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Overexpression of Ste20-Related Proline/Alanine-Rich Kinase Exacerbates Experimental Colitis in Mice

Yutao Yan,* Hamed Laroui,* Sarah A. Ingersoll,* Saravanan Ayyadurai,* Moiz Charania,* Stephen Yang,* Guillaume Dalmasso,* Tracy S. Obertone,* Hang Nguyen,* Shanthi V. Sitaraman,* and Didier Merlin*†

Inflammatory bowel disease, mainly Crohn’s disease and ulcerative colitis, are characterized by epithelial barrier disruption and altered immune regulation. Colonic Ste20-like proline/alanine-rich kinase (SPAK) plays a role in intestinal inflammation, but its underlying mechanisms need to be defined. Both SPAK-transfected Caco2-BBE cells and villin-SPAK transgenic (TG) FVB/6 mice exhibited loss of intestinal barrier function. Further studies demonstrated that SPAK significantly increased paracellular intestinal permeability to FITC-dextran. In vivo studies using the mouse models of colitis induced by dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid showed that TG FVB/6 mice were more susceptible to DSS and trinitrobenzene sulfonic acid treatment than wild-type FVB/6 mice, as demonstrated by clinical and histological characteristics and enzymatic activities. Consistent with this notion, we found that SPAK increased intestinal epithelial permeability, which likely facilitated the production of inflammatory cytokines in vitro and in vivo, aggravated bacterial translocation in TG mice under DSS treatment, and consequently established a context favorable for the triggering of intestinal inflammation cascades. In conclusion, overexpression of SPAK inhibits maintenance of intestinal mucosal innate immune homeostasis, which makes regulation of SPAK important to attenuate pathological responses in inflammatory bowel disease.

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Materials and Methods

Plasmid construction

SPAK/pC DNA6 was cloned in our laboratory previously (16). SPAK small interfering RNA (siRNA) and scramble siRNA were commercially ob-

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tained from Applied Biosystems (Foster City, CA). IL-1β–Luc promoter was a gift from Jesse Roman (Emory University, Atlanta, GA), and TNF-α–Luc, IL-17–Luc, and IFN-γ–Luc plasmids were purchased from Addgene (Cambridge, MA).

Cell culture

The human intestinal cell line Caco2-BBE between passages 20 and 30 was cultured according to the standard protocol and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Real-time PCR

Total RNA from Caco2-BBE cells and mouse colon mucosa were reverse transcribed using the ThermoScript RT-PCR System (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen, Germantown, MD). Real-time PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) with the iCycler sequence detection system (Bio-Rad) with specific primers (Supplemental Table I, Table I). Real-time PCR data were presented using the 2ΔΔCt method (21) with GAPDH or 36B4 gene levels serving as the internal standard.

Western blotting

All Western blots were performed with appropriate Abs based on standard protocols.

Immunocytochemistry and immunohistochemistry

Immunostaining assays were performed according to the standard protocol with relevant primary Abs (SPAK and mucin 2 Abs [Santa Cruz Biotechnology, CA], ZO-1, ZO-2, occludin, claudin-1, -2, and -4 Abs [Invitrogen], Alexa Fluor 488 secondary Ab [Molecular Probes, Carlsbad, CA], and rhodamine/phalloidin [Molecular Probes]) as described previously (22) to visualize actin. Samples were mounted in Prolong Gold Antifade Reagent (Invitrogen) and analyzed by Zeiss Axioskop 2 Plus Microscope (Carl Zeiss Microimaging, Thornwood, NY).

In vitro and ex vivo transepithelial resistance assay

As transepithelial barrier dysfunction is necessary for the development of intestinal inflammation, we studied the effects of SPAK expression on intestinal barrier function by in vitro and ex vivo experiments. For in vitro assay, Caco2-BBE cells grew confluent on snap well filters (Corning Costar, Corning, NY); relative transepithelial resistance (TER) was measured with Ussing chambers (Physiologic Instruments, San Diego, CA). Ex vivo TER assay with distal colonic mucosa was obtained by blunt stripping from muscularis, and serosa was the same as the in vitro TER assay.

