in Tissue-Tek O.C.T. compound (Sakura Finetek),³⁴ stained with the primary antibodies against myeloperoxidase (anti-MPO) (ab 25989) and neutrophil peroxidase (anti-NE) (ab68672) with specific secondary antibodies of goat anti-mouse immunoglobulin G (IgG) coupled with Alexa Fluor 647 (ab 150115) and Alexa Fluor 488 (ab150077) with DAPI (4-,6-diamidino-2-phenylindole) (Sigma-Aldrich). Likewise, the goat anti-mouse IgG coupled with Alexa Fluor 488 (ab150113) was used for detection of immunoglobulin deposition in colon and kidney. While IgG deposition on damaged colon indicates the DSS-induced intestinal injury with wound repairing processes,³⁶ the antibody deposition in kidneys with high anti-dsDNA indicates immune complex deposition and lupus disease activation.¹² Additionally, the neutrophil infiltration in colon was evaluated by anti-Ly6G (BioLegend

127609). The fluorescence intensity was visualized and analyzed by confocal microscope (ZEISS LSM 800, Carl Zeiss, Germany).

Neutrophil Isolation and the *in vitro* Experiments

Mice were intraperitoneally injected with 1 mL of 3% Thioglycolate (Sigma-Aldrich) or PBS (control) and the peritoneal neutrophils were harvested after 2 h post-injection by lavages of the peritoneal cavity with 20 mL of cold PBS after sacrifice with cardiac puncture under isoflurane anesthesia. The cells were centrifuged and washed with PBS and resuspended in Roswell Park Memorial Institute (RPMI) media 1640 with 25 mM HEPES, L-Glutamine (Hyclone, Marlborough, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, Paisley, UK). For human neutrophils, heparinized blood was collected from healthy donors under an approved protocol by the Ethical Institutional Review Board, Faculty of Medicine, Chulalongkorn University, according to the Declaration of Helsinki, with written informed consent. Neutrophils were isolated by density centrifugation with Polymorphprep™ (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. The contaminated erythrocytes were removed by hypotonic lysis buffer. Cells were resuspended in RPMI 1640 supplemented with 10% FBS. Cell viability of human and mouse neutrophils was measured by Trypan blue dye (Sigma-Aldrich) exclusion method. Only viable cells which do not take up the dye were counted by a hemocytometer. Cell purity was assessed by Wright Giemsa staining following the manufacturer's instructions. The cells with segmental lobe nuclei and granules which indicated neutrophil morphology were counted per total 100 cells. Only the samples with cell viability and purity of neutrophils of more than 95% were further processed for the experiments.

Human or mouse neutrophils were placed onto Poly-L-Lysine (Sigma-Aldrich) coated glass coverslips, incubated at 37°C, 5% CO₂ for 1 h with BG, using whole glucan particle (WGP) that was purified from *Saccharomyces cerevisiae* (Biothera, Eagan, MN), at 10 μ g/mL with or without LPS (*Escherichia coli* 026: B6) (Sigma-Aldrich) at 10 μ g/mL. After 2 h of the incubation, the coverslips were fixed with 4% formaldehyde, blocked with Tris Buffered Saline (TBS) in 2% bovine serum albumin (BSA) (Sigma-Aldrich) and permeabilized by TBS with 0.05% Tween 20 (Sigma-Aldrich). Then, NETs formation was detected by (i) immunofluorescence (anti-MPO, anti-NE and DAPI) in human neutrophils as

previously described and the nucleus morphology, using DAPI staining, in mouse neutrophils before mounting on glass slides using ProLong anti-fade (Invitrogen)³⁷ and (ii) free dsDNA representing NETs in supernatant. After neutrophils were incubated for 2 h, 0.1 M CaCl₂ was added, followed by 0.5 unit (U) of micrococcal nuclease (Sigma-Aldrich, Singapore) and incubated at 37°C, 5% CO₂ for 10 min. The nuclease reaction was stopped by 0.5 M ethylenediaminetetraacetic acid (EDTA). Supernatant was collected and centrifuged to remove the cell pellets. Subsequently, the Quant-iT™ PicoGreen reagent (Thermo Fisher Scientific, Auckland, New Zealand) was added to measure dsDNA according to the manufacturer's instructions. In brief, aqueous working solution of the Quant-iT™ PicoGreen® reagent was added into each sample. The mixtures were incubated for 5 min at room temperature, protected from light. The amount of dsDNA in the mixture was measured at 480 nm excitation (520 nm emission) on a fluorescent microplate reader³⁸ to test the possible synergy between LPS with BG.^{22,23,39,40}

Statistical Analysis

Statistical differences among groups were examined using the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with two groups or more than two groups, respectively. Time-point data were analyzed by repeated measures ANOVA. Data were presented as the mean ± standard error (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.

Results

The severity of DSS-induced colitis was enhanced by *Candida* administration which was more prominent in FcGRIIB^{-/-} mice than Pristine mice, partly due to the accelerated NETs formation from the loss of the inhibitory FcGRIIB signaling.

The Prominent Severity of Colitis in DSS-Administered *Candida* Gavage FcGRIIB^{-/-} Mice, the Loss of Inhibitory Signaling Induced Hyper-responsiveness Against Organismal Molecules

Spontaneous lupus nephritis (anti-dsDNA, proteinuria and serum creatinine) and gut permeability defect (serum FIT-C dextran) in the time-course evaluation (Figure 1A–D)

together with the parameters of 40-wk-old mice, including gut translocation of organismal molecules (LPS and BG), systemic inflammation (serum IL-6) and glomerular NETs in FcGRIIB^{-/-} full-blown lupus mice were more prominent than Pristine mice (Figure 1E–J). However, the glomerular neutrophil elastase (NE) showed no difference between FcGRIIB^{-/-} mice and Pristine mice (Figure 1I). Notably, the glomerular NETs, but not the intestinal NETs (data not shown), were detectable only in 40-wk-old lupus mice (FcGRIIB^{-/-} and Pristine) (Figure 1H and I) but not in the 16-wk-old mice of both groups (data not shown). In 40-wk-old full-blown FcGRIIB^{-/-} lupus mice, the spontaneous NETs in glomeruli, but not in colon, indicates a limited impact of colon NETs in the lupus pathogenesis. To explore the impact of gut-leakage in asymptomatic lupus, 16-wk-old mice were used in all experiments.

In 16-wk-old mice, the severity of DSS-induced colitis was significantly increased by *Candida* administration particularly in FcGRIIB^{-/-} mice which was more severe than Pristine mice. The biomarkers of active lupus (serum anti-dsDNA, serum creatinine and urine protein) between WT and lupus mice (FcGRIIB^{-/-} and Pristine) was not different, indicating asymptomatic lupus before the DSS administration (Figure 1A–C, 16-wk-old time-point). At 7 days of DSS, *Candida* accelerated DSS-induced weight reduction in all groups and the weight loss was more severe in FcGRIIB^{-/-} mice (Figure 2A–D). Despite the comparable diarrheal severity in all DSS-administered mice (WT, FcGRIIB^{-/-} and Pristine) with or without *Candida* (Figure 2E–H), DSS-*Candida* FcGRIIB^{-/-} mice demonstrated the most severe colitis (Figure 3A). Of note, *Candida* worsened DSS-induced colitis in all groups (Figure 3A). Also, the injury in DSS-*Candida* FcGRIIB^{-/-} mice was more severe than Pristine mice (Figures 3A and 4), despite a similar lupus-prone condition between lupus models (Figure 1A and B, 16-wk-old time-point).

The Prominent Neutrophil Infiltration and NETs in Colon of DSS-Administered *Candida*-Gavage FcGRIIB^{-/-} Mice, an Impact of Inhibitory FcGRIIB on NETs Formation

Candida accelerated neutrophil infiltration (Ly6G staining) and immunoglobulin deposition, an indicator for the severity of injury, in the colon of DSS lupus mice (more severe in FcGRIIB^{-/-} mice than Pristine mice), but not in WT mice

