博士学位論文

Leucine-rich repeat kinase 2 は
重症急性膵炎の発症を促進する

近畿大学大学院
医学研究科医学系専攻

大塚康生

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Doctoral Dissertation

Leucine-rich repeat kinase 2 promotes the development of experimental severe acute pancreatitis

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Research Article

Leucine-rich repeat kinase 2 promotes the development of experimental severe acute pancreatitis

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Abstract

Translocation of gut bacteria into the pancreas promotes the development of severe acute pancreatitis (SAP). Recent clinical studies have also highlighted the association between fungal infections and SAP. The sensing of gut bacteria by pattern recognition receptors promotes the development of SAP via the production of proinflammatory cytokines; however, the mechanism by which gut fungi mediate SAP remains largely unknown. Leucine-rich repeat kinase 2 (LRRK2) is a multifunctional protein that regulates innate immunity against fungi via Dectin-1 activation. Here, we investigated the role of LRRK2 in SAP development and observed that administration of LRRK2 inhibitors attenuated SAP development. The degree of SAP was greater in *Lrrk2* transgenic (Tg) mice than in control mice and was accompanied by an increased production of nuclear factor-kappaB-dependent proinflammatory cytokines. Ablation of the fungal mycobiome by anti-fungal drugs inhibited SAP development in *Lrrk2* Tg mice, whereas the degree of SAP was comparable in *Lrrk2* Tg mice with or without gut sterilization by a broad range of antibiotics. Pancreatic mononuclear cells from *Lrrk2* Tg mice produced large amounts of IL-6 and TNF-α upon stimulation with Dectin-1 ligands, and inhibition of the Dectin-1 pathway by a spleen tyrosine kinase inhibitor protected *Lrrk2* Tg mice from SAP. These data indicate that LRRK2 activation is involved in the development of SAP through proinflammatory cytokine responses upon fungal exposure.

Keywords: LRRK2, dectin-1, acute pancreatitis, fungus, cytokines

Abbreviations: ABPC: ampicillin; BMT: bone marrow transplantation; DC: dendritic cell; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocyanate; H&E: hematoxylin and eosin; IP: intraperitoneal; ITS1: internal transcribed spacer 1; LRRK2: leucine-rich repeat kinase 2; KM: kanamycin; LPS: lipopolysaccharide; MNZ: metronidazole; NEO: neomycin; NF-κB: nuclear factor-kappaB; NOD1: nucleotide-binding oligomerization domain 1; PAM: Pam_3CSK4; PCoA: Principal coordinate analysis; PE: phycoerythrin; PMNC: pancreatic mononuclear cell; SAP: severe acute pancreatitis; STAT3: signal transducer and activator of transcription 3; Syk: spleen tyrosine kinase; Tg: transgenic; TLR4: toll-like receptor 4; ZymD: zymosan depleted.

Introduction

Acute pancreatitis is one of the most common emerging gastrointestinal diseases, often requiring urgent admission to the hospital [1, 2]. Although most cases of acute pancreatitis are self-limiting, approximately 20% of patients with acute pancreatitis develop severe acute pancreatitis (SAP) accompanied by necrosis of the pancreas and/or multiple organ failure. Intestinal barrier dysfunction following the onset of acute pancreatitis could increase the risk for SAP development [3]. Moreover, bacterial infection in the necrotic pancreatic tissue is one of the major causes of morbidity and mortality in SAP [4, 5]. Recent studies have highlighted the importance of both bacterial and fungal infections in SAP development [6–9]. Rasch et al. reported that candida-infected pancreatic necrosis was found in approximately 40% of necrotizing acute pancreatitis cases and was associated with mortality [7]. Furthermore, multiple organ failure has been implicated as an indicator of prophylactic anti-fungal therapy in acute pancreatitis [9, 10]. Thus, fungal infections in patients with SAP are increasingly recognized as possible risk factors for increased lethality associated with SAP. However, the molecular mechanisms underlying the development of SAP by fungal infections remain poorly understood.

Intrapancreatic activation of trypsinogen followed by autodigestion of pancreatic tissue has been shown to underlie the pathogenesis of acute pancreatitis [3, 11]. This mechanism is supported by human studies in which gene mutations involved in the activation of trypsinogen increased patient sensitivity to pancreatitis [12]. Gain-of-function mutations in *PRSS1*, encoding trypsinogen, or loss-of-function mutations in *serine protease inhibitor Kazal type I*, encoding pancreatic secretory trypsin inhibitor, are associated with hereditary pancreatitis [12]. However, mice deficient in the T7 isoform of trypsinogen also experienced experimental acute and chronic pancreatitis even in the absence of trypsinogen [13, 14]. Thus, the pathogenesis of pancreatitis cannot be fully explained by intrapancreatic activation of trypsinogen alone. Recent studies have shown that proinflammatory cytokine and chemokine responses against microorganisms promote the development of experimental acute and chronic pancreatitis [15, 16].

