Journal of Inflammation Research Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

Gain-of-Function Variant in Spleen Tyrosine Kinase Regulates Macrophage Migration and Functions to Promote Intestinal Inflammation

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Purpose: Spleen tyrosine kinase (Syk) is a widely-expressed cytoplasmic non-receptor tyrosine kinase involved in regulating various signaling pathways and plays an important role in chronic inflammation and autoimmune diseases. Gain-of-function *SYK* variants have been implicated in pediatric inflammatory bowel diseases. This study aimed to investigate the effects of gain-of-function *SYK* variants on the susceptibility to experimental colitis and macrophage function.

Methods: Colitis was induced using dextran sodium sulfate and dinitrobenzene sulfonic acid in mice harboring a gain-of-function variant in *SYK* (Syk^{S544Y}). Intestinal inflammation was assessed via disease activity index, histological analysis, and Western blotting. The frequencies of macrophages, phagocytosis, and reactive oxygen species (ROS) production in bone marrow-derived macrophages (BMDM) were measured via flow cytometry. Chemokines and BMDM chemotaxis were analyzed using real-time quantitative reverse transcription polymerase chain reaction and Transwell assays. The expression of nucleotide-binding domain leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome-related proteins were detected using immunohistochemistry, enzyme-linked immunoassay and Western blotting.

Results: Syk^{S544Y} mice exhibited increased susceptibility to experimental colitis, and macrophage infiltration in colon tissues significantly increased. We observed increased expression of macrophage chemokines in colon tissues and enhanced chemotaxis in Syk^{S544Y} BMDM. Additionally, we detected increased levels of fluorescent microspheres and 2.7-dichloride-hydro fluorescein diacetate-labeled ROS in Syk^{S544Y} BMDM. Moreover, enhanced levels of NLRP3 inflammasome-related proteins were observed in the colon tissues and BMDM from Syk^{S544Y} mice.

Conclusion: Gain-of-function variant in *SYK* may contribute to the pathogenesis of pediatric inflammatory bowel diseases by promoting macrophage migration, phagocytosis, ROS production and activation of NLRP3 inflammasomes.

Keywords: inflammatory bowel disease, SYK, gain-of-function variant, macrophage

Introduction

Spleen tyrosine kinase (Syk) is a cytoplasmic non-receptor tyrosine kinase expressed in various cell types, including hematopoietic (such as B lymphocytes, T lymphocytes, macrophages, dendritic cells and mast cells) and non-hematopoietic cells (such as intestinal epithelial cells).[1] Syk transmits signals by phosphorylating tyrosine residues of downstream signaling molecules, activating various signaling pathways crucial in chronic inflammation or autoimmune diseases, such as rheumatoid arthritis, allergic asthma, and rhinitis.[2] Syk inhibitors, including R406, fostamatinib (R788) and piceatannol, have shown promise in treating autoimmune and allergic diseases.[3]

Inflammatory bowel diseases (IBD) are a heterogeneous group of chronic gastrointestinal inflammatory disorders comprising ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis.[4] It is wildly recognized that IBD is

Received: 27 July 2024
Accepted: 5 November 2024
Published: 12 November 2024

caused by an inappropriate immunological response to intestinal microbiota triggered by environmental factors in genetically susceptible individuals.[5] Syk plays an important role in IBD pathogenesis. DSS or 2,4,6-trinitrobenzene sulfonic acid-induced colitis in $Syk^{-/-}$ mice was milder than that in WT mice.[6] Furthermore, piceatannol and fostamatinib alleviate experimental colitis in mice.[7,8] In $IL10^{-/-}$ mice with spontaneous IBD, deficiency of stomach-cancer-associated protein tyrosine phosphatase 1 (SAP-1) results in severe colitis. The association of carcinoembryonic antigen-related cell adhesion molecule 20, a substrate for SAP-1, with Syk, promoted interleukin (IL) 8 production by activating nuclear transcription factor-κB (NF-κB).[9] The kinase inhibitor TOP1210, targeting Syk, reduces pro-inflammatory cytokines in UC biopsies and various cell types, including epithelial, innate, and adaptive immune cells.[10] Additionally, anti-tumor necrosis factor antibodies, including infliximab and adalimumab, suppress IL12/IL23 production via Syk.[11] Moreover, Syk mediates cytokine production in dendritic cells through caspase recruitment domain family member 9, which has been associated with IBD.[12,13]

The point mutation of *SYK* (Syk^{Y317F}) has been reported to result in a gain of function, increasing histamine release in mast cells.[14] Our previous study reported that gain-of-function variants in *SYK* caused IBD, and patients presented with immune deficiency and multi-organ inflammatory diseases such as colitis, arthritis, dermatitis and diffuse large B cell lymphomas, which conformed to autosomal dominant inheritance. However, the knock-in (Syk^{S544Y}) mouse model, corresponding to the gain-of-function variant (Syk^{S550Y}) in humans, is characterized by spontaneous arthritis and can be alleviated with the Syk inhibitor R406 or transplantation of bone marrow from wild-type (WT) mice.[15] Thus, the role of Syk^{S544Y} in susceptibility to experimental colitis in mice needs to be further investigated.

In our previous study, the frequency of macrophages in the ankle tissues from Syk^{S544Y} mice with idiopathic arthritis increased.[15] Macrophages are important components of innate immunity and play a crucial role in establishing and maintaining intestinal homeostasis.[16] As a pattern recognition receptor, macrophage-inducible C-type lectin Mincle activates Syk upon recognizing damage-associated molecular patterns or pathogen-associated molecular patterns.[17,18] Mincle signaling is up-regulated in patients with CD and mice with induced colitis. Reduced CD11b+ monocytes/macrophages, especially M1 proinflammatory macrophages, and pyroptosis-related proteins such as nucleotide-binding domain leucine-rich-containing family, pyrin domain-containing-3 (NLRP3), were observed in colonic mucosa from mice with Mincle deficiency or Syk inhibitor.[19] Additionally, myeloid differentiation primary response 88 (MyD88) activated the downstream NF-κB signaling pathway by interacting with Syk in RAW264.7 cells. The Syk-induced inflammatory response and reactive oxygen species (ROS) production were significantly inhibited in $MyD88^{-/-}$ RAW264.7 cells.[20] Piceatannol ameliorates experimental colitis by targeting C-C motif chemokine receptor 5 (CCR5) expressing macrophages. This effect is achieved by delivering the compound encapsulated with poly-(lactic-co-glycolic acid) nanoparticles, which are conjugated with chemokine C-C motif ligand (CCL) 4.[21] However, the impact of gain-of-function variant in *SYK* on macrophage function and IBD remains unclear.

In this study, based on the findings that gain-of-function in *SYK* caused pediatric IBD, mice with gain-of-function in *SYK* (Syk^{S544Y}) were induced with experimental colitis for the first time. We used mice to further investigated the effects of Syk^{S544Y} on macrophage function. Our study contributes to understanding the effects of Syk on macrophage function and the potential mechanisms involved in IBD development.