In vitro and in vivo permeability assays

In vitro and in vivo permeability assays were performed using an FITC-labeled dextran method to assess barrier function. For in vitro permeability assay, confluent and polarized Caco2-BBE cells grown on filters were treated with FITC-labeled dextrans (4 kDa; Sigma-Aldrich) on the upper chamber for 2 h at 37°C. The medium in the lower chamber was collected. In vivo permeability assays were performed as described previously (20). Briefly, food and water were withdrawn from the mice for 4 h and then gavaged with permeability tracer FITC-labeled dextrans (60 mg/100 g body weight; Sigma-Aldrich). Serum was collected retro-orbitally 4 h after gavage; fluorescence intensity of each sample was measured (485 excitation/520 emission; Cytofluor 2300; Millipore, Billerica, MA; Waters Chromatography), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran.

In vitro and ex vivo ion selectivity assays

The ion selectivity of tight junctions was determined by measurement of dilution potentials (Caco2-BBE monolayer) or short-circuit current (mouse colonic mucosa) by replacing either the apical or basolateral solution while keeping the other side (basolateral or apical) bathed in Kreb’s solution. For 2:1 NaCl dilution potentials/short-circuit current, the 128 mM NaCl solution was replaced with 52 mM NaCl in Kreb’s solution, and osmolarity was maintained with mannitol.

SPAK translocation, immunoprecipitation, and kinase assays

Nuclear proteins were extracted from Caco2-BBE cells as described previously (23). Immunoprecipitation was performed using the Catch and Release Reversible Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions. Exogenous substrate phosphorylation assays with myelin basic protein (MBP; Upstate, Charlottesville, VA) as substrate were performed as described previously (16). To confirm these results, the N terminus of SPAK was cloned into the vector PCR11 (Invitrogen) and subjected to TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) to express the N terminus protein in vitro for the kinase assay. All kinase assays were visualized by Western blot with anti–phospho-MBP Ab (Upstate) and anti–phospho-threonine Ab (Sigma-Aldrich).

Mouse model

FVB/6 mice (8–10 wk, 18–22 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). In collaboration with the Transgenic Mouse and Gene Targeting Core Facility (Emory University), we established the SPAK TG mouse model in FVB/6 mice. All animal experiments were approved by The Institutional Animal Care and Use Committee of Emory University and in accordance with the guide for the Care and Use of Laboratory Animals published by the U.S. Public Health Service.

Induction and assessment of colitis

Colitis was induced by the addition of 3.5% (w/v) dextran sodium sulfate (DSS) (m.w. 50,000; ICN Biochemicals, Aurora, OH) to the drinking water or by colonic injection of 150 mg/kg body weight of trinitrobenzene sulfonic acid (TNBS; Sigma-Aldrich) dissolved in 50% ethanol. Colitis was assessed 8 d after DSS treatment or 48 h after TNBS administration as described previously (24) (n = 6 mice/group). Direct visualization of the colon was performed using the Coloview system (Karl Storz Veterinary Endoscopy, Culver City, CA). Neutrophil infiltration into the colon was quantified by measurement of myeloperoxidase (MPO) activity, as described previously (17). To study the effects of SPAK on the healing phase of intestinal inflammation, we monitored the survival status of the mice for another week after DSS/TNBS withdrawal.

Array analysis

Microarray hybridizations were performed in collaboration with Dr. Andrew Neish (Emory University) using the Vanderbilt Microarray Shared Resource Human 30k Oligonucleotide Microarray Chip. To confirm and narrow the expression gene targets regulated by SPAK, we used the human inflammatory cytokines and receptor RT2 Profiler PCR array (SA Biosciences, Frederick, MD) with the iCycler sequence detection system (Bio-Rad). To minimize variability, the mean of three independent experiments for each gene was calculated and used for final data clustering. Only genes...
that showed significant change (>2-fold difference) were selected for further characterization. DNASTAR ArrayStar 2 analytic software packages were used for scatter plotting.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays identifying binding sites of the proinflammatory cytokines TNF-α, IL-1β, IL-17, and IFN-γ were performed using a ChIP assay kit (Upstate) according to the manufacturer’s instructions using the primers listed in Table I.