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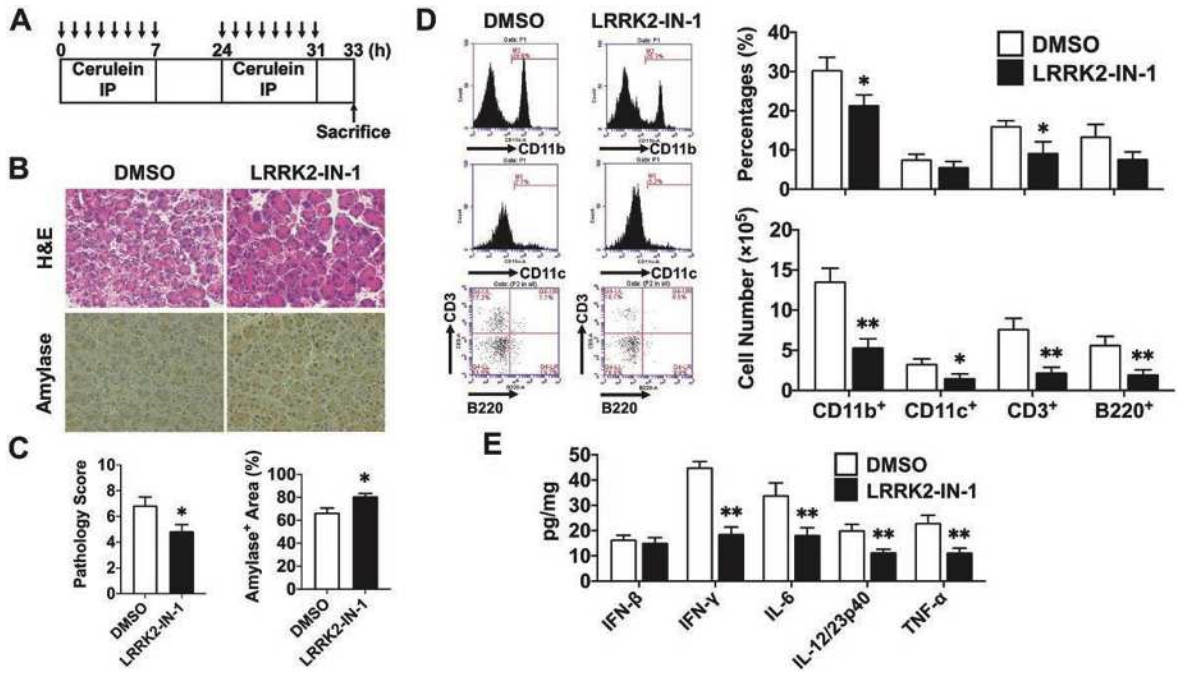

Figure 1. Administration of LRRK2 inhibitor attenuates the development of severe acute pancreatitis. (A) Experimental protocol of severe acute pancreatitis by repeated injections of cerulein. C57BL/6 mice were treated with leucine-rich repeat kinase 2 (LRRK2) inhibitor (LRRK2-IN-1, 0.25 mg, $n = 7$) or DMSO ($n = 7$). Hematoxylin and eosin (H&E) staining and amylase expression of the pancreas. The degree of pancreatitis was lower in C57BL/6 mice treated with LRRK2 inhibitor than those with DMSO. Magnification, ×400 (B). Pathological scores for acute pancreatitis and positive areas for amylase staining (C). (D) Pancreatic accumulation of $CD11b^+$ myeloid cells, $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $B220^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (E) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results shown are combined data obtained from two independent experiments with similar results (DMSO-treated mice; $n = 4$ and $n = 3$, total $n = 7$, LRRK2-IN-1-treated mice; $n = 5$ and $n = 2$, total $n = 7$). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

Intestinal barrier dysfunction followed by the translocation of gut bacteria into the pancreas activates innate immune cells residing in the pancreas to induce proinflammatory cytokine responses [15, 16]. Indeed, mice deficient in Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain 1 (NOD1), both of which recognize components of gut bacteria, are resistant to experimental acute and chronic pancreatitis [15–17]. These studies support the involvement of innate immune responses against gut bacteria in the development of acute pancreatitis. Intestinal barrier dysfunction allows for the translocation of both bacteria and fungi into the pancreas; thus, innate immune responses against fungi could also be associated with the development of pancreatitis.

Leucine-rich repeat kinase 2 (LRRK2) is a multifunctional intracellular protein that induces autophagy and innate immune responses [18–20], and mutations in *Lrrk2* are associated with familial and sporadic Parkinson's disease [18–20]. In addition to Parkinson's disease, polymorphisms in *Lrrk2* are linked to the development of Crohn's disease, tuberculosis, and leprosy [18–20]. Thus, polymorphism-induced LRRK2 dysfunction can contribute to chronic inflammatory disorders. However, the association between *Lrrk2* polymorphisms and pancreatitis is not clear. LRRK2 activation could be involved in the development of acute pancreatitis as excessive proinflammatory cytokines and/or reduced autophagic responses are known to induce acute acinar injury [3]. Moreover, the recognition of β-glucans derived from fungal wall components by Dectin-1 activates LRRK2 in myeloid and dendritic cells (DCs) to induce proinflammatory cytokine responses [21, 22]. Therefore, sensing of fungi, which translocate into the pancreas in acute pancreatitis due to intestinal barrier dysfunction, might accelerate pancreatic injury through the activation of LRRK2. In this study, we aimed to investigate the role of LRRK2 in SAP development.

Materials and methods

Mice

BAC FLAG-*Lrrk2* transgenic (*Lrrk2* Tg) mice [21, 23] were obtained from Dr. T. Takagawa (Department of Inflammatory Bowel Disease, Hyogo College of Medicine). We used 6–8-week-old wild-type C57BL/6 mice and *Lrrk2* Tg mice. The mice were reared at the Animal Facility of the Kindai University Faculty of Medicine. All animal experiments were approved by the Review Board of the Kindai University Faculty of Medicine and adhered to the ARRIVE guidelines.