Material and Methods

Mice

Male Syk^{S544Y} and WT C57BL/6 mice were obtained from Professor Dali Li's research group at East China Normal University, Shanghai, China (SCXK 2016–0004). The mice were individually housed in specific pathogen-free facilities at the Experimental Animal Center at Children's Hospital of Fudan University, Shanghai, China. Age-matched mice (4–8 weeks-old) were used in the animal experiments, which were approved by the Ethics Committee of Children's Hospital of Fudan University, Shanghai, China. Animal welfare was in accordance with Laboratory animal-Guideline for ethical review of animal welfare (GB/T 35892–2018).

Experimental Models of Colitis

The experimental model of acute colitis was induced by administering mice with 2% dextran sodium sulfate (DSS, molecular weight: 36–50kDa, 160110, MP Biomedicals, Santa Ana, CA, USA) dissolved in drinking water for 7 days or via intra-rectal administration of dinitrobenzene sulfonic acid (DNBS, 556971, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 150 mg/kg (dissolved in 0.05mL 50% ethanol). The disease activity index (DAI) was calculated daily based on weight loss, stool consistency, and the degree of intestinal bleeding.[22] Moreover, chronic colitis was induced by three cycles of 2% DSS (7 days of DSS followed by 14 days of water).

Histological Analyses

Mouse colon tissues were fixed in 4% paraformaldehyde and dehydrated in ethanol and xylene. The dehydrated tissues were embedded in paraffin wax and cut into 5μm slices. Sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or Masson's trichrome (Biossci, Hubei, China). For immunohistochemical staining, the sections were dewaxed in xylene and rehydrated in an ethanol gradient. NLRP3 proteins in the sections were examined using a primary antibody against NLRP3 (DF7438, 1:150, Affinity Biosciences, Cincinnati, OH, USA). Staining was visualized using diaminobenzidine system (abs957, Absin, China) according to manufacturer's protocol. Histopathological changes were observed under a light microscope.

Western Blot Analysis

Mouse intestinal tissues and cells were homogenized in ice-cold radioimmunoprecipitation assay buffer (89900, Thermo Fisher Scientific, Waltham, MA, USA) containing a protease and phosphatase inhibitor cocktail (P002, New Cell & Molecular Biotech, Suzhou, China). The Supernatants were collected after being lysed on ice for 30 min, followed by centrifugation at 12000 *g* for 10 min at 4 °C. The lysates were boiled for 10 min after adding a protein-loading buffer (WB2001, New Cell & Molecular Biotech). Total proteins were separated by 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (IPVH00010, Immobilon®-P, Darmstadt, Germany), which was blocked in protein-free rapid blocking buffer (PS108P, EpiZyme, Cambridge, MA, USA) for 20 min at 25 °C. The membrane was further incubated overnight at 4 °C with primary antibodies against Syk (1:1000, 2712T, Cell Signaling Technology, Danvers, MA, USA), pSyk (1:1000, AF8404, Affinity Biosciences), NLRP3 (1:1000, DF7438, Affinity Biosciences), GSDMD (1:1000, AF4012, Affinity Biosciences), caspase1 (1:1000, GB11383, Servicebio, Wuhan, China), cleaved-caspase1 (1:1000, AF4005, Affinity Biosciences), IL1β (1:1000, DF6251, Affinity Biosciences), cleaved-IL1β (1:1000, AF4006, Affinity Biosciences), ERK1/2 (1:2000, AF0155, Affinity Biosciences), pERK1/2 (1:2000, 4370T, Cell Signaling Technology), JNK (1:3000, 66,210–1, Proteintech, Rosemont, IL, USA), pJNK (1:1000, 4668T, Cell Signaling Technology), cjun (1:1000, 9165T, Cell Signaling Technology), pp38 MAPK (1:1000, 5175T, Cell Signaling Technology), p65 (1:1000, 8242T, Cell Signaling Technology), pp65 (1:1000, 3033T, Cell Signaling Technology) or GAPDH (1:5000, 60,004–1, Proteintech). The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:5000, S0001, Affinity Biosciences) or anti-mouse immunoglobulin G (1:5000, S0002, Affinity Biosciences) secondary antibodies for 1h at 25 °C. Target proteins were visualized using NcmECL ultra reagent (P10100, New Cell & Molecular Biotech).

Flow Cytometry

For single cell suspension, minced intestinal tissues were incubated in digestion buffer (Dulbecco's modified Eagle medium (C11995500BT, Gibco, Thermo Fisher Scientific) with 2.5% fetal bovine serum (FBS, F8318, Sigma-Aldrich), penicillin/streptomycin (C100C5, New Cell & Molecular Biotech) and 1mg/mL collagenase/dispase (10269638001, Roche) at 37 °C for 1 h. The supernatant was filtered with a 70 μm strainer and centrifuged at 300 *g* at 25 °C for 5 min. Cells were isolated by 40%/80% Percoll (17089102, Cytiva, Marlborough, MA, USA) gradient centrifugation at 800 *g* for 20 min and washed with phosphate-buffered saline (PBS) containing 1% FBS. Moreover, spleens from WT and Syk^{S544Y} mice were prepared as previously described.[15]

For flow cytometry, the stained were stained with fluorescent-labeled antibodies against CD45 (103,154), F4/80 (123,149), CD11b (101205), Ly6C (128031) and CX3CR1 (149005) at 4 °C for 20 min, all purchased from BioLegend (San Diego, CA, USA). Stained cells were analyzed using a flow cytometer, and data were analyzed using FlowJo version 10 software.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total ribonucleic acid (RNA) from mouse intestinal tissues was extracted using RNAiso Plus (9108, Takara Bio, San Jose, CA, USA) and reverse-transcribed into complementary deoxyribonucleic acid using *Evo M-MLV* reverse transcription kit (AG11728, Accurate Biology, Hunan, China). Real-time PCR was performed using an SYBR Green Premix *Pro* Taq HS qPCR kit (AG11701, Accurate Biology). TATA box-binding protein was selected as an internal reference to calculate the relative expression of target genes. Supplementary Table 1 shows the list of the primers used.

Bone Marrow-Derived Macrophages (BMDM)

Mouse bone marrow was flushed from the femur and tibia with a 1mL syringe containing ice-cold PBS. The suspension was filtered through a 70-μm strainer and red blood cells were removed using a lysis buffer (SL1070, Coolaber, Beijing, China). After being centrifuged at 300 *g* for 5 min at 4 °C, the cells were re-suspended and cultured in cell culture medium containing Roswell Park Memorial Institute 1640 medium (SH30809.01, Cytiva), 10% FBS, penicillin/streptomycin and 20ng/mL M-CSF (315–02, PeproTech, Cranbury, NJ, USA). Fresh culture medium was added on the third and fifth days. After 2 days, the adherent cells were differentiated into mature macrophages for subsequent experiments.