**Transactivation assay**

The transactivation assays were performed as described previously with minor modifications (24). Renilla (phRL-CMV; 5 ng), cytokine Luciferase promoter constructs (4 μg), and SPANK/pcDNA6 (4 μg) were cotransfected into Caco2-BBE cells with Lipofectamine 2000 (Invitrogen). After 48 h, cells were collected with the Dual Luciferase Reporter Assay System (Promega), and the luminescence was measured in a luminometer (Luminoskan; Thermal Labsystems). Luciferase activity was normalized based on the control renilla luciferase activity. Extracts were analyzed in triplicate, and each experiment was performed at least three times.

**Bacterial translocation assay**

CFU in freshly isolated small intestine, colon, spleen, and liver tissues were determined via homogenization of material in PBS/0.01% Triton X-100 followed by serial dilution plating on nonselective Luria-Bertani agar as described previously (25). In parallel, DNA was prepared with the Wizard SV Genomic DNA Purification System (Promega) from three mice of each group. DNA was analyzed by PCR for 20 cycles with universal primers directed against a region of the 16S rRNA gene common to most bacteria: forward, 5'-CCATGAAGTCGGAATCGCTAG-3' and reverse, 5'-ACTC-CCATGGTGTGACGG-3' (bp 1302–1394 in bacteria EU622773). The PCR products were analyzed by electrophoresis.

**Fluorescence in situ hybridization**

The fluorescence in situ hybridization (FISH) assay was performed using an Alexa Fluor 555-conjugated EUB (bp 337–354); the NON-EUB–Alexa Fluor 555 probe was used as a negative control.

**Statistical analysis**

Values are expressed as mean ± SEM with unpaired two-tailed Student t test by InStat v3.06 (GraphPad, San Diego, CA) software. A p value < 0.05 was considered statistically significant.

**Results**

**Colonic SPANK reduces TER in vitro**

Using real-time PCR and Western blot analyses, we found significant increases in SPANK expression in SPANK/pcDNA6-transfected cells and significant decreases of SPANK expression in siRNA-transfected cells at both mRNA and protein levels in comparison with controls (Fig. 1A,1B, Table I). Furthermore, immunostaining showed increased SPANK expression in both cytosolic and nuclear pools (Fig. 1C). We have previously shown that SPANK expression was increased in the colonic mucosa of CD (20) and UC (17) patients and DSS colitic mice (16). Also, SPANK synthesis was increased in IECs treated with proinflammatory signals such as TNF-α (17) or hyperosmolarity (20). Therefore, it is of interest to study the involvement of SPANK in epithelial barrier...
function. As shown in Fig. 1D, overexpression of SPAK significantly reduced TER to 69.6 ± 14.3 ohms.cm² (SPAK-transfected Caco2-BBE cells) from 132.1 ± 20.1 ohms.cm² in vector-transfected cells. Knockdown of SPAK expression significantly increased TER from 119.9 ± 23.6 ohms.cm² to 234.9 ± 31.0 ohms.cm². In addition, we found that Caco2-BBE cells over-

FIGURE 2. SPAK TG mice display loss of intestinal barrier function. SPAK expression is enhanced in colonic mucosa in TG mice by real-time PCR (A), Western blot (B), and immunofluorescence (C) (SPAK, green; β-actin, red). D, TER assay with Ussing chamber (Physiologic Instruments) in mouse colonic mucosa. TG mice have lower TER compared with WT mice in ex vivo experiments. E, WT and TG mice were starved for 4 h and then gavaged with FITC-dextran (4 kDa). Serum was collected retro-orbitally 4 h after gavage, fluorescence intensity of each sample was measured, and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. F, In 2:1 NaCl short-circuit current assay in mouse colonic mucosa, the 128 mM NaCl solution was replaced with 52 mM NaCl in Kreb’s solution, and osmolarity was maintained with mannitol. Transepithelial current was monitored and recorded at 10-s intervals, with the voltage continuously clamped at zero. Data are expressed as means ± SEM (n = 9 mice per group). Statistical differences of TG versus WT mice are reported. Data are pooled from three independent experiments. *p < 0.05, **p < 0.01.