Induction of SAP

C57BL/6 mice and *Lrrk2* Tg mice at 6–8 weeks of age were treated with hourly intraperitoneal (IP) injections of cerulein (100 μg/kg, Sigma-Aldrich, St. Louis, MO, USA), as depicted in Fig. 1A. Mice received hourly injections of cerulein eight times over 2 consecutive days to induce SAP, in accordance with previous reports [24]. In some experiments, mice

received IP injections of LRRK2 inhibitor (LRRK2-IN-1, 0.25 mg, Calbiochem, San Diego, CA, USA) [21], spleen tyrosine kinase (Syk) inhibitor (piceatannol, 1.25 mg, Abcam, Cambridge, MA, USA) [25], or DMSO prior to the initial injection of cerulein each day.

Bone marrow transplantation

Bone marrow (BM) transplantation (BMT) experiments were performed as previously described [15, 16]. Briefly, recipient mice were irradiated with 10 Gy and received 2×10^6 BM cells from donor mice via the tail vein. BM chimeric mice were used to induce SAP 2 months after BMT.

Abrogation of fungal mycobiome or gut bacterial microbiota

Lrrk2 Tg mice were treated with ampicillin (ABPC, 1 g/L), neomycin (NEO, 1 g/L), kanamycin (KM, 0.5 g/L), and metronidazole (MNZ, 1.0 g/L) in drinking water for 2 weeks prior to the induction of SAP to sterilize the gastrointestinal tract, as previously described [15]. Antibiotics were purchased from Wako Chemical Industries (Osaka, Japan). Gut bacterial sterilization was continued during the experimental period. *Lrrk2* Tg mice were treated with fluconazole (Sigma-Aldrich, 0.5 mg/mL) in drinking water to deplete commensal fungi [26, 27]. Anti-fungal therapy was initiated 3 weeks before the induction of SAP and continued throughout the experimental period. In addition to fluconazole, amphotericin B (Sigma-Aldrich, 0.25 mg) was administered daily via the oral route to the mice, which was initiated 5 days before the induction of SAP.

Pathological analysis

The pancreatic tissues were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm thickness were subjected to hematoxylin and eosin (H&E) staining. The degree of acute pancreatitis was semi-quantitatively determined as previously described [15, 24]. Briefly, pancreatic morphological changes were graded using a scale of 0–4 based on the degree of edema, acinar necrosis, hemorrhage with fat necrosis, and infiltration of immune cells. Total pathology score was given as the sum of each scale of edema, acinar necrosis, hemorrhage with fat necrosis, and infiltration of immune cells.

Flow-cytometric analysis

Pancreatic mononuclear cells (PMNCs) were isolated as previously described [16, 28–31]. Briefly, pancreatic tissues were cut into small pieces using scissors, washed with hanks' balanced salt solution without Ca^{2+} and Mg^{2+}, and subjected to collagenase digestion at 37°C for 45 min as previously described [16, 28–31]. After collagenase digestion, cell debris was eliminated by low-speed centrifugation (30 × *g*, 5 min, 4°C). Ammonium-chloride-potassium lysis buffer was used for the lysis of red blood cells. PMNCs were stained with fluorescein isothiocyanate (FITC)-conjugated CD11b or B220 antibodies (eBioscience, San Diego, CA, USA), in combination with phycoerythrin (PE)-conjugated CD11c or CD3 antibodies (eBioscience). In some experiments, PMNCs were stained with FITC-conjugated CD11b or CD11c antibodies (eBioscience) in combination with PE-conjugated Dectin-1 antibody (BioLegend, San Diego, CA, USA). Flow-cytometric analysis was performed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and CFlow Plus software (BD Biosciences) [28, 29, 32].

Fungal mycobiome analyses

Fecal samples were prepared from C57BL/6 mice and *Lrrk2* Tg mice before SAP induction, and DNA was extracted using QIAamp stool knit (Qiagen, Hilden, Germany) as previously described [29, 33]. Nuclear ribosomal repeat gene profiling with an Illumina library targeting the internal transcribed spacer 1 (ITS1) region was performed using DNA isolated from stool samples [34]. The MiSeq system was used for ITS1 sequencing. Sequence data were processed using the Trimmomatic, Cutadapt, and Fastq-join programs, and the obtained fungal sequences were clustered into operational taxonomic units. The resulting fungal sequences were aligned with the Greengenes microbial gene database with 97% sequence similarity, using QIIME (1.8.0). Species richness, alpha diversity, and beta diversity were calculated as previously described [29, 33]. Differences in beta diversity were visualized in three-dimensional plots obtained using principal coordinate analysis (PCoA). Differential abundance analysis at the genus and species levels was performed using R package ALDEx2. False discovery rate-corrected *P*-values were calculated as per the Benjamini–Hochberg procedure. The effect size was determined using the aldex. glm effect. $P < 0.05$ or effect size > 1 was considered significant in accordance with previous studies [35, 36].

Stimulation of PMNCs with Dectin-1 ligands

Isolated PMNCs were stimulated with Pam_3CSK4 (PAM, a TLR2 ligand, 10 μg/ml, InvivoGen, San Diego, CA, USA), lipopolysaccharide (LPS, a TLR4 ligand, 1 μg/ml, InvivoGen), Zymosan (TLR2 and Dectin-1 ligands, InvivoGen, 10 μg/ml), and Zymosan Depleted (ZymD, Zymosan lacking TLR ligands, a Dectin-1 ligand, InvivoGen, 100 μg/ml). ZymD, which was created by treating conventional Zymosan with hot alkali to remove its TLR2-stimulating properties, is a Dectin-1-specific ligand [37]. PMNCs (2×10^6/mL) were cultured in complete RPMI medium for 24 h [16, 28].