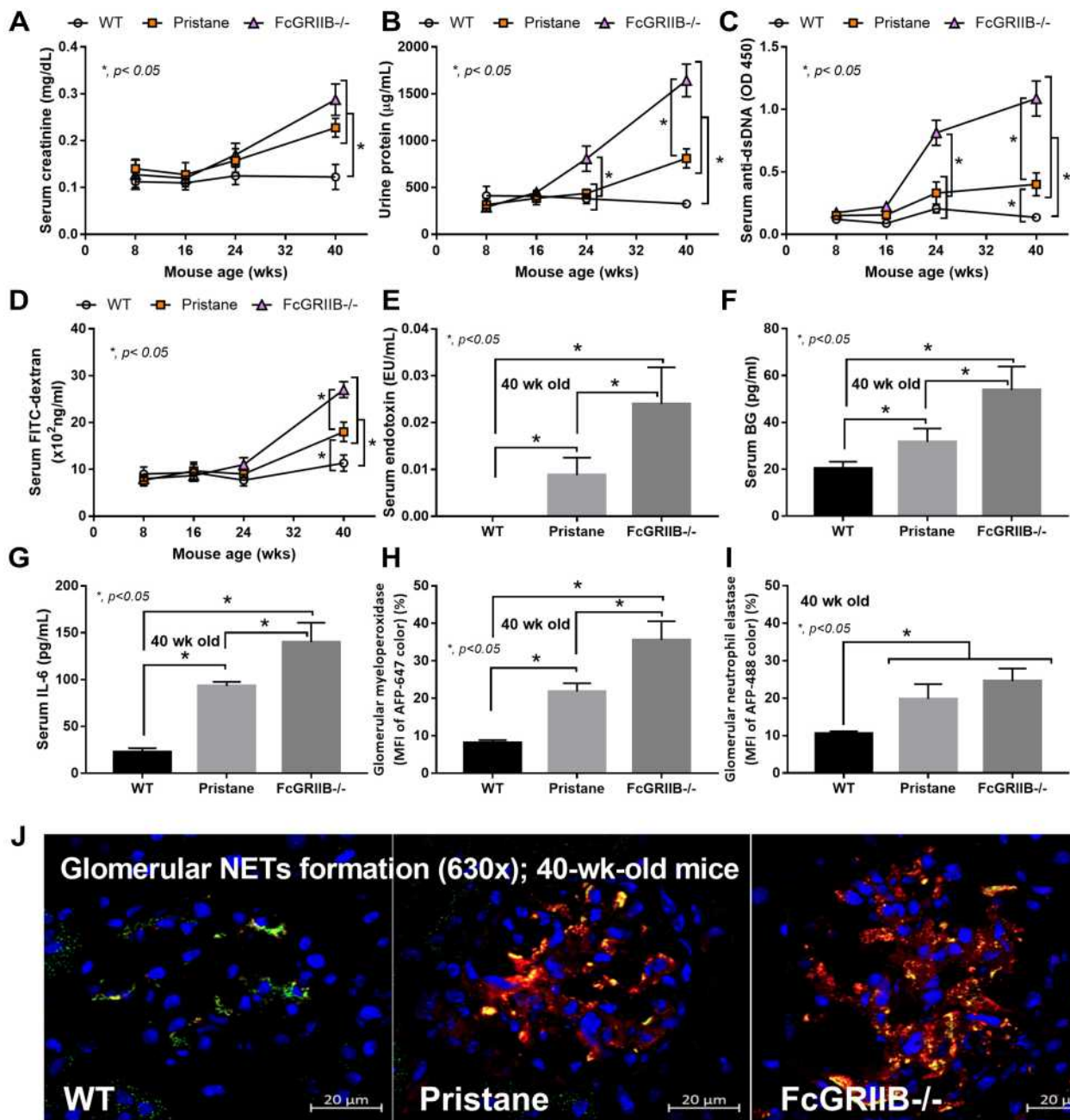


Figure 1 Time-course of mouse characteristics in wild-type (WT), Pristane and FcGRIIB^{-/-} groups as determined by lupus characteristics (serum creatinine, urine protein and anti-dsDNA) (A–C) and gut permeability defect (FITC-dextran assay) (D) are demonstrated (n = 5–7/time-point). Additionally, the parameters of mice at 40 wks old, including gut translocation of serum lipopolysaccharide (endotoxemia) and serum (1→3)-β-D-glucan (BG) (E and F), systemic inflammation (serum IL-6) (G) and neutrophil extracellular traps (NETs) in glomeruli using immunofluorescent staining of myeloperoxidase (MPO) (red color of Alexa Fluor 647) and neutrophil elastase (NE) (green color of Alexa Fluor 488) as indicated by the percentage of mean fluorescent intensity (MFI) (H and I) and the representative pictures (J) are demonstrated (n = 7–9/groups for E–I). *p < 0.05.

(Figures 3B and C–5). In colon, *Candida* more prominently enhanced NETs (MPO, NE and *PAD4* expression) (Figures 3D–F and 6) and inflammation (gene expression of *IL-1β*, *IL-6* and *TNF-α*) in both lupus models (more severe in FcGRIIB^{-/-} mice) compared with WT (Figure 3G–I) which implied the high *Candida*-induced NETs in the immune hyper-responsive

condition of lupus. Meanwhile, *Candida* alone without DSS did not induce intestinal injury in all models (data not shown), indicating the protective property of the healthy gut. Because of the dominant intestinal injury in DSS-*Candida* FcGRIIB^{-/-} groups, the inflammation might be severe enough to induce systemic inflammatory responses.

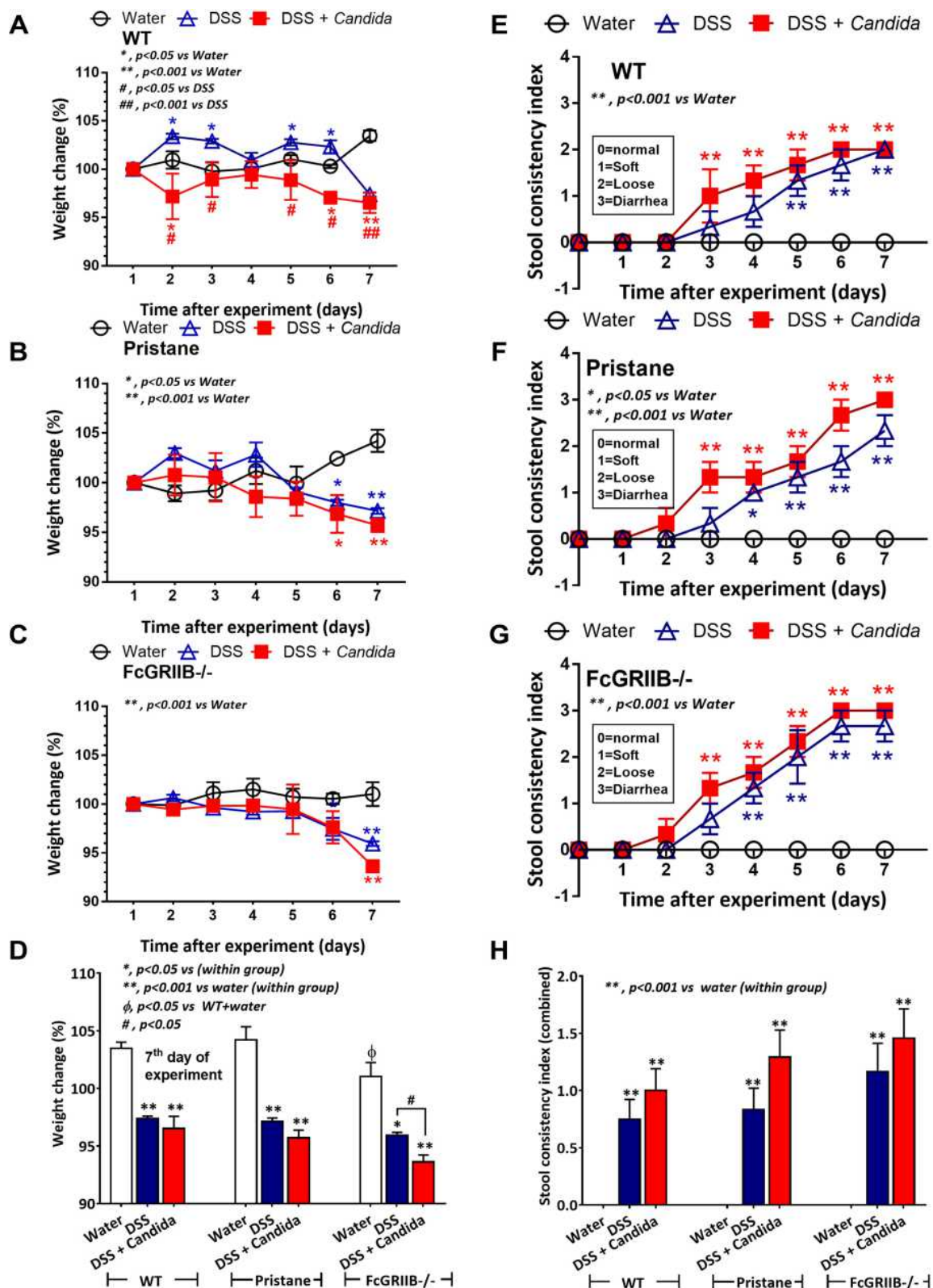


Figure 2 Characteristics of dextran sulfate solution (DSS) administered mice from wild-type (WT), Pristane and FcGRIIB^{-/-} group as determined by body weight alteration in time-points (A–C) and at 7 days post-DSS (D) together with stool consistency index in time points (E–G) and at 7 days post-DSS (H) are demonstrated (n = 6–9/time-point or group). **p* < 0.05 vs water; ***p* < 0.001 vs water; #*p* < 0.05 vs DSS; ##*p* < 0.001 vs DSS; φ *p* < 0.05 vs WT + water.