FIGURE 3. SPAK TG mice were more susceptible to DSS- and TNBS-induced mouse colitis. A, Phenotypic assay of WT and TG mice with 3.5% DSS treatment for 10 d or with 150 mg/kg body weight of TNBS treatment for 48 h. B, Body weight measurement in WT and TG mice with 3.5% DSS treatment for 10 d or with 150 mg/kg body weight of TNBS treatment for 48 h. C, Colonic phenotypic assay in WT and TG mice with 3.5% DSS treatment for 10 d or with 150 mg/kg body weight of TNBS treatment for 48 h. D, Measurement of mouse colon length in WT and TG mice with 3.5% DSS treatment for 10 d or with 150 mg/kg body weight of TNBS treatment for 48 h. Data are expressed as means ± SEM (n = 4 mice per group). Statistical differences of TG versus WT mice are reported. Data are pooled from three independent experiments. *p < 0.05, **p < 0.01.
expressing SPAK did not demonstrate increased levels of apoptosis (data not shown), indicating the increase of intestinal permeability by SPAK expression is not due to apoptosis.

The total TER is represented by two resistances in parallel: the transcellular resistance ($R_{tc}$) and the paracellular resistance ($R_p$). The paracellular pathway is formed mainly by the resistance of the tight junction ($R_t$). In the circuit model, TER is represented by: $1/TER = (1/R_{tc}) + (1/R_t)$. This value of TER across a leaky epithelium, such as that of the intestine, reflects principally the resistance afforded by $R_t$. Because TER is determined by tight junction size and ion selectivity (2, 26, 27), the reduction in TER caused by SPAK may be attributable to alteration of tight junction size or ion selectivity. To further investigate this hypothesis, we examined transepithelial permeability using the 4-kDa FITC-dextran method. As shown in Fig. 1E, vector-transfected cells showed a FITC-dextran flux (ng/ml/min) of $12.1 \pm 2.6$. In comparison, an $\sim$2-fold increase in FITC-dextran flux was observed in SPAK-transfected cells ($23.8 \pm 4.03$), with no significant change in scrambled siRNA-transfected cells ($13.9 \pm 3.2$). In addition, an $\sim$3.2-fold decrease in SPAK siRNA-transfected cells ($4.3 \pm 1.3$) was observed. To further evaluate the effects of SPAK on IEC barrier function, ion selectivity was first examined in vitro. As shown in Fig. 1F, the dilution potential (mV) did not differ on both the apical side (vector: $-6.2 \pm 1.03$; SPAK: $-5.7 \pm 0.85$; siRNA: $-6.4 \pm 1.93$; scrambled siRNA: $-5.64 \pm 1.33$) and basolateral side (vector: $4.2 \pm 1.29$; SPAK: $3.6 \pm 1.58$; siRNA: $3.9 \pm 0.48$; scrambled siRNA: $3.96 \pm 0.97$).

**FIGURE 4.** SPAK TG mice exhibit aggravated inflammation. A, Representative photomicrographs of paraffin-embedded, hematoxylin-stained sections of the distal colon. Original magnification ×10 (upper panels) and ×20 (lower panels). B, Intestinal inflammation was evaluated macroscopically in vivo using a murine miniature endoscope. Representative images of six different mice are shown. C, WT and TG mice were given water or 3.5% DSS for 10 d or 100 mg/kg body weight of TNBS for 48 h as described in Fig. 3, and distal colon tissue was collected and subjected for MPO activity measurement. Data are expressed as means ± SEM ($n$ = 9 mice per group). Statistical analysis was performed using an unpaired two-tailed Student t test. D, After 10 d of 3.5% DSS or 48 h of TNBS, mice were given tap water and followed for mortality during recovery phase. *$p < 0.05$, **$p < 0.01$. Colonic SPAK reduces TER ex vivo