Enzyme-linked immunosorbent assay

Pancreatic expression of the cytokines (IFN-γ, IL-6, IL-12/23p40, and TNF-α) was measured using enzyme-linked immunosorbent assay (ELISA) kits from eBioscience as previously described [16, 28–31, 38, 39]. Briefly, pancreatic tissue was homogenized and whole pancreatic protein was extracted using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Pancreatic protein extracts isolated from each mouse were applied to the wells for ELISA assays after the measurement of protein concentrations. IFN-β expression was determined using an ELISA kit from R&D Systems (Minneapolis, MN, USA) [16]

Immunohistochemical analyses

Deparaffinized pancreatic sections were incubated with rabbit anti-amylase (Sigma-Aldrich) or anti-LRRK2 antibodies (GeneTex, Irvine, CA), and protein expression was visualized using the Dako Envision + system (DAKO, Tokyo, Japan) as previously described [16]. Intact pancreatic acinar cells are positive for cytoplasmic amylase staining. Induction of pancreatitis reduces the percentages of cells positive for cytoplasmic amylase staining due to acinar cell death and

thus the decrease in amylase positive areas reflects the degree of acinar injury [3]. At least two images were taken from each slide, and areas positive for amylase were calculated using the hybrid cell count software (Keyence, Osaka, Japan) [28].

Transcription factor assay

Nuclear extracts were isolated from the pancreas using a nuclear extraction kit (Active Motif). The binding activity of pancreatic nuclear extracts (10 μg) to the consensus promoter sequence of nuclear factor-κB (NF-κB) subunits and signal transducer and activator of transcription 3 (STAT3) was assessed using a Transam kit (Active Motif) [32].

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Two-tailed Student's *t*-tests were used, and a *P*-value of <0.05 was considered statistically significant.

Results

LRRK2 inhibitors attenuate the development of SAP

Although LRRK2 activation is involved in the development of experimental colitis and experimental autoimmune uveitis [21, 40, 41], the role of LRRK2 in the development of experimental pancreatitis is poorly defined. Given that bacterial and fungal infections are among the strongest risk factors for SAP [1], LRRK2-mediated innate immunity might be involved in SAP development. In this study, we investigated whether LRRK2 activation promoted SAP development by inducing SAP in C57BL/6 mice through eight hourly injections of supramaximal doses of cerulein for 2 consecutive days, as previously reported [24] (Fig. 1A). We examined the involvement of LRRK2 activation by treating C57BL/6 mice with an LRRK2 inhibitor or DMSO [21]. Pathological examinations revealed destruction of the pancreatic acinar architecture and infiltration of immune cells in mice with induced SAP and DMSO treatment (Fig. 1B). In contrast, treatment with the LRRK2 inhibitor attenuated pancreatic injury. Moreover, mice treated with the LRRK2 inhibitor showed lower pathological scores of acute pancreatitis than those with DMSO treatment. Immunohistochemical analysis revealed that consistent with reduced pathological scores, pancreatic expression of amylase was lower in mice treated with DMSO than in those treated with the LRRK2 inhibitor, reflecting the release of intracellular amylase due to the acinar cell death in the former mice (Fig. 1C).

Flow-cytometric analysis using PMNCs revealed that the percentages and numbers of $CD11b^+$ myeloid cells, $CD11c^+$ DCs, and $CD3^+$ T cells were greater in the pancreas of mice treated with DMSO than in those treated with the LRRK2 inhibitor (Fig. 1D). The numbers and percentages of $CD11b^+$ myeloid cells, $CD11c^+$ DCs, or $CD3^+$ T cells were comparable in the pancreas of mice treated with DMSO or the LRRK2-inhibitor in the absence of SAP induction (Supplementary Fig. 1A). Thus, activation of LRRK2 is necessary for the recruitment of immune cells into the pancreas in response to SAP induction.

Experimental pancreatitis is characterized by proinflammatory cytokine responses including TNF-α and IL-6 [3]. The expression of innate immunity cytokines produced by myeloid cells and DCs, such as IL-6, IL-12/23p40, and TNF-α, was significantly higher in the pancreas of mice treated with DMSO than in those treated with the LRRK2 inhibitor (Fig. 1E). In addition, expression of IFN-γ, a prototypical T helper type 1 cytokine, was also higher in the pancreas of mice treated with DMSO than in those treated with the LRRK2 inhibitor. In contrast, the expression of type I IFN (IFN-β) was comparable in these mice. We then analyzed serum cytokine responses in mice treated with DMSO or the LRRK2 inhibitor. Serum concentrations of IL-6 and TNF-α were significantly lower in mice treated with the LRRK2 inhibitor than in those treated with DMSO (Supplementary Fig. 1B). Thus, activation of LRRK2 induced both pancreatic and systemic inflammatory responses. These data suggest that the activation of LRRK2 promotes SAP through the induction of proinflammatory cytokine responses.

*Lrrk2*Tg mice are susceptible to SAP

We next investigated whether *Lrrk2* Tg mice were sensitive to SAP induction. As shown in Fig. 2A, *Lrrk2* Tg mice displayed a higher degree of pancreatitis than that in littermate control mice (C57BL/6 mice). The pancreas of *Lrrk2* Tg mice showed degraded acinar architecture and accumulation of immune cells. In contrast, amylase-positive areas were significantly smaller in the pancreas of *Lrrk2* Tg mice than in control C57BL/6 mice (Fig. 2B).