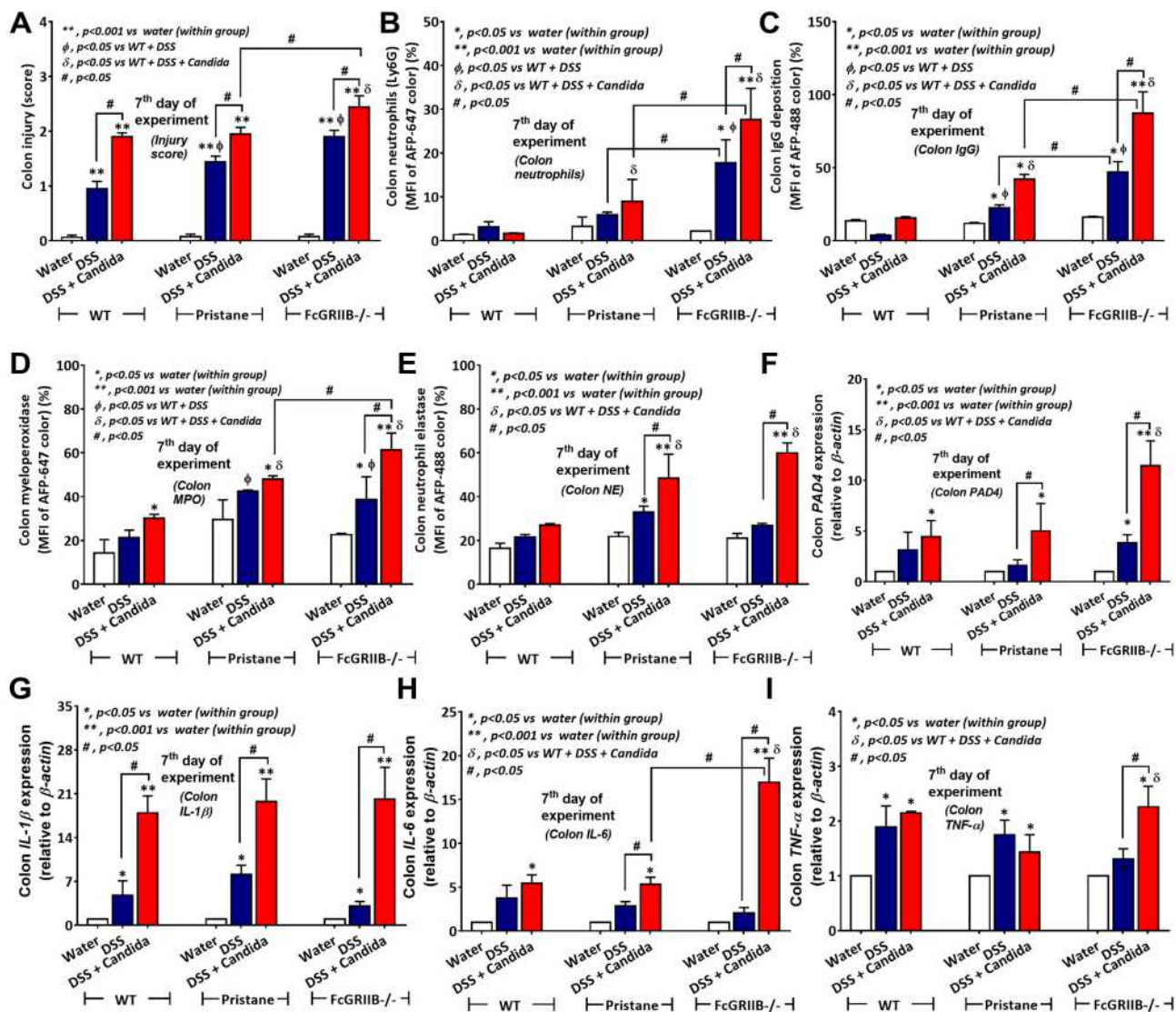


Figure 3 Characteristics of colon injury in mice from wild-type (WT), Pristane and FcGRIIB^{-/-} groups after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS+*Candida*) as determined by colon injury score (A), colon immunofluorescent stains for neutrophils (Ly6G), immunoglobulin G (IgG) deposition, myeloperoxidase (MPO) and neutrophil elastase (NE) (B–E) and gene expression by polymerase chain reaction (PCR) for *PAD4* and tissue cytokines (*IL-1 β* , *IL-6* and *TNF- α*) (F–I) are demonstrated (n = 6–9/group). *p < 0.05 vs water (within group); **p < 0.001 vs water (within group); ϕ p < 0.05 vs WT + DSS; δ p < 0.05 vs WT + DSS + *Candida*; #p < 0.05.

The Lupus Disease Exacerbation Through Gut-Leakage Induced Systemic Inflammation, an Enhanced NETs Formation by Endotoxin and (1 \rightarrow 3)- β -D-Glucan in Synergy

Accordingly, *Candida* worsened DSS-induced gut-leakage as indicated by FITC-dextran assay and damaged intestinal mucosa that was severe enough for the translocation of LPS and BG, the major organismal components, from gut into blood circulation in all groups (Figure 7A–C). However, the level of endotoxemia, a potent pro-

inflammatory activator, was highest in DSS-*Candida* FcGRIIB^{-/-} mice (Figure 7B). In parallel, the circulating organismal molecules (LPS and BG) prominently increased peripheral blood neutrophils of DSS-*Candida* mice in comparison with DSS activation alone (similar severity between WT and lupus mice) (Figure 7D). The circulatory NETs, as determined by serum dsDNA, increased only in DSS FcGRIIB^{-/-} mice (with or without *Candida*), but not in DSS WT mice (Figure 7E). The non-detectable NETs in DSS WT mice with the presentation of NETs inducers (LPS and BG)^{41,42} in serum supported an impact of Fc gamma receptors (FcGRs) on

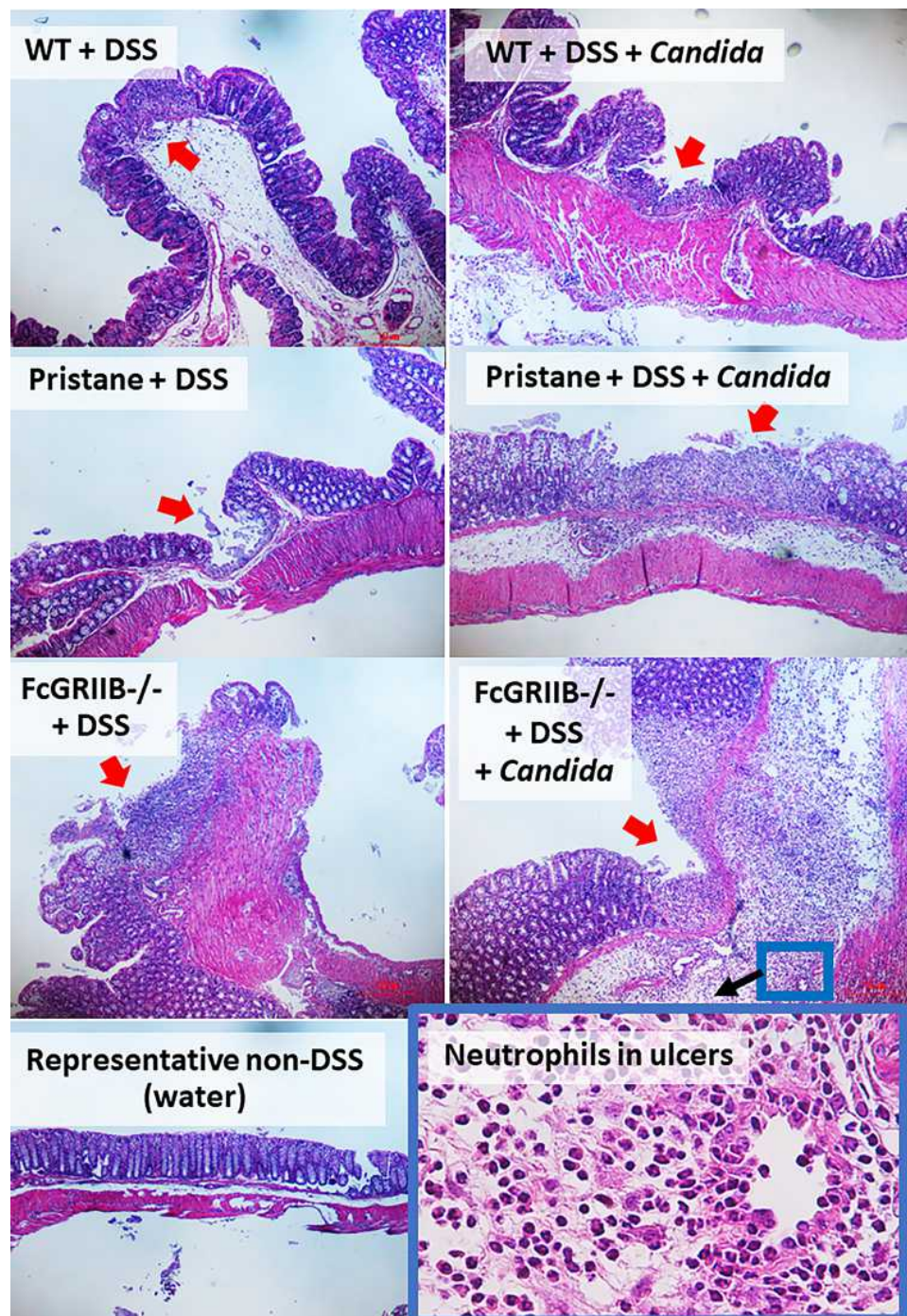


Figure 4 Representative pictures of hematoxylin and eosin (H&E) stained sections from colons of mice from wild-type (WT), Pristane and FcGRIIB^{-/-} groups after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS+*Candida*) and FcGRIIB^{-/-} with water control (water) (original magnification 200x) are demonstrated. A high magnification (400x) picture at the ulcer-base in FcGRIIB^{-/-} mice with DSS + *Candida* (the inset picture with blue outline) indicates neutrophil infiltration in the colon ulcer. Colon pictures of mice with control water from WT and Pristane group are not presented due to the similarity to FcGRIIB^{-/-} with water control. Red arrows indicate ulcer lesions.