To investigate whether SPAK operates as a barrier function regulator in vivo, we generated villin-SPAK TG mice, which target SPAK overexpression to IECs. SPAK TG mice had a 7-fold increase of mRNA transcripts (Fig. 2A, Table I) and a marked increase of SPAK protein expression (Fig. 2B) in colonic mucosa compared with wild-type (WT) mice. Immunostaining (Fig. 2C) of colon sections detected SPAK mainly in the muscularis, muscularis mucosa, and epithelial layers of WT mice. However, in comparison with WT mice, TG mice showed increased SPAK expression only in the epithelium, including different cell lineages, such as absorptive enterocytes, enteroendocrine cells, and goblet cells. Goblet cells are the principal cell lineage to express SPAK in TG mice. A similar degree of expression in the muscularis and muscularis mucosa layers was observed. In ex vivo assays, the mucosa of SPAK TG mice exhibited a TER (214.5 ± 43.2 ohms.cm$^2$) that was significantly lower than that of WT mice (361.9 ± 18.3 ohms.cm$^2$) (Fig. 2D). These data indicate that SPAK affects colonic TER ex vivo. In vivo size selectivity assays using 4 kDa FITC-dextran yielded similar results as in vitro experiments. WT mice had a flux level of 86.5 ± 21.0 ng/mg protein. In comparison, an increased flux (≥2-fold) was observed in TG mice (180.3 ± 40.4 ng/mg protein). These results indicated that SPAK increased transepithelial permeability in vivo. But in ex vivo experiments, the transepithelial current (mA) did not differ significantly between SPAK TG and
WT colon mucosa (Fig. 2F) on either the apical (−10.63 ± 4.54 versus −10.01 ± 2.05) or basolateral side (5.47 ± 2.36 versus 5.12 ± 1.22).

**SPAK overexpression deteriorates experimental colitis**

We then examined clinical and histological changes from phenotypic and pathologic perspectives (28). TG mice showed a marked increase in diarrhea and more serious rectal bleeding (Fig. 3A) than WT mice during DSS treatment, body weight decreased significantly in both TG and WT mice, but weight loss was more severe in TG mice (20 versus 6%) (Fig. 3B). In addition, DSS caused a significant reduction in colon length, as shown in Fig. 3C and 3D, which was more severe in TG mice compared with WT mice. The body weight loss and colon length reduction in the TNBS colitis model are consistent with the DSS colitis model (Fig. 3).

Histological staining (Fig. 4A) showed an intact epithelium, a well-defined crypt length, no edema, no neutrophil infiltration into the mucosa or submucosa, and no ulcers or erosions in untreated WT mice. Untreated TG mouse colon sections (Fig. 4A) exhibited an intact epithelium with a shorter crypt length, a thicker submucosa or lamina propria, noticeable edema, slight neutrophil infiltration into the mucosa, and no ulcers or erosions. Colon tissue from DSS-treated WT mice had extensive inflammatory lesions throughout the mucosa (Fig. 4A); both ulcers and shortening/loss of crypts were focally apparent. In TG mice treated with DSS, ulcers and shortening and loss of crypts progressed to more extensive areas of mucosa; submucosal edema became more severe with DSS treatment, and a large infiltration of immune cells was seen in DSS-treated TG mice. Similar histological characteristics were noticed in the TNBS colitis mouse model (Fig. 4A). Colonscopic analysis demonstrated that both WT and TG animals showed no evidence of macroscopic inflammation, displaying a semitranslucent mucosa characteristic of a healthy colon (Fig. 4B, left two panels). However, both DSS and TNBS induced a rapid and progressive severe, ulcerating, colonic inflammation with bloody diarrhea in both WT and TG mice (Fig. 4B, right two panels). Furthermore, TG mice exhibited more extensive intestinal inflammation, including increased colonic inflammation with prominent mucosal edema and spontaneous bleeding compared with WT mice.

MPO activity was measured as an indicator of tissue damage and the extent of infiltration by inflammatory cells. No significant MPO changes were noticed between untreated WT (0.06 ± 0.03 mUnits/µg protein) and untreated TG mice (0.095 ± 0.05 mUnits/µg protein) (Fig. 4C). MPO values increased in DSS-treated mice compared with controls, but DSS-treated TG mice showed significantly higher MPO values (0.67 ± 0.167 mUnits/µg protein).