Transwell Cell Migration Assay

Transwell chamber with 8 μm pores (ICS003024, Jet Bio-Filtration, Guangzhou, China) was employed to analyze the cell migration assay. WT or Syk^{S544Y} BMDM (5×10^4 cells) were added to the upper chamber with serum-free 1640 medium, and 100 ng/mL CCL2 (250–10, PeproTech) or CCL5 (250–07, PeproTech) was added to the lower chamber with 1640 medium containing 10% FBS for overnight culture. The cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet (S0288, Bioss) for 30 min. After gently removing cells from the upper surface with a cotton swab, the migrated cells were counted under a microscope.

Phagocytosis Assay

BMDM were cultured in six-well plates at a density of 8×10^5 cells per well and treated with or without 200 ng/mL lipopolysaccharides (LPS, L4516, Sigma-Aldrich) overnight in the presence or absence of 2uM R406 (S2194, Selleck Chemicals LLC, Boston, MA, USA) pretreatment for 1 h. FluoSpheres™ carboxylate-modified fluorescent microspheres (2μm diameter, F8827, Thermo Fisher Scientific) were suspended in 1640 medium (10 μL/mL) containing 1% bovine serum albumin (BS114, Biosharp, Hefei, China), incubated at 25 °C in the dark for 30 min, and sonicated for 5 min. A total of 0.5 mL microsphere suspension was added to the culture medium of each well for 1 h. After washing twice with PBS, the cells were harvested using 0.25% trypsin (C100C1, New Cell & Molecular Biotech) for 7–8 min. The cell suspensions were centrifuged at 300 *g* for 5 min, and the cell pellets were gently re-suspended with PBS. The suspensions were subjected to flow cytometry, and the phagocytic activity was assessed using the phagocytic rate (percentage of fluorescein isothiocyanate-positive cells) and phagocytic index (ratio of engulfed microspheres to total macrophages).

ROS Detection

BMDM were cultured in six-well plates at a density of 8×10^5 cells per well and treated or not treated with 200 ng/mL LPS for 4 h in the presence or absence of 2 uM R406 pretreatment for 1 h. After being harvested with trypsin, the cells were resuspended and incubated with serum-free 1640 medium containing 10 uM 2.7-dichloride-hydro fluorescein

diacetate (DCFH-DA, S0033S, Beyotime Biotech, Shanghai, China) for 20 min. The cells were then washed three times with PBS, and ROS were detected using flow cytometry according to the manufacturer's protocol.

Enzyme-Linked Immunosorbent Assay (ELISA)

BMDM were cultured in six-well plates at a density of 8×10^5 cells per well and treated with or without 200 ng/mL LPS for 6 h. The supernatant was collected and centrifuged at 1000 *g* for 5 min. Murine IL1β in the cell-free supernatant was detected with ELISA kit (E-EL-M0037, Elabscience Biotechnology, Houston, TX, USA) according to the manufacturer's protocol.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9 software. The statistics of different groups were compared using the unpaired *t*-test or Mann–Whitney *U*-test, as indicated in the figure legends. *P* values less than 0.05 were considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Gain-of-Function Variant in SYK Increases Susceptibility to DSS or DNBS-Induced Colitis

The role of gain-of-function *SYK* variant in colitis was examined by assessing the susceptibility of WT and Syk^{S544Y} mice to induced colitis. First, acute colitis was induced using DSS and DNBS. Figure 1A shows that WT and Syk^{S544Y} mice that were not treated with DSS exhibited no difference in body weight. DSS treatment led to weight loss in all mice by day 5, with Syk^{S544Y} mice showing more pronounced weight loss than WT mice by day 7. Induction was halted when weight loss in Syk^{S544Y} mice exceeded 20% of their initial weight. In contrast, DSS-treated Syk^{S544Y} mice exhibited higher DAI and shorter colon length than DSS-treated WT mice (Figure 1B and C). Consistent with these results, more severe histological inflammation was observed in the DSS-treated Syk^{S544Y} mice, particularly transmural infiltration of inflammatory cells, epithelial cell destruction, crypt distortion, and goblet cell loss (Figure 1D). The intestinal inflammation was further confirmed by increased NF-κB and MAPK signaling pathway-related proteins (Figure 1E). Similar results were obtained in DNBS-induced colitis in WT and Syk^{S544Y} mice (Figure 2).

Moreover, we induced sustained intestinal inflammation with DSS in mice, resulting in a significant decrease in the survival rate of Syk^{S544Y} mice (Supplementary Figure 1A). After three cycles of 2% DSS, statistical analyses of body weight loss, colon length, and spleen weight between WT and Syk^{S544Y} mice were not performed because of the reduced number of Syk^{S544Y} mice. Compared with WT mice, only two surviving Syk^{S544Y} mice showed splenomegaly, but no significant difference was observed in colon length (Supplementary Figure 1B–D). Histological staining revealed pronounced epithelial cell destruction, crypt distortion, and fibrosis in the colon of Syk^{S544Y} mice (Supplementary Figure 1E).

Gain-of-Function Variant in SYK Increased Macrophage Infiltration in Colon Tissues from Mice with Colitis

In view of earlier findings of increased macrophages in the ankle tissues of Syk^{S544Y} mice,[15] we investigated the role of gain-of-function *SYK* variant in intestinal inflammation. Flow cytometry revealed significantly higher frequencies of Cd11b+ and F4/80+ macrophages in the colon and spleen tissues of Syk^{S544Y} mice with acute colitis (Figure 3A and B). We further classified intestinal macrophages of mice with DSS-induced acute colitis. A significant increase in the number of $Ly6C^{hi}CX3CR1^{int}$ macrophages was observed in the colonic tissue of Syk^{S544Y} mice (Figure 3C). $Ly6C^{hi}CX3CR1^{int}$, a pro-inflammatory macrophage, aggravates intestinal inflammation and plays a critical role in the pathogenesis of CD and UC.[23,24] Moreover, the GSE123141 dataset obtained from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/gds) were reanalyzed. We screened the RNA-seq data of purified CD14+/CD163+ intestinal macrophages from patients with UC (n=9), CD (n=10), and healthy donors (n=9). The expression of *SYK* was significantly increased in the UC group and had a tendency to increase in the CD group (Supplementary Figure 2), suggesting that Syk may be involved in IBD pathogenesis through macrophage modulation.