The percentage and number of pancreatic $CD11b^+$ myeloid cells were significantly higher in *Lrrk2* Tg mice than in control C57BL/6 mice (Fig. 2C). In addition, the numbers of pancreatic $CD11c^+$ DCs and $CD3^+$ T cells were higher in the pancreas of *Lrrk2* Tg mice, although the difference was not statistically significant. Consistent with the pancreatic accumulation of $CD11b^+$ myeloid cells and $CD3^+$ T cells, pancreatic expression of IL-6, IL-12/23p40, TNF-α, and IFN-γ, but not IFN-β, was significantly higher in the pancreas of *Lrrk2* Tg mice with induced SAP than in those of control C57BL/6 mice (Fig. 2D).

The expression of proinflammatory cytokines and the development of experimental pancreatitis is regulated by the activation of transcription factors including NF-κB and STAT3 [3]. We determined NF-κB and STAT3 activation using a transfactor assay on pancreatic nuclear extracts. As shown in Fig. 2E, activation of NF-κB subunits p65, p50, and c-Rel, but not RelB or p52, was significantly higher in the pancreas of *Lrrk2* Tg mice than in control C57BL/6 mice, suggesting that overexpression of LRRK2 induces SAP through the activation of canonical NF-κB signaling pathways. Moreover, *Lrrk2* Tg mice also displayed pancreatic STAT3 activation compared with control C57BL/6 mice. Collectively, these results suggest that overexpression of LRRK2 induces SAP through NF-κB-mediated proinflammatory cytokine responses.

LRRK2 expressed in hematopoietic cells mediates SAP

We next determined the type of cells overexpressing LRRK2 by analyzing pancreatic tissues obtained from *Lrrk2* Tg mice and control C57BL/6 mice challenged with SAP and then subjected to immunohistochemical analyses. As shown in Supplementary Fig. 2, endogenous expression of LRRK2 was observed in both immune cells and acinar cells in the pancreas of control C57BL/6 mice. However, LRRK2 expression was higher in pancreatic immune cells and acinar

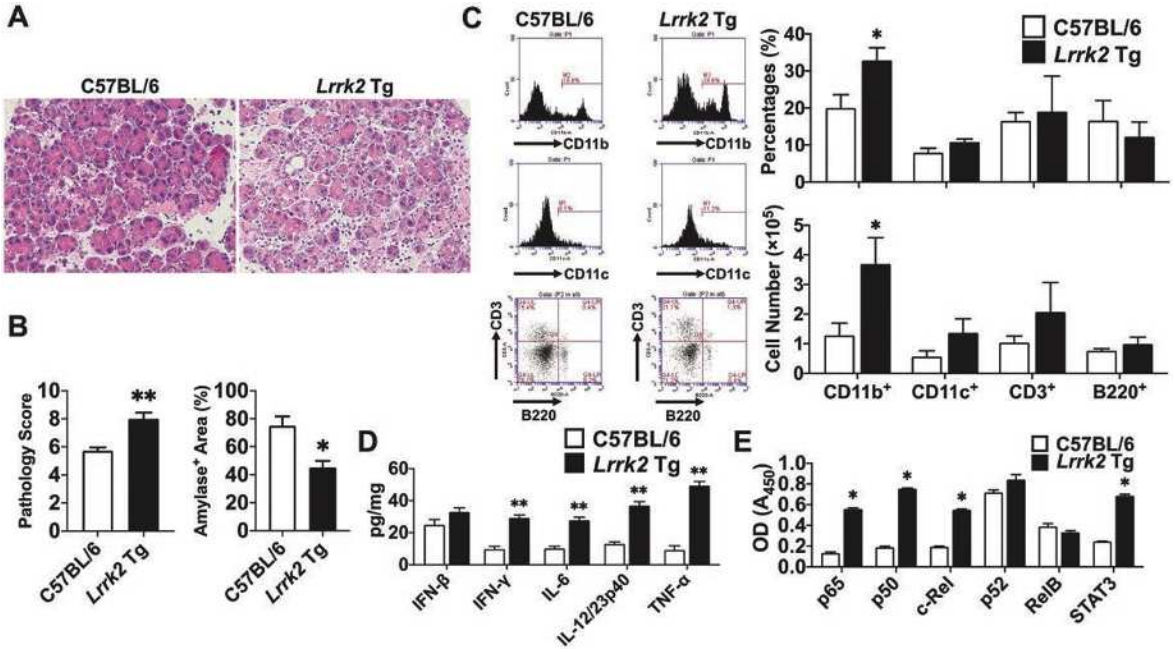

Figure 2. *Lrrk2* Tg mice are susceptible to severe acute pancreatitis. *Leucine-rich repeat kinase 2* transgenic (*Lrrk2* Tg) mice ($n = 6$) and littermate control C57BL/6 mice ($n = 7$) were subjected to cerulein-induced severe acute pancreatitis protocols. Hematoxylin and eosin staining of the pancreas. The degree of pancreatitis was greater in *Lrrk2* Tg mice than in C57BL/6 mice. Magnification, ×400 (A). Pathological scores for acute pancreatitis and positive areas for amylase staining (B). (C) Pancreatic accumulation of $CD11b^+$ myeloid cells, $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $B220^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (D) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. (E) Activation of transcription factors was assessed by transfactor assays. Results are combined data obtained from three independent experiments with similar results (*Lrrk2* Tg mice; $n = 2$, $n = 3$, $n = 1$, total $n = 6$, C57BL/6 mice; $n = 2$, $n = 3$, $n = 2$, total $n = 7$). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

cells in *Lrrk2* Tg mice than in control C57BL/6 mice. Thus, consistent with previous reports [22, 40, 42], LRRK2 was expressed in both hematopoietic and non-hematopoietic cells.