**FIGURE 5.** SPAK facilitates the production of proinflammatory cytokines. A, Microarray hybridizations using the Affymetrix GeneChip (Affymetrix) exhibited alteration of expression of hundreds of different genes in human IECs. The mean of three independent experiments for each gene was calculated and used for data clustering. Only genes that showed significant change (>2-fold difference) were selected for further characterization. DNASTAR ArrayStar 2 analytic software packages were used for scatter plotting. B, PCR array of cytokines demonstrated increase of proinflammatory cytokines, including TNF-α, IL-1β, and IL-17 in human IECs. C, Real-time PCR was performed in WT and TG mice for proinflammatory cytokines, including TNF-α, IL-1β, and IL-17, with or without DSS treatment. IFN-γ acted as a negative control. Data are expressed as means ± SEM (n = 9 mice per group). Statistical differences of TG versus WT mice are reported. *p < 0.05, **p < 0.01.
than DSS-treated WT mice (0.27 ± 0.095 mUnits/μg protein), indicating more neutrophil infiltration into the mucosa and submucosa of TG mice than WT mice. Fig. 4D shows the survival curves of WT and TG mice after DSS and TNBS treatment; 38% of TG mice died during this period, whereas no WT mice succumbed, indicating a higher mortality of TG mice during recovery. The pattern of MPO activities and the pattern of recovery were also seen in TNBS-induced colitis (Fig. 4C, 4D).

**SPAK is involved in production of inflammatory cytokines**

Microarray analysis showed that SPAK changed the expression of 631 genes >2-fold (Fig. 5A), and 35 of these 631 genes have increased expression by >4-fold in comparison with controls, including some proinflammatory cytokines, such as TNF-α, IL-1β, and IL-17. These data have been deposited in the Gene Expression Omnibus with the accession number GSE25641 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25641). Given that proinflammatory cytokines play central roles in the pathogenesis of IBD, we confirmed these results using a PCR array (Fig. 5B). The white solid squares (A, B, and C) represent cytokines TNF-α, IL-1β, and IL-17, respectively. We then performed real-time PCR with specific primers (Table I) to monitor the levels of proinflammatory cytokines in mouse colon tissue. As shown in Fig. 5C, the levels of IL-1β (a 6.4-fold increase), TNF-α (a 4.8-fold increase), and IL-17 (a 5.3-fold increase) in TG animals were significantly higher than seen in WT mice. But we did not find any of these four inflammatory cytokines detectable by ELISA, which suggests any effect of SPAK on the intestinal barrier in vitro is not mediated by cytokine secretion. No significant difference in IFN-γ level was observed between TG and WT mice. After DSS treatment, production of proinflammatory cytokines was significantly increased in both WT and TG mice. The IL-1β transcripts showed a 17.5-fold increase in WT mice and a 20.6-fold increase in TG mice; the TNF-α transcripts increased 7.6-fold in WT mice and 18.8-fold in TG mice; the IL-17 transcripts showed a 2.6-fold increase in WT mice and a 13.7-fold increase in TG mice; and the IFN-γ transcripts increased 11.2-fold in WT mice and 11.8-fold in TG mice.

**SPAK N terminus can undergo translocation into the nucleus and retain kinase activity**

Because SPAK increases the production of cytokines TNF-α, IL-1β, and IL-17, we were prompted to study the underlying mechanism. First, we found that N terminus of SPAK expressed by the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) can phosphorylate the substrate MBP and autophosphorylate itself (Fig. 6A), which indicates that the N terminus of SPAK maintains its kinase activity in vitro. Next, we studied the kinase activity of SPAK in the cell nucleus. Immunoprecipitation by Xpress Ab, kinase assays, and Western blot analyses showed (Fig. 6B) that the N-terminal catalytic domain underwent translocation to the nucleus and retained kinase activity by phosphorylating MBP and autophosphorylation.