NETs formation.⁴³ Moreover, the profound NETs in DSS-*Candida* FcGRIIB^{-/-} mice over Pristane mice resulted in the more prominent inflammatory cytokines and serum anti-dsDNA (Figure 7F–I), the biomarkers of lupus disease activity. Notably, there was neutrophilia in DSS-

Candida WT mice, but neither NETs nor serum cytokines (Figure 7E–H), highlighting the impact of inhibitory FcGRIIB on NETs attenuation in WT mice. Therefore, the increased susceptibility to inflammation of FcGRIIB^{-/-} lupus mice toward gut *Candida* provided

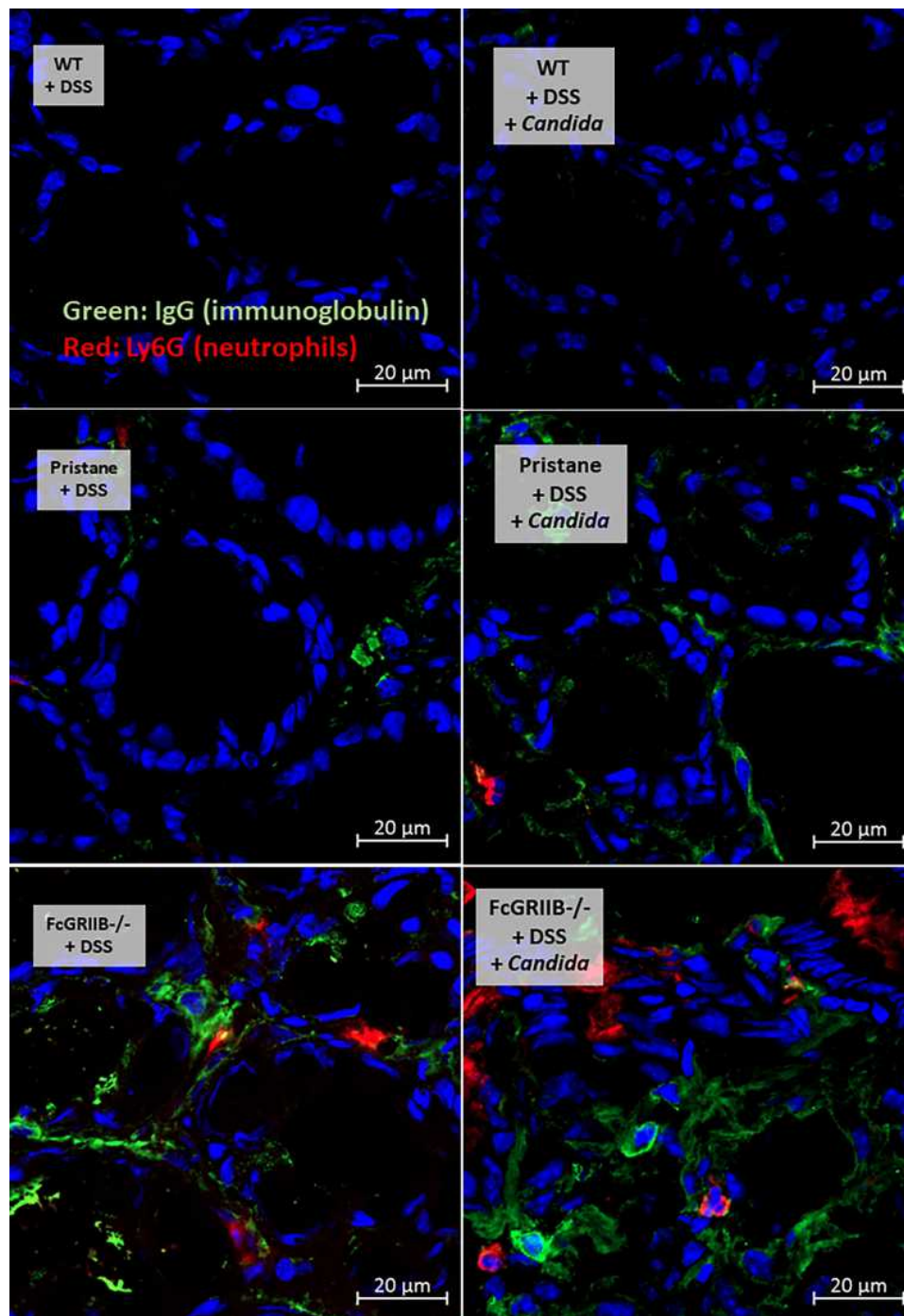


Figure 5 Representative pictures of immunofluorescent stained sections for colon injury as determined by immunoglobulin G (IgG deposition) (green color of Alexa Fluor 488) and neutrophil accumulation (Ly6G) (red color of Alexa Fluor 647) of mice from wild-type (WT), Pristane and FcGRIIB^{-/-} group after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS + *Candida*) (original magnification 630x) are demonstrated. Colon pictures from mice with control water in WT, Pristane and FcGRIIB^{-/-} group are not presented due to the similarity to the represented pictures of WT+DSS and WT+DSS+Candida.

several effects, including (i) the worsened intestinal injury (intestinal neutrophils and NETs) (Figure 3B–F), (ii) the enhanced gut translocation of organismal

molecules (Figure 7B and C), (iii) the induction of systemic inflammation (Figure 7F and G) and (iv) the aggravation of lupus disease activity (anti-dsDNA) (Figure 7I).

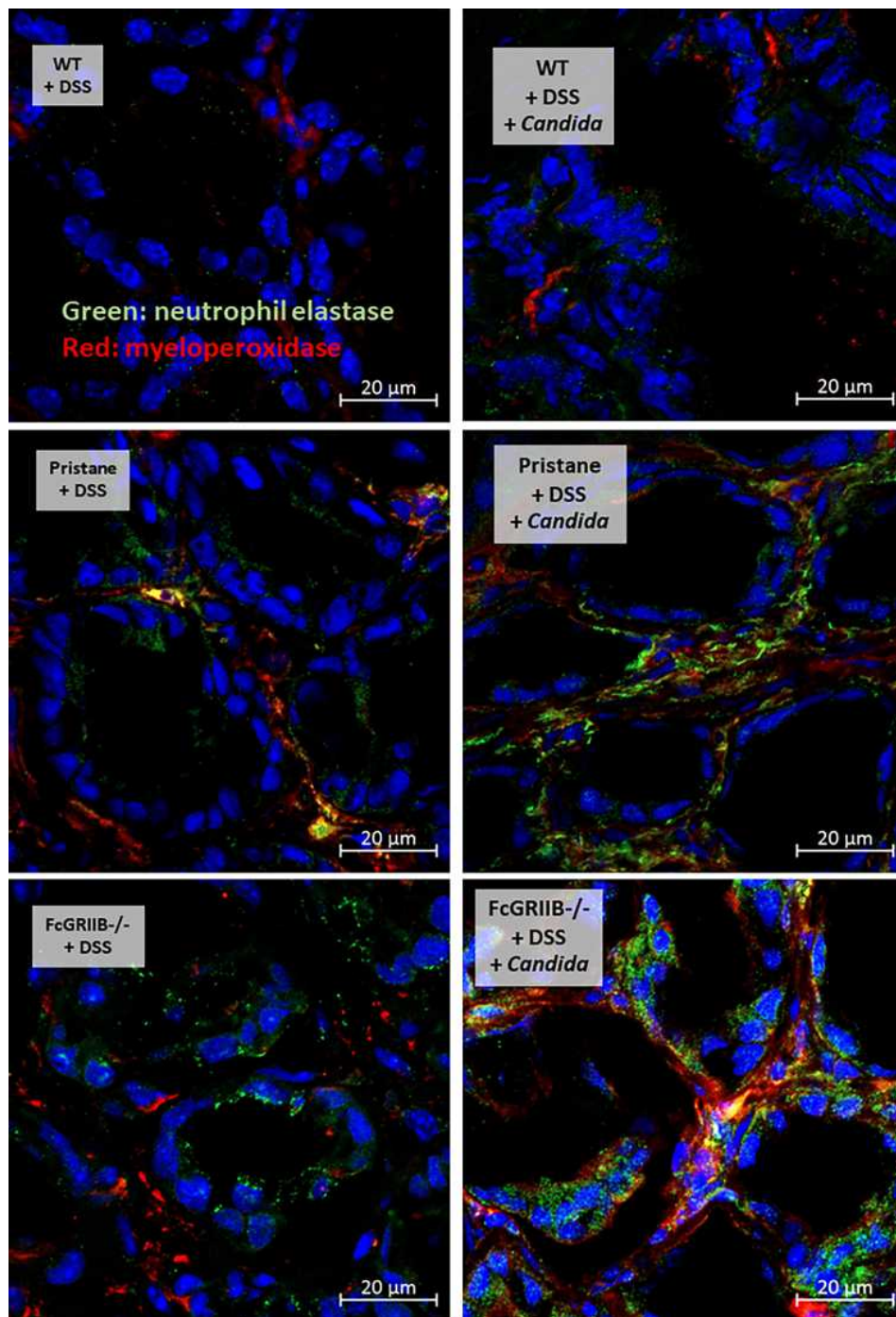


Figure 6 Representative picture of immunofluorescent stained sections for neutrophil extracellular traps (NETs) in colons as determined by neutrophil elastase (NE) (green color of Alexa Fluor 488) and myeloperoxidase (MPO) (red color of Alexa Fluor 647) of mice from wild-type (WT), Pristane and FcGRIIB^{-/-} groups after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS + *Candida*) (original magnification 630x) are demonstrated. Colon pictures from mice with control water in WT, Pristane and FcGRIIB^{-/-} group are not presented due to the similarity to the represented pictures of WT+DSS.