Lrrk2 Tg mice overexpress LRRK2 in both hematopoietic and non-hematopoietic cells; thus, the cellular contribution of LRRK2 in SAP induction remains unknown. Therefore, we created BM chimeric mice to determine the type of LRRK2-overexpressing cells responsible for SAP development. Two types of BM chimeric mice were generated: irradiated control C57BL/6 mice transplanted with BM cells from control C57BL/6 mice (C57BL/6 > C57BL/6 mice) and irradiated C57BL/6 mice transplanted with BM cells from *Lrrk2* Tg mice (*Lrrk2* Tg > C57BL/6 mice). As shown in Fig. 3A, the degree of acute pancreatitis was significantly greater in *Lrrk2* Tg > C57BL/6 mice than in C57BL/6 > C57BL/6 mice. The pancreas of *Lrrk2* Tg > C57BL/6 mice displayed significantly degraded acinar architecture and accumulation of immune cells. Moreover, amylase-positive areas were significantly smaller in the pancreas of *Lrrk2* Tg > C57BL/6 mice than in C57BL/6 > C57BL/6 mice (Fig. 3B).

The percentages of $CD11b^+$ myeloid cells, $CD11c^+$ DCs, and $CD3^+$ T cells were significantly higher in the pancreas of *Lrrk2* Tg > C57BL/6 mice than in C57BL/6 > C57BL/6 mice (Fig. 3C). Furthermore, the pancreatic expression of IL-12/23p40, TNF-α, and IFN-γ was markedly higher in the pancreas of *Lrrk2* Tg > C57BL/6 mice than in C57BL/6 > C57BL/6 mice (Fig. 3D). These findings support the idea that LRRK2 expressed in hematopoietic cells, but not in acinar cells, mediates the development of SAP through the induction of proinflammatory cytokine responses.

LRRK2 expressed in non-hematopoietic cells does not aggravate acute pancreatitis

To clarify roles played by LRRK2 in non-hematopoietic cells, we created two types of BM chimeric mice: irradiated control C57BL/6 mice transplanted with BM cells from *Lrrk2* Tg mice (*Lrrk2* Tg > C57BL/6 mice) and irradiated *Lrrk2* Tg mice transplanted with BM cells from *Lrrk2* Tg mice (*Lrrk2* Tg > *Lrrk2* Tg mice). As shown in Fig. 4A and B, the degree of acute pancreatitis was comparable between *Lrrk2* Tg > C57BL/6 mice and *Lrrk2* Tg > *Lrrk2* Tg mice as assessed by pathological analyses. No significant differences were observed in the percentages or numbers of $CD11b^+$ myeloid cells, $CD11c^+$ DCs, and $CD3^+$ T cells in the pancreas of *Lrrk2* Tg > C57BL/6 mice and *Lrrk2* Tg > *Lrrk2* Tg mice (Fig. 4C). Furthermore, the pancreatic expression of proinflammatory cytokines including IL-6, IL-12/23p40, TNF-α, IFN-β, and IFN-γ was comparable between the two types of BM chimeric mice (Fig. 4D). These findings from BM chimeric mice strongly support the idea that LRRK2 expressed in hematopoietic cells, but not in acinar cells, mediates the development of SAP through the induction of proinflammatory cytokine responses. Further, these observations suggest that SAP development can be achieved in the presence of LRRK2 overexpression in immune cells and that synergistic effects of LRRK2 expression in both immune and acinar cells are unlikely to be operating.