**SPAK may associate with and transactivate cytokine genes**

To confirm the importance of SPAK in the increased production of TNF-α, IL-1β, and IL-17 in vivo, we performed ChIP analyses with specific primers (Table I). As shown in Fig. 6C, under resting conditions, SPAK involvement in the production of proinflammatory cytokines: vector-transfected Caco2-BBE cells (1); and SPAK-transfected Caco2-BBE cells (2). D, Transient transfection of different constructs into Caco2-BBE cells and transactivation assays of SPAK and the genes related to cytokines TNF-α, IL-1β, and IL-17. The results are representative of three independent experiments performed in triplicate, and error bars represent SD analyzed by Student t test by InStat v3.06 (GraphPad) software. *p < 0.05, **p < 0.01.
conditions, SPAK was associated with genes encoding TNF-α, IL-1β, and IL-17, but not with the IFN-γ gene. Overexpression of SPAK increased the association of the SPAK protein with these genes. To further study the linkage between enhanced proinflammatory cytokine production and SPAK expression, we investigated whether SPAK could stimulate transactivation of target cytokines. The results (Fig. 6D) indicate that transfection of cytokine reporters leads to increased basal level reporter activity levels of TNF-α (20-fold), IL-1β (7-fold), and IL-17 (3-fold) compared with empty vector. However, cotransfection of SPAK and cytokine reporter genes results in a marked increased luciferase activity for TNF-α (38-fold), IL-1β (17-fold), and IL-17 (11-fold) compared with cotransfection of control vectors. We used the IFN-γ reporter as a control. These results indicate that SPAK is able to trigger transactivation of certain proinflammatory cytokines including TNF-α, IL-1β, and IL-17.

**SPAK aggravates commensal bacterial translocation in DSS-induced colitis**

The imbalance between innate and adaptive immunity caused by luminal bacteria is thought to be a main contributor to the onset of IBD (29). We examined the translocation of luminal bacteria in TG and WT mice. First, no significant difference in CFU levels was seen in any organs tested in TG and WT mice. However, CFU levels increased markedly in all DSS-treated mice, and TG animals showed significantly higher CFU levels than WT in all organs tested (Fig. 7A). PCR analysis using primers directed toward highly conserved bacterial 16S rRNA sequences confirmed these results. Twenty cycles of amplification did not generate visible products in either untreated WT or TG mice; however, DSS-treated samples yielded strong DNA bands in both WT and TG mice. Furthermore, TG mice exhibited significantly more intense product bands than WT mice (Fig. 7B). We then performed FISH and immunostaining on the same slides to visualize translocated bacteria (Fig. 7C). We obtained results similar to those shown by CFU analysis, with no noticeable bacterial translocation in either untreated TG or WT mice. Bacterial translocation was apparent upon DSS treatment in both TG and WT mice and significantly higher in TG mice.

**Discussion**

In the current study, we have established for the first time, to our knowledge, a colonic SPAK TG mouse model to highlight the importance of SPAK in the regulation of intestinal barrier function, which describes substantial new mechanistic insights into the pathogenesis of IBD.

Unlike other experimental colitis models with TG IL-17 (30), STAT-4 (31), HLA B27 (32), or Nod2 (33), which are mainly involved in the imbalance of innate and adaptive immune systems, SPAK TG mice primarily cause collapse of barrier function by increasing epithelial permeability, then enhance production of inflammatory cytokines and translocation of luminal bacteria. Similar observations have been reported in junctional adhesion molecule knockout mice, which demonstrated increased mucosal permeability with enhanced expression of claudin-10, claudin-15, and inflammatory cytokines in colonic mucosa (34, 35). Additionally, myosin L chain kinase TG mice demonstrated significant barrier loss and accelerated onset and severity of immune-mediated colitis (36). The tight junction, unlike the epithelial cells themselves, forms a selectively dynamic permeable barrier with the ability to alter its permeability in response to extracellular stimuli. The sustained enhancement of paracellular permeability under certain circumstances may result in an uncontrolled leaky tight junction barrier, facilitating the constant passage of luminal bacteria.
pathogens and/or Ags through the mucosa and leading to inflammation in susceptible individuals (37). In fact, increased intestinal permeability is implicated in the pathogenesis of CD and UC as an initiating factor, which leads to a secondary mucosal inflammatory response (3–6). The SPAK TG mouse model mimics the natural pathogenesis of IBD, primarily affecting the intestinal barrier function, rendering IECs more susceptible to stress factors.

Mechanisms underlying the intestinal barrier dysfunction caused by SPAK are complicated. We found that epithelial barrier dysfunction is the primary effect of SPAK, which then leads to the increased production of inflammatory cytokines (Supplemental Figs. 1–3). The increased expression of inflammatory cytokines could in turn cause intestinal barrier function collapse, and these cytokines are of great importance not only in the pathogenesis of colitis, but also as intervention targets against colitis (9, 38).