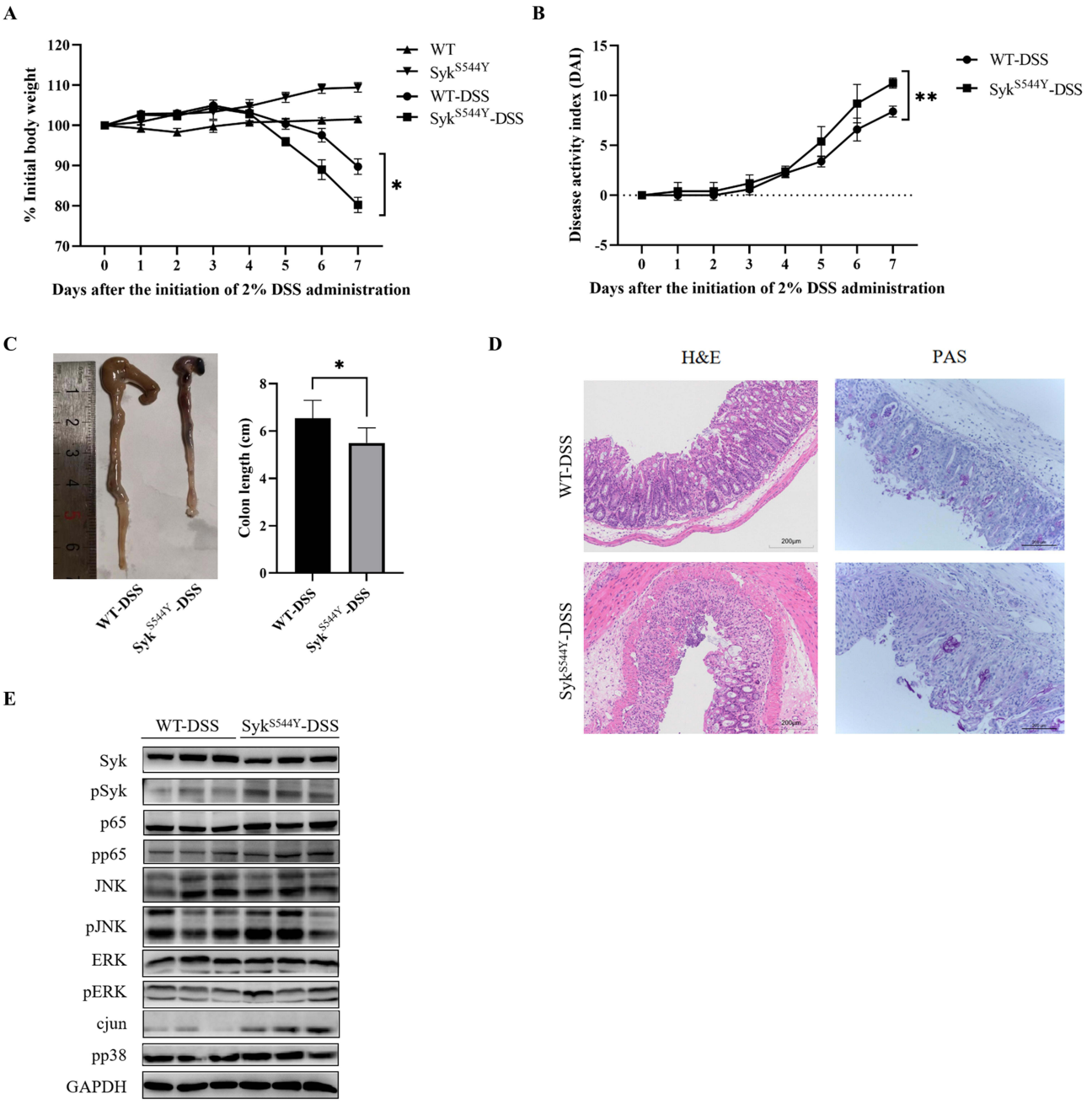

Figure 1 Syk^{S544Y} mice were susceptible to DSS-induced acute colitis. WT and Syk^{S544Y} mice (n=5/group) were treated with 2% DSS in the drinking water for 7 days. The colitis was evaluated using (**A**) body weight loss, expressed as a percentage of the initial body weight, (**B**) DAI, and (**C**) colon length on day 7. (**D**) Representative images of mucosal inflammation assessed via H&E and PAS staining. Scale bar: 200μm. (**E**) Western blot analysis of total Syk, pSyk (Y525/526), NF-κB, and MAPK signaling pathway-related proteins in colon tissues. Data are presented as mean values ± SEM; *P < 0.05, **P < 0.01.

Abbreviations: DSS, dextran sodium sulphate; DAI, disease activity index; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; SEM, standard error of the mean.

Gain-of-Function Variant in SYK Promotes Macrophage Migration

To explore whether gain-of-function variant in *SYK* regulate macrophage migration, we first examined the expression of CCL2 and CCL5, which regulates macrophage migration, in colon tissues of WT and Syk^{S544Y} mice via qRT-PCR.[25,26] The mRNA levels of these chemokines were significantly upregulated in the colon of Syk^{S544Y} mice with induced acute colitis (Figure 4A and B). A transwell assay was performed to investigate the effect of Syk on macrophage responsiveness to chemokines. We induced the migration of BMDM from WT and Syk^{S544Y} mice using CCL2 or CCL5. Compared