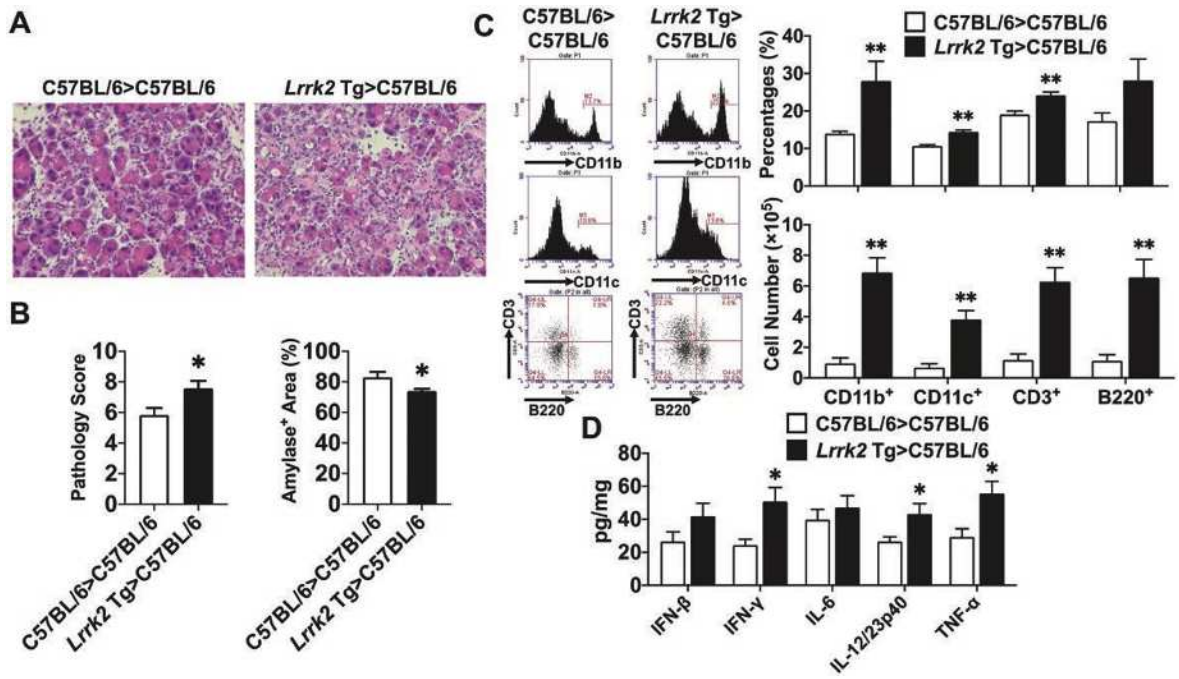

Figure 3. LRRK2 expressed in hematopoietic cells mediates severe acute pancreatitis. Bone marrow chimeric mice were generated by bone marrow transplantation. Irradiated control C57BL/6 mice received bone marrow cells from *leucine-rich repeat kinase 2* transgenic (*Lrrk2*Tg) mice (*Lrrk2*Tg > C57BL/6, $n = 6$) or control mice (C57BL/6 > C57BL/6, $n = 6$) and then these mice were subjected to cerulein-induced severe acute pancreatitis protocols. Hematoxylin and eosin staining of the pancreas. The degree of pancreatitis was greater in *Lrrk2*Tg > C57BL/6 mice than in C57BL/6 > C57BL/6 mice. Magnification, ×400 (A). Pathological scores for acute pancreatitis and positive areas for amylase staining (B). (C) Pancreatic accumulation of CD11b$^+$ myeloid cells, CD11c$^+$ dendritic cells, CD3$^+$ T cells, and B220$^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (D) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results are expressed as mean ± SEM. Results are combined data obtained from two independent experiments with similar results (*Lrrk2*Tg > C57BL/6 mice; $n = 3$ and $n = 3$, total $n = 6$, C57BL/6 > C57BL/6 mice; $n = 3$ and $n = 3$, total $n = 6$). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

Depletion of gut microbiota does not affect the development of SAP

Bacteria and fungi sensing by TLRs and/or Dectin-1 induce proinflammatory cytokine responses via activation of LRRK2 [21, 43, 44]. We next examined whether overexpression of LRRK2 in myeloid cells alters the sensitivity to SAP through recognition of bacteria and/or fungi. We initially examined the involvement of immune responses against gut bacteria in the development of SAP in *Lrrk2* Tg mice by depleting gut bacteria via the administration of a broad range of antibiotics (ABPC/NEO/KM/MNZ), as previously described [15] (Fig. 5A). Bowel sterilization by antibiotics did not alter the severity of acute pancreatitis in *Lrrk2* Tg mice, as assessed by semiquantitative analyses of the pathology score and amylase$^+$ areas (Fig. 5B and C). In addition, no significant difference in the number of immune cells or expression of proinflammatory cytokines was observed in *Lrrk2* Tg mice irrespective of antibiotic treatment (Fig. 5D and E). Thus, SAP development in *Lrrk2* Tg mice does not require proinflammatory cytokine responses driven by intestinal bacteria.

Ablation of fungal mycobiome inhibits the development of SAP in *Lrrk2*Tg mice

Next, we addressed whether depletion of the gut mycobiome inhibited the development of SAP in *Lrrk2* Tg mice by treating *Lrrk2* Tg mice with anti-fungal agents, as previously described (Fig. 6A) [26, 27]. As shown in Fig. 6B and C, the severity of acute pancreatitis was significantly lower and amylase-positive areas were significantly higher in *Lrrk2* Tg mice treated with anti-fungal agents than in untreated *Lrrk2* Tg mice. Ablation of fungal mycobiome markedly decreased pancreatic accumulation of CD11b$^+$ myeloid cells, CD11c$^+$ DCs, and CD3$^+$ T cells in SAP-induced *Lrrk2* Tg mice compared with untreated SAP-induced *Lrrk2* Tg mice (Fig. 6D). Attenuation of SAP by depletion of fungal mycobiome was accompanied by reduced production of proinflammatory cytokines. Pancreatic expression of IL-6, IL-12/23p40, TNF-α, and IFN-γ was markedly reduced in the pancreas of *Lrrk2* Tg mice treated with anti-fungal agents compared with those from untreated *Lrrk2* Tg mice (Fig. 6E). Taken together, these data suggest that LRRK2 expressed in hematopoietic cells mediates the development of SAP through fungi-driven proinflammatory cytokine responses.

Treatment with antimicrobials alone may exert some influence on host immune cells [45–47]. To exclude off-target effects mediated by antibiotics or anti-fungal agents, *Lrrk2* Tg mice were treated with antibiotics or anti-fungal agents alone and pancreatic tissues were subjected to immune cell population analysis. As shown in Supplementary Fig. 3, the numbers and percentages of CD11b$^+$ myeloid cells, CD11c$^+$ DCs, and CD3$^+$ T cells were comparable in the pancreas of mice whether mice were treated with or without antimicrobials.