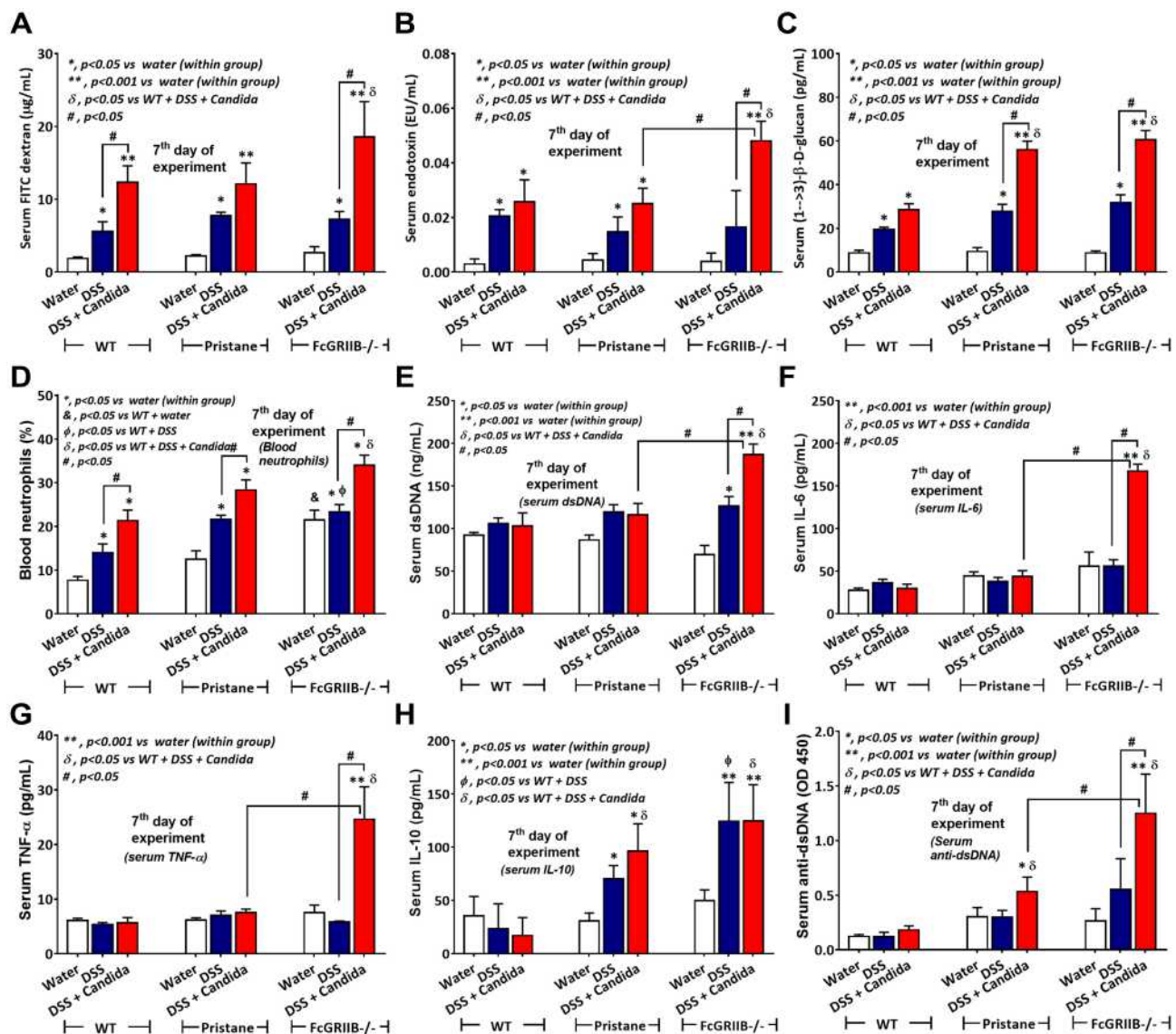


Figure 7 Characteristics of systemic impact on colon injury in mice from wild-type (WT), Pristane and FcGRIIB $^{-/-}$ groups after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS+Candida) as determined by gut permeability defect (gut-leakage) including FITC-dextran assay, endotoxemia and serum (1 \rightarrow 3)- β -D-glucan (A–C) and systemic inflammation as indicated by peripheral blood neutrophils (D), serum dsDNA (a marker of neutrophil extracellular traps) (E), serum cytokines (IL-6, TNF- α and IL-10) (F–H) and anti-dsDNA (a major lupus auto-antibody) (I) are demonstrated (n = 6–9/group). *p < 0.05 vs water (within group); **p < 0.001 vs water (within group); δ , p < 0.05 vs WT + DSS + Candida; #p < 0.05.

The Induction of Lupus Nephritis in DSS-Administered *Candida*-Gavage FcGRIIB $^{-/-}$ Mice Due to Prominent Immune Complex Deposition and NETs in Glomeruli

There were no characteristics of lupus nephritis (proteinuria, kidney injury and anti-dsDNA) in control 16-wk-old FcGRIIB $^{-/-}$ mice (Figures 8A and B and 7I). However, the induction by DSS-*Candida* (but not DSS alone) induced lupus nephritis only in FcGRIIB $^{-/-}$ mice, but neither in WT mice nor Pristane mice, as indicated by proteinuria,

glomerular mesangial expansion, immune complex deposition and renal NETs formation (MPO and NE) (Figure 8A–H). These data supported an influence of immune complex deposition and NETs formation in lupus.⁴⁴ In contrast, DSS-*Candida* Pristane mice demonstrated only a slight increase in anti-dsDNA (Figure 7I) without lupus nephritis (Figure 8B) compared with DSS-*Candida* FcGRIIB $^{-/-}$ mice, possibly due to the intact FcGRIIB inhibitory signals in Pristane mice. Since LPS and BG induced NETs,^{41,42} the effect of combined LPS and BG (LPS+BG) on NETs formation was explored both in human and in mouse neutrophils. As such, LPS+BG