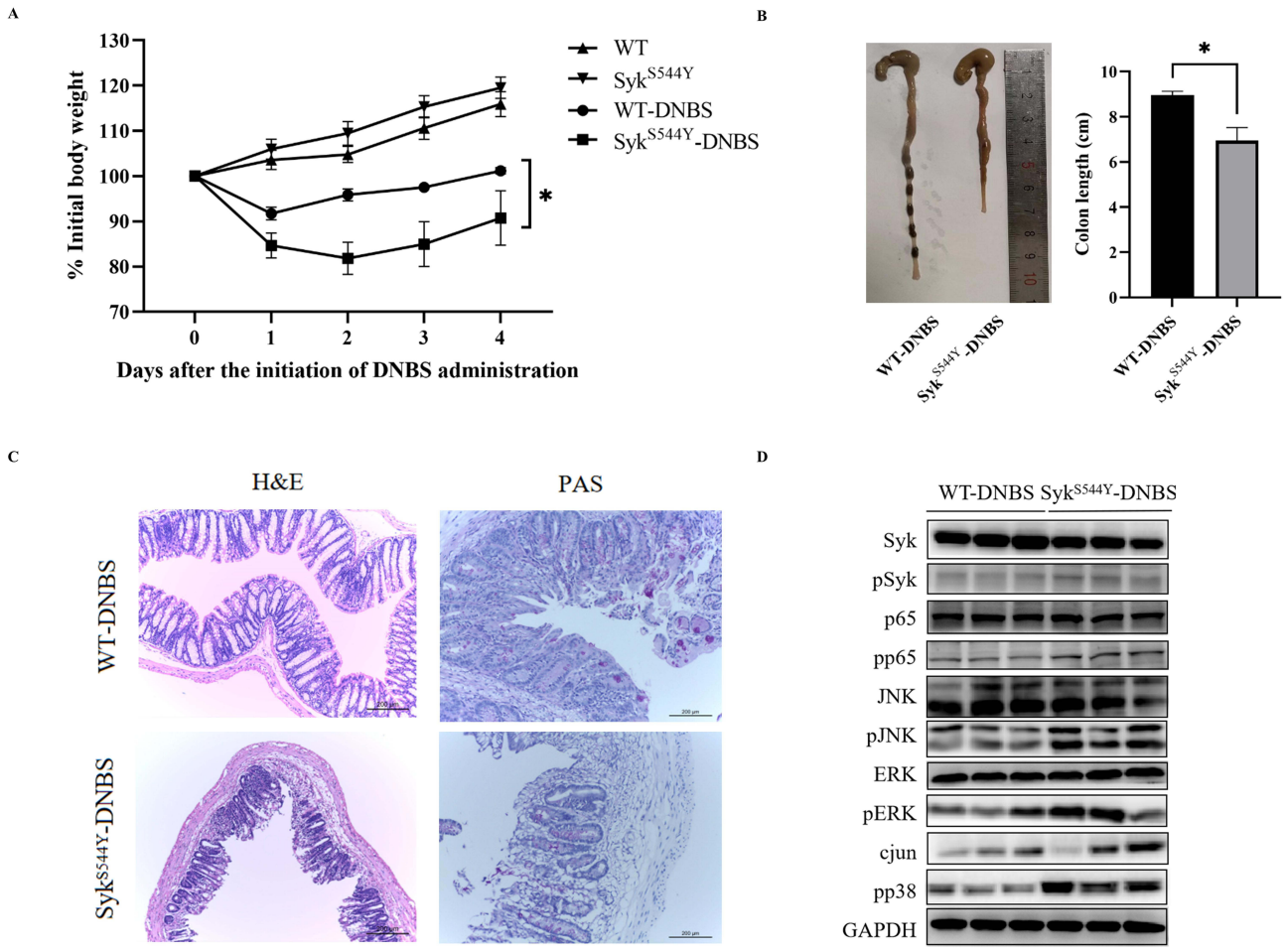

Figure 2 Syk^{S544Y} mice were susceptible to DNBS-induced acute colitis. WT and Syk^{S544Y} mice (n=5/group) received intra-rectal administration of dinitrobenzene sulfonic acid at a dose of 150 mg/kg. The colitis was evaluated using (**A**) body weight loss, expressed as a percentage of the initial body weight, and (**B**) colon length on day 4. (**C**) Representative images of mucosal inflammation assessed via H&E and PAS staining. Scale bar: 200μm. (**D**) Western blot analysis of total Syk, pSyk (Y525/526), NF-κB, and MAPK signaling pathway-related proteins in colon tissue. Data are shown as mean values ± SEM; *$P < 0.05$.
Abbreviations: DNBS, dinitrobenzene sulfonic acid; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; SEM, standard error of the mean.

with WT BMDM, Syk^{S544Y} BMDM had significantly increased mobility upon stimulation with CCL2 or CCL5 (Figure 4C).

Gain-of-Function Variant in SYK Promotes Macrophage Phagocytosis and ROS Production

To investigate the impact of gain-of-function *SYK* variant on the function of macrophages, fluorescent microspheres with a diameter of 2 μm were used to examine the phagocytic ability of macrophages (Supplementary Figure 3A). The results suggested that treatment with LPS or R406 could promote and inhibit the phagocytosis of BMDM, respectively (Figure 5A and B). Under these two conditions, the phagocytic rate and phagocytic index of Syk^{S544Y} BMDM were higher than those of WT BMDM. When LPS and R406 were simultaneously administered, the phagocytosis index of Syk^{S544Y} BMDM remained higher than that of WT BMDM, although no significant difference was observed in the phagocytic rate between WT and Syk^{S544Y} BMDM.

To further explore whether gain-of-function variant in SYK play a role in ROS production in macrophages, ROS were detected using DCFH-DA dye in WT and Syk^{S544Y} BMDM (Supplementary Figure 3B). Syk^{S544Y} BMDM treated with or without LPS produced more ROS than WT BMDM, and no significant difference was observed in ROS levels between