Another possible mechanism is that SPAK facilitates the translocation of luminal bacteria into mucosa after DSS treatment. It has been accepted that luminal bacteria are involved in innate immune homeostasis and the intestinal barrier function (39). We also observed that ZO-1, ZO-2, claudin-1, -2, and -4 were expressed at the same levels in SPAK TG and WT animals (Supplemental Figs. 4, 5, 7–9, Supplemental Table 1). In contrast, the level of occludin expression (Supplemental Fig. 6, Supplemental Table 1) was significantly lower in SPAK TG mice compared with WT littermates. These results suggest that the lower level of occludin observed in SPAK TG animals could be responsible for the increase in paracellular flux of small m.w. tracers, thus modulating the overall intestinal barrier defect observed in SPAK TG mice. These results are in agreement with studies showing that occludin affected both tight junction size selectivity and electrical resistance (8, 40–42). The studies underlying the involvement of occludin in SPAK overexpression-induced barrier defects are ongoing. In addition, it is known the cytoskeleton factors actin and myosin (43) play important roles in the regulation of intestinal barrier function. Some stress factors, such as hyperosmolarity, which regulate the expression and activity of SPAK, can modulate intestinal barrier function by modulating redistribution of actin and phosphorylation of the myosin L chain (43, 44). Because we did not investigate in vivo ion selectivity, we thus cannot draw any definitive conclusions on the in vivo ion selectivity of tight junctions. It is known that ion selectivity of tight junctions plays an important role in the integrity of epithelial cells, thus in TER (27, 40). The claudin tight junction protein family plays an important role in the regulation of ion selectivity to mediate TER (45–47). IFN-γ and IL-1 regulate TER not only through size selectivity but also ion selectivity by regulating claudins (48, 49). TNF-α increases size selectivity without altering ion selectivity, whereas IL-13 alters ion selectivity without affecting size selectivity (50). In this study, we did not see significant differences in the expression of claudin-1, -2, or -4 (Supplemental Figs. 7–9, Supplemental Table 1); however, more studies are needed to determine the claudin expression profile during the alteration of SPAK expression. Also, our data indicate that barrier function loss caused by SPAK does not result from reduced cell viability and increased cytoxicity, decreased proliferation, or increased apoptosis (data not shown), which is consistent with reports that TNF-α causes epithelial dysfunction in an apoptosis-independent manner (51).

Tremendous evidence shows that kinases are involved in the production of proinflammatory cytokines. For example, p38 regulates the biosynthesis of proinflammatory cytokine IL-1β (52); MAPK 6 can stimulate the production of cytokine IL-6 (53); and MAPK-activated protein kinase 2 regulates TNF-α expression (54). Protein kinase Cδ is involved in the expression of IL-2, IFN-γ, IL-6, and IL-17 (55–58). Interestingly, SPAK is the first Ste20-like kinase, to our knowledge, to stimulate the production of the proinflammatory cytokines IL-1β, TNF-α, and IL-17. The underlying mechanisms involved in regulating the expression of proinflammatory cytokines by kinases could be at the transcriptional level. For example, calmodulin-dependent kinase II regulates expression of GM-CSF by phosphorylating transcription factor Ets1 (59). The data from our ChIP and transcriptional assays demonstrate that SPAK could be associated with the IL-1β, TNF-α, and IL-17 genes, either directly or indirectly. However, we cannot exclude the possibility that SPAK phosphorylates another protein, perhaps an enzyme, kinase, or transcription factor, which in turn stimulates production of IL-1β, TNF-α, and IL-17.

In summary, SPAK regulates IEC barrier function in vitro and in vivo by modulating the size selectivity of tight junctions. Furthermore, SPAK dramatically facilitates the secretion of proinflammatory cytokines and luminal bacterial translocation in SPAK TG mice in the presence of DSS or TNBS. We believe that SPAK establishes a context favorable for the triggering of intestinal inflammation cascades induced by DSS and TNBS. In conclusion, SPAK TG mice are more susceptible to induced colitis due to deregulated intestinal mucosal innate immune homeostasis.

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Disclosures
The authors have no financial conflicts of interest.

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