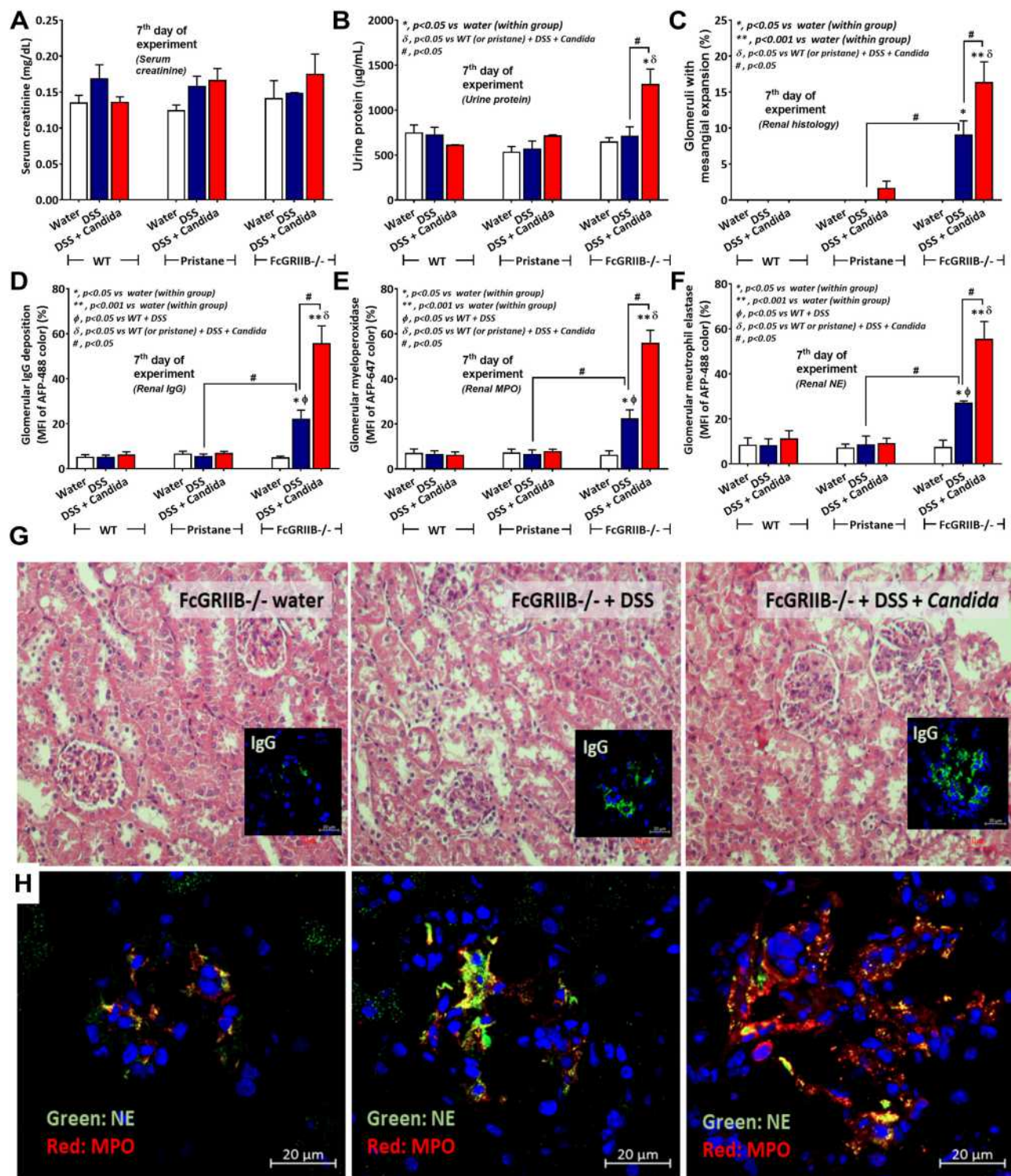


Figure 8 Characteristics of renal injury in FcGRIIB^{-/-} mice after the administration of dextran sulfate solution (DSS) alone or with *Candida* gavage (DSS+*Candida*) as determined by serum creatinine (A), urine protein (B), glomerular injury (mesangial expansion) from the histology (C), immunoglobulin G (IgG) deposition in glomeruli (D) and glomerular neutrophil extracellular traps (NETs) as indicated by immunofluorescence of myeloperoxidase (MPO) (red color of Alexa Fluor 647) and neutrophil elastase (NE) (green color of Alexa Fluor 488) (E and F) are demonstrated (n = 6–9/group). Additionally, representative pictures of hematoxylin and eosin (H&E) stained section from kidney with immunofluorescence of glomerular IgG deposition (inset pictures) (G) and glomerular NETs formation (MPO and NE) (H) are demonstrated (original magnification 400x for H&E stain and 600x for immunofluorescence). Pictures from WT and Pristane mice are not presented due to the non-difference from FcGRIIB^{-/-} mice with water control. *p < 0.05 vs water (within group); **p < 0.001 vs water (within group); ϕ p < 0.05 vs WT+DSS; δ p < 0.05 vs WT (or pristane) + DSS + *Candida*; #p < 0.05 vs WT (or pristane) + DSS + *Candida*; #p < 0.05.

more prominently induced NETs, when compared with LPS alone, as indicated by dsDNA in supernatant and the percentage of cells with NETs (human neutrophils) (Figures 9C and D and 10). In mouse neutrophils, NETs in LPS+BG activated FcGRIIB^{-/-} neutrophils was more prominent than the WT cells (Figures 9A and B and 10) supporting an influence of LPS+BG (from gut translocation) and an impact of the loss of inhibitory FcGRIIB in neutrophils on lupus. In LPS+BG activation (but not the activation by each molecule alone), the gene expression of *Syk*, *NFκB* and activating-*FcGRIV*, but not inhibitory-*FcGRIIB*, were prominently upregulated in FcGRIIB^{-/-} neutrophils compared with WT cells (Figure 11A–G). These data imply a role of FcGRs, Syk and NFκB in neutrophilic NETs formation.

Discussion

A more easily induced NETs in DSS-administered *Candida* gavage FcGRIIB^{-/-} mice than Pristine mice

implied an impact of gut fungi and inhibitory-FcGRIIB signaling on NETs formation.

Prominent Enteropathy in DSS-Administered *Candida*-Gavage FcGRIIB^{-/-} Mice Over Pristine Mice, an Impact of Gut Fungi and FcGRs in NETs Formation

Since (i) gastrointestinal symptoms in patients with SLE⁴⁵ are due to silent intestinal vasculitis⁴⁶ and immunosuppression-induced colitis,^{47,48} (ii) there is possible co-existence of lupus and acute diarrhea in the developing countries,^{10,11} (iii) there is a lower abundance of fungi in mouse guts than the human intestines²⁶ and (iv) the double-edged sword of NETs in the pathogen control versus the enhanced autoantigen exposure is reported,⁴⁹ the impact of gut mucosal injury and fungi on lupus using a model of DSS with *Candida* gavage (DSS-*Candida*) was performed. The lupus models were performed in 16-wk-old mice in FcGRIIB^{-/-} mice and Pristine mice (12 wks

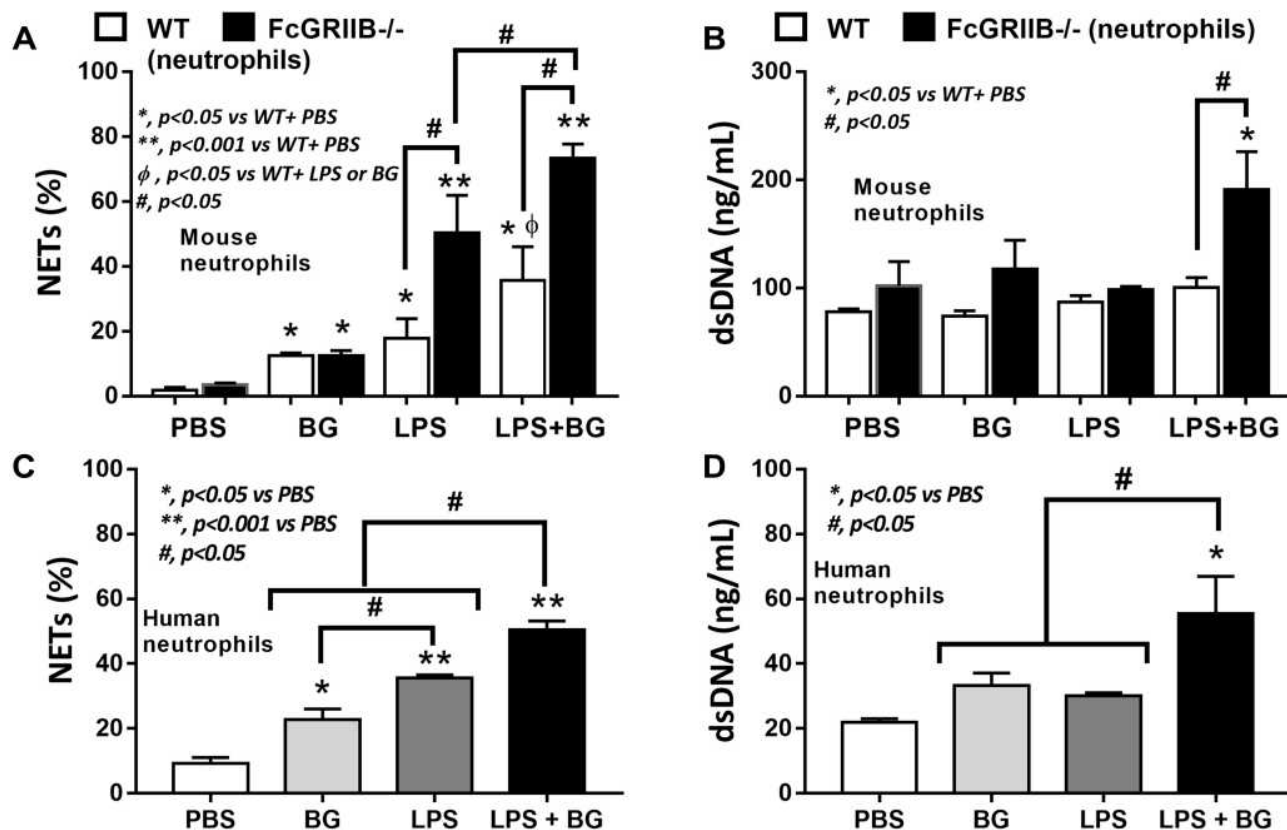


Figure 9 Neutrophil extracellular traps (NETs) in mouse neutrophils, from wild-type (WT) and FcGRIIB^{-/-} mice, and human neutrophils after the 2 h activation by phosphate buffer solution (PBS) or (1→3)-β-D-glucan (BG) or lipopolysaccharide (LPS) or LPS with BG (LPS+BG) are demonstrated. In mouse neutrophils, NETs were determined by the percentage of cells with NETs nucleus morphology using 4,6-diamidino-2-phenylindole (DAPI), a nucleus stained color, with supernatant dsDNA by PicoGreen assay (A and B). In human neutrophils, NETs using the percentage of cells with positive staining for both myeloperoxidase (MPO) together with neutrophil elastase (NE) (merge color) (C) and the supernatant dsDNA (D) are demonstrated. All experiments were independently performed in triplicate. *p < 0.05 vs WT + PBS; **p < 0.001 vs WT + PBS; φp < 0.05 vs WT + LPS or BG; #p < 0.05.