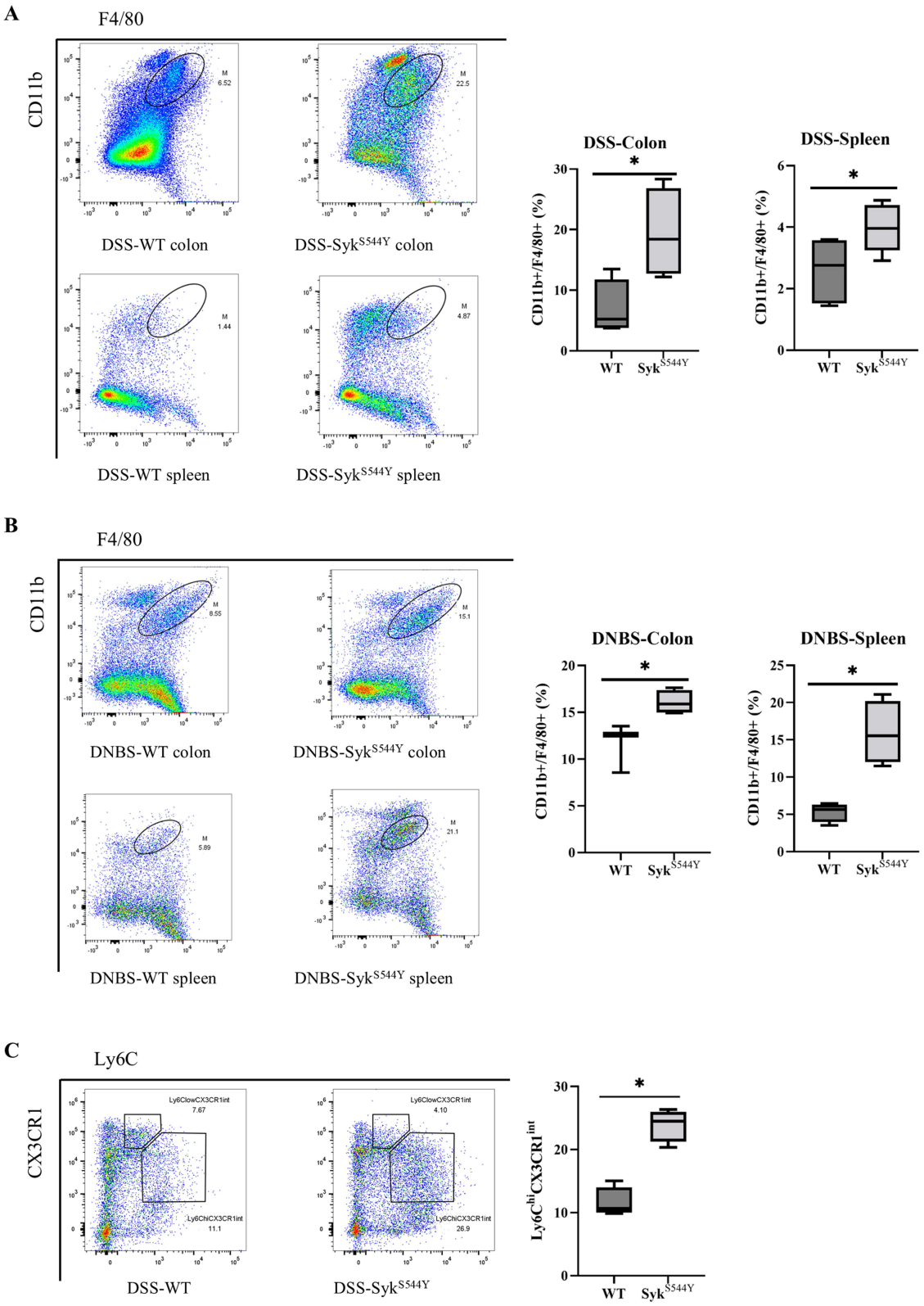

Figure 3 Gain-of-function variant in SYK increased macrophage frequency in mice with acute colitis. (**A** and **B**) The frequency of CD11b+F4/80+ macrophages in WT or Syk^{S544Y} mice with DSS or DNBS-induced acute colitis (n=4/group). (**C**) The frequency of Ly6C^{hi}CX3CR1^{int} macrophages in WT or Syk^{S544Y} mice with DSS-induced acute colitis (n=4/group). Data are presented as mean values ± SEM; *P < 0.05.

Abbreviations: DSS, dextran sodium sulphate; DNBS, dinitrobenzene sulfonic acid; SEM, standard error of the mean.

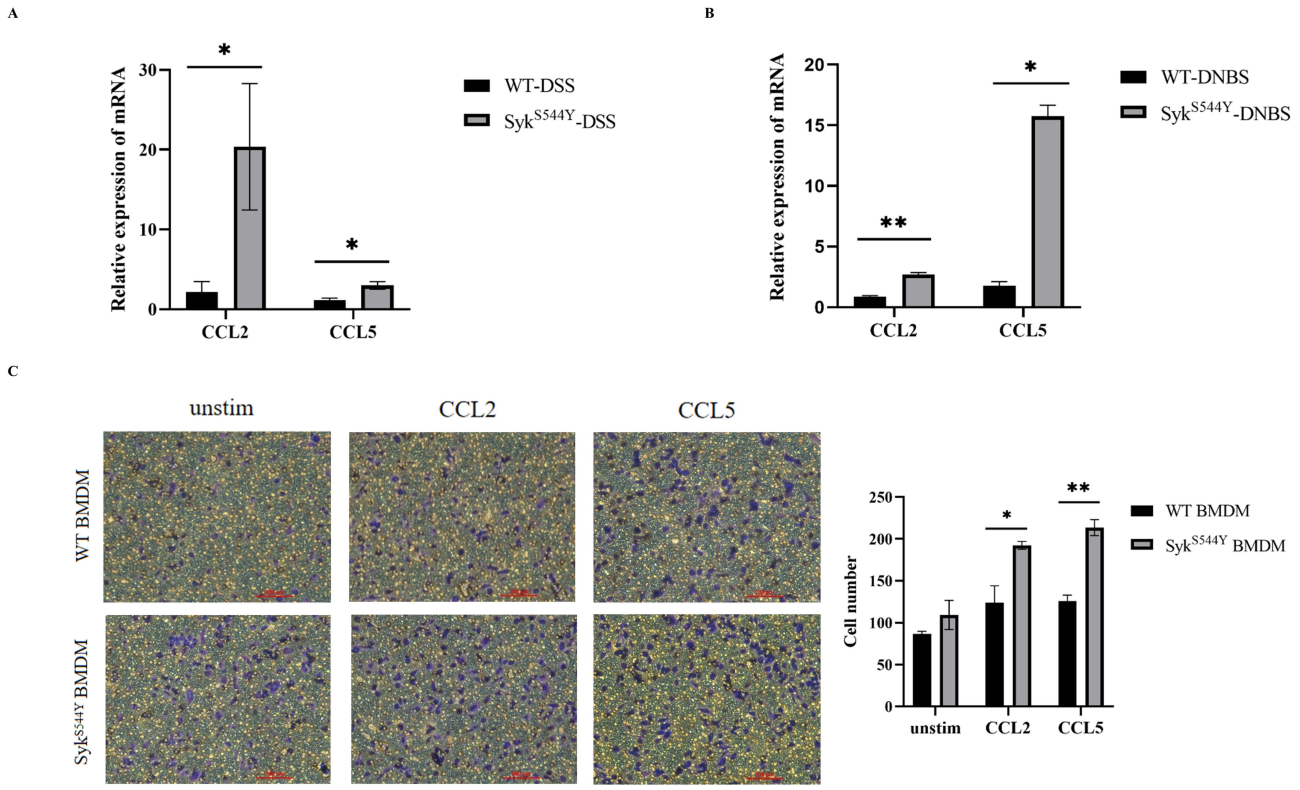

Figure 4 Gain-of-function variant in *SYK* promoted the migration of macrophages. (**A** and **B**) qRT-PCR analysis of chemokine expression (CCL2 and CCL5) in colon tissues from WT and Syk^{S544Y} mice with DSS and DNBS-induced acute colitis (n=5/group). (**C**) Transwell migration assay of migration abilities of macrophages to chemokines. WT and Syk^{S544Y} BMDM (5×10^4) were incubated with 100 ng/mL CCL2 or CCL5 overnight (n=4/group). Scale bar: 200μm. Data are presented as mean values ± SEM; *$P < 0.05$, **$P < 0.01$.

Abbreviations: qRT-PCR, quantitative reverse transcription polymerase chain reaction. CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; DSS, dextran sodium sulphate; DNBS, dinitrobenzene sulfonic acid; BMDM, bone marrow-derived macrophages; SEM, standard error of the mean.

WT and Syk^{S544Y} BMDM treated with R406 alone or in combination with LPS (Figure 5C). These results suggest that the gain-of-function variant in *SYK* promotes the phagocytic function and ROS production in macrophages.

Gain-of-Function Variant in SYK Promotes NLRP3 Activation

NLRP3 inflammasome is an important pattern recognition receptor in the cytoplasm of macrophages and can be assembled to inflammasome involved in the pathogenesis of IBD.[27] The activation of NLRP3 inflammasome increases the active form of caspase1, cleaving the precursors of the inflammatory cytokines such as IL1β and IL18,[28] and the pyroptosis-related protein GSDMD, leading to downstream inflammation.[29] To further investigate the effect of gain-of-function *SYK* variant on NLRP3 activation, we detected the expression of NLRP3 inflammasome-related proteins, including GSDMD, caspase1, and IL1β. We discovered that the expression of NLRP3 inflammasome-related proteins was significantly increased in the colonic tissues of Syk^{S544Y} mice with induced acute colitis (Figure 6A and B), which was also confirmed via the immunohistochemistry of NLRP3 (Figure 6C). Moreover, the ELISA assay of the supernatant of BMDM medium showed a higher concentration of IL1β released by Syk^{S544Y} BMDM with LPS stimulation than that of WT BMDM (Figure 6D). Western blotting further demonstrated that the expression of NLRP3-related proteins in Syk^{S544Y} BMDM was significantly higher than in WT BMDM after LPS treatment (Figure 6E). Collectively, these results suggest that gain-of-function variant in *SYK* promotes NLRP3 inflammasome activation.