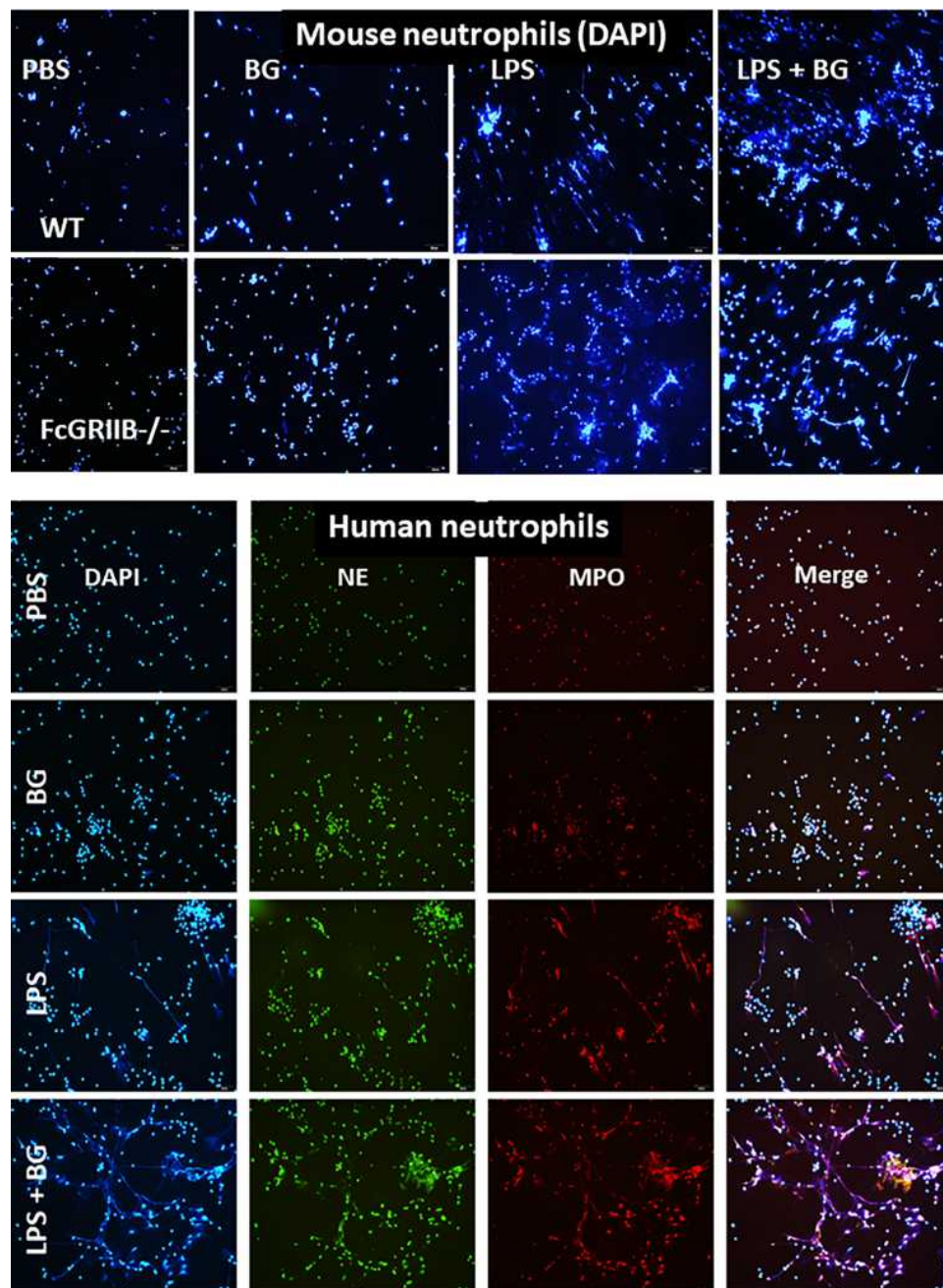


Figure 10 Representative immunofluorescent pictures for neutrophil extracellular traps (NETs) from wild-type (WT) or FcGRIIB^{-/-} neutrophils after the 2 h activation by phosphate buffer solution (PBS) or (1→3)- β -D-glucan (BG) or lipopolysaccharide (LPS) or LPS with BG (LPS+BG) as determined by the branching of nucleus morphology using 4-,6-diamidino-2-phenylindole (DAPI), a nucleus stained color (blue), in mouse neutrophils (upper part) and the co-localization (merge purple color) of myeloperoxidase (MPO) (red) with neutrophil elastase (NE) (green) in human neutrophils (lower part) are demonstrated.

post-Pristane injection) as the representative models of asymptomatic lupus prone mice caused by a genetic defect and an external stimulation in the mice with normal genetic background, respectively. As such, Pristane (a hydrocarbon from shark oil) induces chronic peritonitis, recruits peritoneal neutrophil accumulation, causes NETs-induced diffuse alveolar hemorrhage (DAH) in some

mice⁵⁰ and also directly triggers the neutrophilic NETs *in vitro*.⁵¹ However, lung hemorrhage, a moribund complication of Pristane, was not detectable in our Pristane mice (data not shown). Although the pathophysiology and the clinical presentations are different between FcGRIIB^{-/-} mice and Pristane mice⁵ including the hyper-responsive inflammation with dominant lupus nephritis and the potent

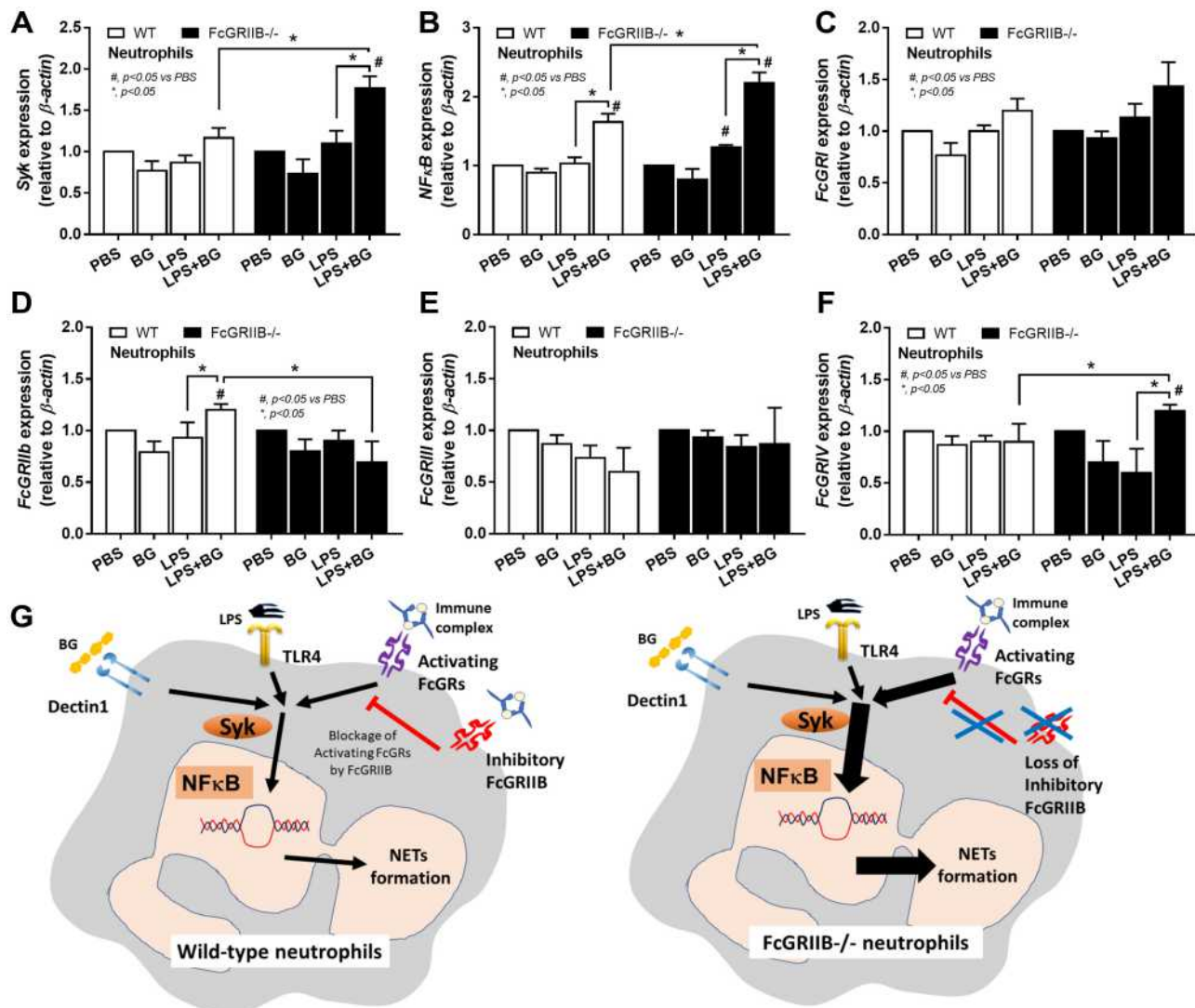


Figure 11 Characteristics of neutrophils from wild-type (WT) and FcGRIIB^{-/-} mice after the 2 h activation by phosphate buffer solution (PBS) or (1 \rightarrow 3)- β -D-glucan (BG) or lipopolysaccharide (LPS) or LPS with BG (LPS+BG) as determined by the gene expression of *Spleen tyrosine kinase* (Syk) (A), *Nuclear factor kappa B* (NF κ B) (B) and activating FcGRs (FcGRI, FcGRIII, FcGRIV) and inhibitory FcGRIIB (C–F) are demonstrated. All experiments were independently performed in triplicate. Additionally, the proposed hypothesis (G) indicates a possible difference in NETs formation between WT and FcGRIIB^{-/-} neutrophils that proposes Syk as a common downstream signaling of TLR-4, Dectin-1 and activating-FcGRs that could be partly blocked by the inhibitory FcGRIIB. #p < 0.05 vs WT + PBS; *p < 0.05.

activation of interferon (IFN) type I with injury in several organs, respectively, both lupus models could be used to compare the lupus severity between the DSS model with versus without *Candida* administration. Notably, a dominant impact of gut-leakage in FcGRIIB^{-/-} mice over Pristane mice using a non-diarrheal 1% DSS model through macrophage activation¹² is demonstrated; however, the influence of symptomatic colitis on neutrophils in these models has never been tested.

With 3% DSS for 7 days, obvious diarrhea with similar clinical manifestations (weight loss and loose stool consistency) presented in all groups (WT, FcGRIIB^{-/-} and Pristane), but DSS induced the more severe pathological

injury (ulcers and neutrophil infiltration) in lupus models (more severe in FcGRIIB^{-/-} mice than Pristane mice), possibly through the mild inflammatory status in lupus.^{12,15} While the intestinal NETs, using MPO and NE with *PAD4* (a chromatin de-condensation enzyme), in DSS alone were far less in all groups, DSS-*Candida* induced more NETs^{52–55} with severe pathological injury (neutrophil accumulation^{56,57}) in all groups (more prominent in FcGRIIB^{-/-} mice than Pristane mice). These data supported an impact of gut *Candida* in DSS-induced non-infectious colitis model.²⁶ Likewise, the more severe intestinal NETs in DSS-*Candida* FcGRIIB^{-/-} mice than Pristane mice supported a possible impact of FcGRs on

NETs formation,⁴³ partly through the enhancement of several activating FcGRs due to the loss of inhibitory FcGRIIB.^{4,15} Meanwhile, the limited intestinal NETs in DSS-*Candida* Pristine mice implied a balance against the pro-inflammatory signals through an intact inhibitory FcGRIIB due to a normal genetic background. In 40-wk-old FcGRIIB^{-/-} mice (a full-blown lupus), NETs were spontaneously detectable only in kidneys, but not in the intestines, suggesting that NETs did not originate from the intestines in active lupus. In contrast, with DSS-induced gut inflammation, NETs formation started at the intestines and accelerated NETs elsewhere (in blood circulation and in kidneys), implying lupus disease exacerbation through gut-leakage.

Prominent Gut-Leakage Induced Inflammation Aggravated Lupus Nephritis in DSS-Administered *Candida*-Gavage FcGRIIB^{-/-} Mice

The more severe gut translocation of organismal molecules (LPS and BG) in DSS-*Candida* FcGRIIB^{-/-} mice prominently activated systemic inflammation^{6,12,58} (blood neutrophils, circulating NETs and serum cytokines). Since (i) NETs could be induced by LPS, BG and immunoglobulin (Ig) through TLR-4, Dectin-1 and FcGRs,¹⁶ (ii) NETs are present in active lupus⁵⁹⁻⁶¹ and (iii) the collaboration among TLR-4, Dectin-1 and FcGRs is mentioned,^{6,62} LPS+BG activation might profoundly enhance NETs in FcGRIIB^{-/-} neutrophils. Accordingly, LPS+BG, when compared with LPS alone, more prominently enhanced NETs in FcGRIIB^{-/-} neutrophils than WT cells, which implies the importance of TLR-4, Dectin-1 and FcGRs on NETs formation.⁴³ Furthermore, the more severe inflammation in DSS-*Candida* FcGRIIB^{-/-} mice also exacerbated lupus disease activity (increased anti-dsDNA and proteinuria), supporting inflammation-aggravating lupus activity. The high level of anti-dsDNA resulted in an accelerated Ig deposition in glomeruli that induced glomerular mesangial expansion, glomerular NETs and proteinuria in FcGRIIB^{-/-} mice, but not in Pristine mice. Indeed, proteinuria is a risk factor of renal progression⁶³ and is also a poor prognostic factor of lupus. Hence, *Candida* presentation in FcGRIIB^{-/-} lupus prone mice exacerbated lupus activity through the induction of intestinal NETs that were severe enough to cause systemic inflammation and the inflammation aggravated lupus nephritis. Also, gut-leakage induced inflammation in 16-wk-old FcGRIIB^{-/-} mice (asymptomatic lupus)

also implied a possibility of lupus exacerbation from acute diarrhea (non-infectious and infectious causes) in full-blown lupus. Although DSS is not administered in 40-wk-old FcGRIIB^{-/-} full-blown lupus mice, DSS-induced gut leakage in the active lupus with a pre-formed Ig deposition in gut might worsen the lupus activity. Moreover, there are several limitations in our lupus mouse models for use as a representative of patients with lupus, including the absence of other important lupus characteristics (polyarthritis, skin manifestations and serositis⁶⁴) and the diversity of genetic defects in patients. An impact of gut-leakage in other lupus models might be different.

Prominent Inflammatory Responses in FcGRIIB^{-/-} Neutrophils Over Wild-Type Cells, an Inhibitory Effect of FcGRIIB

Since (i) spleen tyrosine kinase (Syk) is a common downstream signaling from the activation of TLR-4, Dectin-1 and FcGRs^{4,15} that activates NFκB, a NETs-associated transcriptional factor,^{65,66} (ii) LPS and BG could activate NETs through TLR-4 and Dectin-1, respectively,^{67,68} and (iii) FcGRs are also correlated with NETs formation,^{43,69} these signals might be associated with the prominent NETs in LPS+BG activated FcGRIIB^{-/-} neutrophils. Here, we hypothesize that the mechanism of NETs formation in neutrophils might be similar to the macrophages due to the presence of all receptors in both cell types.^{2,3} In FcGRIIB^{-/-} neutrophils, only LPS+BG, but not LPS or BG alone, upregulated *Syk*, *NFκB* and activating-*FcGRIV*. Meanwhile, LPS+BG in WT neutrophils, but not by each molecule alone, upregulated only *NFκB* and inhibitory-*FcGRIIB*. In contrast, *FcGRI* and *FcGRIII* expression after activation by LPS and/or BG were not different, implying a limited role of these receptors in NETs formation. Notably, mouse FcGRIV is possibly more active than FcGRIII, because FcGRIV recognizes 3 out of 4 isoforms of mouse IgG (IgG1, IgG2a, and IgG2b), while FcGRIII recognizes only mouse IgG1 (mouse IgG3 is non-recognizable by any mouse FcGRs).^{70,71} Because NETs formation occurs after the activation on TLR-4, Dectin-1 and activating-FcGRs is previously mentioned-^{16,65,69} and NFκB is also associated with NETs formation.-^{65,66} We hypothesize the activation of TLR-4, Dectin-1 and activating-FcGRs (especially FcGRIV), without the inhibitory FcGRIIB, in FcGRIIB^{-/-} neutrophils, though Syk and NFκB is responsible for the prominent NETs

(Figure 11G). Additionally, the increased-NETs enhanced systemic inflammation in DSS-*Candida* FcGRIIB^{-/-} mice.

Then, gut fungi prominently enhanced intestinal NETs in FcGRIIB^{-/-} mice (a genetic prone lupus), compared with Pristane mice (a chemical-induced lupus), supporting the importance of FcGRs and gut fungi in NETs formation. Gut fungi increased BG in gut contents and LPS+BG from gut translocation exacerbated lupus nephritis in FcGRIIB^{-/-} lupus mice through the profound NETs formation in FcGRIIB^{-/-} neutrophils. Because the increase in both BG and LPS in serum is reported in patients with active lupus,^{6,7} our results demonstrate a possible impact of LPS+BG in lupus exacerbation through the induction of NETs formation.

In conclusion, the evaluation of gut fungi, gut-leakage and NETs formation in lupus are clinically interesting and the exploration of the association between gut and kidney (gut-kidney axis) in lupus might lead to new treatment strategies. Further studies are warranted.

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Disclosure

The authors report no conflicts of interest in this work.

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