Discussion

Activation of Syk regulating intestinal homeostasis is by promoting the production of inflammatory cytokines and chemokines.[30,31] Syk deficiency or inhibition leads to mild colitis in mice.[6–8] Our previous study revealed that gain-of-

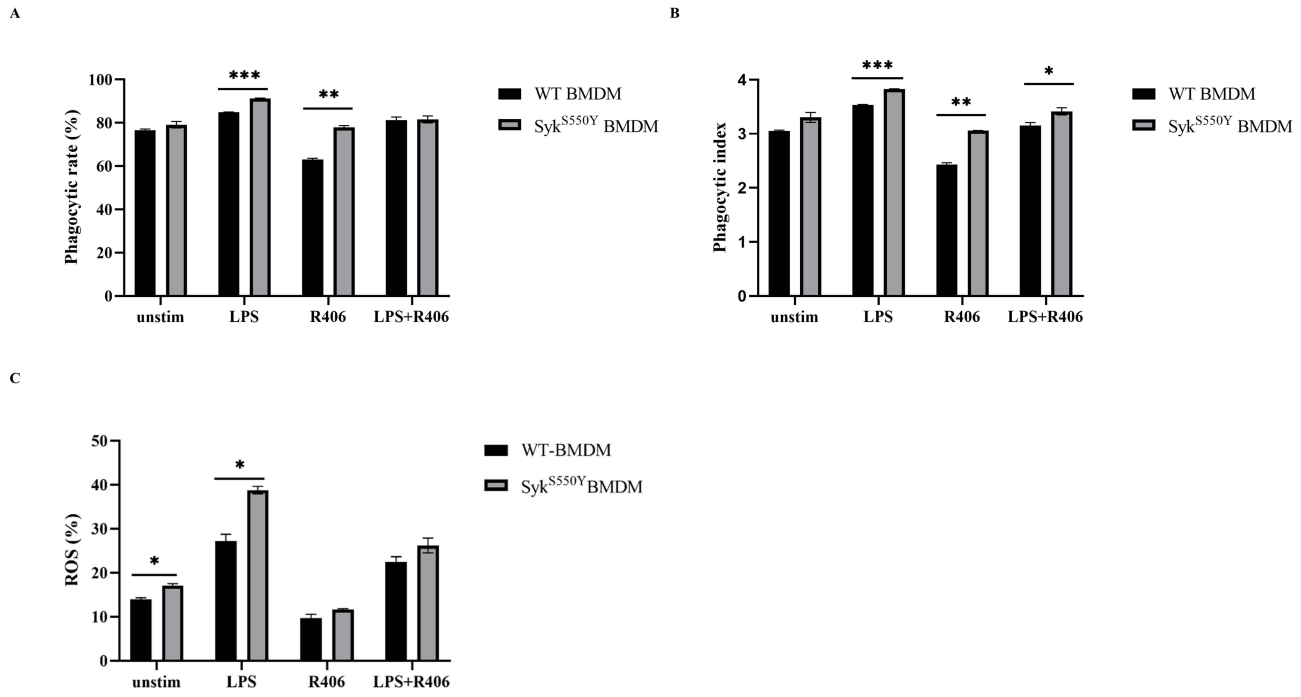

Figure 5 Gain-of-function variant in *SYK* promoted phagocytic function and ROS production of Macrophages. WT and Syk^{S544Y} BMDM (8×10^5) were treated with or without 200 ng/mL LPS overnight for phagocytosis or treated for 3 h for ROS detection in the presence or absence of 2 uM R406. (**A** and **B**) Phagocytosis rate and index of BMDM using fluorescent microspheres with a diameter of 2 μm in different groups (n=4/group). (**C**) ROS production in macrophages measured via DCFH-DA (n=4/group). Data are presented as mean values ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Abbreviations: ROS, reactive oxygen species; BMDM, bone marrow-derived macrophages; LPS, Lipopolysaccharides; DCFH-DA, 2,7-dichloride-hydro fluorescein diacetate; SEM, standard error of the mean.

function variants promote Syk autophosphorylation and activate downstream NF-κB and MAPK signaling pathways, leading to immune dysregulation and systematic inflammation in humans and mice.[15] In line with previous studies, we discovered that compared with WT mice, Syk^{S544Y} mice manifested with more severe symptoms and activated NF-κB and MAPK signaling pathway in colon tissues after acute colitis induction. A reduced survival rate in Syk^{S544Y} mice following chronic colitis induction further supports the role of Syk in exacerbating intestinal inflammation. These results suggest that constitutive phosphorylation of Syk aggravates DSS- or DNBS-induced colitis, aligning with our previous findings that gain-of-function variants in *SYK* cause multi-organ inflammatory diseases, including colitis.

The role of Syk in IBD and other intestinal diseases has primarily focused on signal transduction and cytokine production in intestinal epithelial and innate immune cells, including monocytes/macrophages, dendritic cells, mast cells and B cells.[32–34] Consistent with the higher macrophage infiltration in the affected joints of Syk^{S544Y} mice,[15] we discovered that Syk^{S544Y} mice with induced acute colitis had increased macrophage infiltration, especially pro-inflammatory Ly6C^{hi}CX3CR1^{int} macrophages in colonic tissue. Gain-of-function variants in *SYK* promoted macrophage migration by promoting chemokine expression and responsiveness to chemokines. Additionally, phagocytosis and ROS production were elevated in macrophages from Syk^{S544Y} mice, suggesting that Syk contributes to colitis pathogenesis by altering macrophage function. Notably, the gain-of-function variants in *SYK* identified in patients with IBD were recently found to cause dysregulation of podosomes in macrophages.[35]

The NLRP3 inflammasome plays an important role in the pathogenesis of IBD. For example, the activation of NLRP3 inflammasome was promoted in the colonic mucosa of patients with UC,[36] and the single nucleotide mutation in NLRP3 is associated with CD susceptibility.[37] NLRP3 deficiency or inhibition can alleviate colitis in mice.[38] Moreover, Syk inhibitor can reduce intestinal inflammation by decreasing the expression of pyroptosis-related proteins such as NLRP3, IL-1β, and IL-18.[19] The up-regulation of inflammatory cytokines caused by NLRP3 activation can promote the polarization of macrophages to M1 type.[39] In this study, we discovered that the expression levels of NLRP3 inflammasome-related proteins was significantly increased in colon tissue and BMDM from mice with gain-of-function variant in *SYK*.

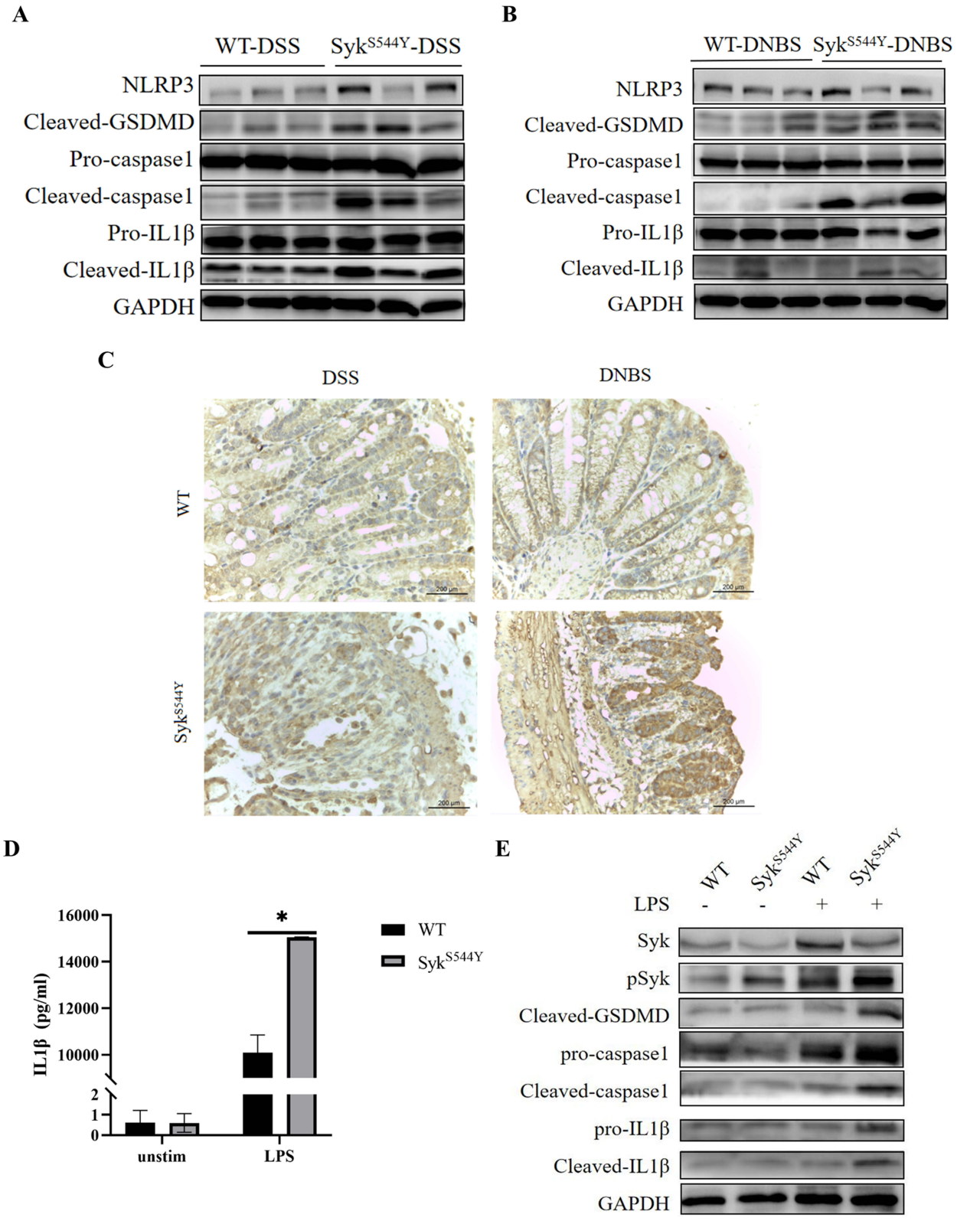

Figure 6 Gain-of-function variant in *SYK* promoted NLRP3 inflammasome activation. (**A** and **B**) Western blot analysis of NLRP3-related proteins in colon tissues from WT and Syk^{S544Y} mice with DSS or DNBS-induced acute colitis (n=3/group). (**C**) Representative images of expression of NLRP3 detected via immunohistochemical staining of colon sections (n=5/group). Scale bar: 200μm. (**D**) ELISA analysis of IL1β in supernatant of BMDM from WT and Syk^{S544Y} mice (n=3/group). BMDM was stimulated with 200 ng/mL LPS for 6 h. (**E**) Western blot analysis of Syk, pSyk (Y525/526), and NLRP3 inflammasome-related proteins in BMDM (n=3/group). Data are presented as mean values ± SEM; *$P < 0.05$.
Abbreviations: DSS, dextran sodium sulphate; DNBS, dinitrobenzene sulfonic acid; BMDM, bone marrow-derived macrophages; LPS, Lipopolysaccharides; SEM, standard error of the mean.

The study had some limitations. First, Syk inhibitor treatment or Syk knock-down/knockout mouse models could not be controlled to further investigate the role of Syk in colitis development in mice; however, phagocytosis and ROS production in BMDM were studied with R406. Second, the significance of gain-of-function variants in *SYK* on macrophage function in the IBD pathogenesis needs further investigation through adoptive transfer of macrophages or by constructing a mouse model with a selective gain-of-function variant in *SYK* gene in macrophages. Finally, the effect of the gain-of-function variant in *SYK* on NLRP3 activation must be confirmed in THP1 cell lines; however, the recombinant adenovirus overexpressing the variant failed to be packaged for undetermined reasons.

Conclusion

Our study demonstrated that a gain-of-function variant in *SYK* increases susceptibility to experimental colitis and contributes to intestinal mucosal inflammation by promoting migration, phagocytosis, and ROS production of macrophages, and NLPR3 activation, supporting the involvement of gain-of-function variants of *SYK* in the pathogenesis of IBD.

Abbreviations

Syk, Spleen tyrosine kinase; IBD, inflammatory bowel diseases; UC, ulcerative colitis; CD, Crohn's disease; SAP-1, stomach-cancer-associated protein tyrosine phosphatase 1; IL, interleukin; NF-κB, nuclear transcription factor-κB; WT, wild-type; NLRP3, nucleotide-binding domain leucine-rich-containing family, pyrin domain-containing-3; MyD88, myeloid differentiation primary response 88; ROS, reactive oxygen species; CCL, chemokine C-C motif ligand; DSS, dextran sodium sulfate; DNBS, dinitrobenzene sulfonic acid; DAI, disease activity index; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA, ribonucleic acid; BMDM, bone marrow-derived macrophages; LPS, lipopolysaccharides; ELISA, enzyme-linked immunosorbent assay.

Data Sharing Statement

All data analyzed in this study are available from the corresponding author at yhuang815@163.com.

Ethics Approval

This study was approved by the Ethics Committee of Children's Hospital of Fudan University, Shanghai, China.

Acknowledgments

The authors express their gratitude to Professor Dali Li for providing the mice.

Funding

This study was supported by National Natural Science Foundation of China (Grant Number: 82101953), Natural Science Foundation of Shanghai Municipality (Grant Number: 21ZR1410300), Shanghai Rising-Star Program (Grant Number: 22QA1401400), and National Key Research and Development Program of China (Grant numbers: 2023YFC2706501).

Disclosure

The authors report no conflict of interest in this work.

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