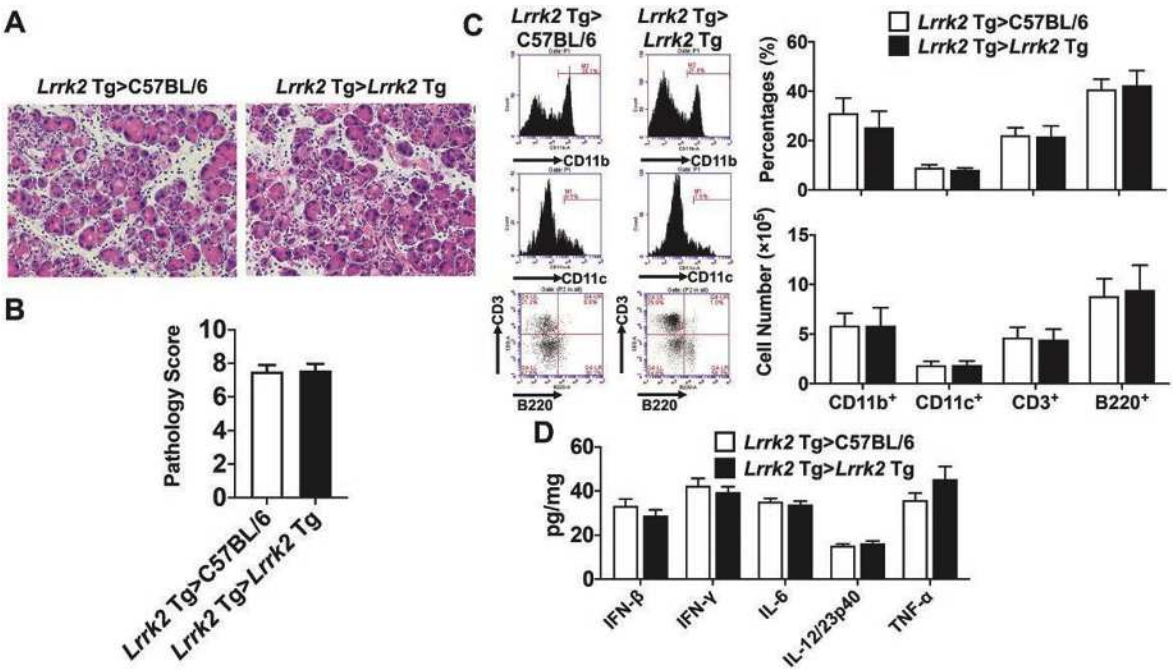

Figure 4. LRRK2 expressed in non-hematopoietic cells does not aggravate acute pancreatitis. Two types of bone marrow (BM) chimeric mice, irradiated control C57BL/6 mice transplanted with BM cells from *leucine-rich repeat kinase 2* transgenic mice (*Lrrk2*Tg > C57BL/6 mice, $n = 6$) and irradiated *Lrrk2*Tg mice transplanted with BM cells from *Lrrk2*Tg mice (*Lrrk2*Tg > *Lrrk2*Tg mice, $n = 6$), were prepared. BM chimeric mice were subjected to cerulein-induced severe acute pancreatitis protocols. Hematoxylin and eosin staining of the pancreas. Magnification, ×400 (A). Pathological scores for acute pancreatitis (B). (C) Pancreatic accumulation of CD11b$^+$ myeloid cells, CD11c$^+$ dendritic cells, CD3$^+$ T cells, and B220$^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (D) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results are combined data obtained from two independent experiments with similar results (*Lrrk2* Tg > C57BL/6 mice; $n = 3$ and $n = 3$, total $n = 6$, *Lrrk2*Tg > *Lrrk2*Tg mice mice; $n = 3$ and $n = 3$, total $n = 6$). Data are expressed as mean ± SEM.

Fungal mycobiome composition is different in *Lrrk2* Tg and C57BL/6 mice

Inhibition of SAP by the administration of anti-fungal agents led us to examine whether alterations in fungal mycobiome components predispose mice to SAP susceptibility. We subjected fecal samples from untreated control C57BL/6 mice and *Lrrk2* Tg mice to next-generation sequencing analyses targeting the fungal ITS1 region. We performed differential abundance analyses to identify the fungal species associated with the development of SAP in *Lrrk2* Tg mice. The R package ALDEx2 was used to determine the statistical significance of fungal mycobiome composition in the stool of C57BL/6 and *Lrrk2* Tg mice. Sequence read counts were comparable between C57BL/6 and *Lrrk2* Tg mice albeit much lower compared with those in next-generation sequencing analyses targeting bacterial 16S ribosomal RNA [29, 33] (Supplementary Fig. 4A). Fungal mycobiome diversity, as judged by the rare fraction analyses of alpha and beta diversity, showed no significant difference when the latter analysis was visualized using a three-dimensional scatter plot by PCoA (Supplementary Fig. 4B).

At the genus level, as judged by the effect size of ALDEx2, we identified fungi that were significantly increased or decreased in the stool of *Lrrk2* Tg mice (Supplementary Fig. 4C). The relative abundance of *Inocybe* species was significantly greater in the stool of *Lrrk2* Tg mice than in that of C57BL/6 mice (Supplementary Fig. 4D). In contrast, colonization by *Claviceps* and *Pseudotomentella* species was enriched in C57BL/6 mice. These data indicate that alterations in fungal mycobiome composition may be associated with increased sensitivity to SAP. However, these data need careful interpretation as colonization differences have been observed in mushroom-forming *Inocybe* species and plant-derived *Claviceps* and *Pseudotomentella* species [48–50]. Thus, we have not identified commensal fungi species associated with the development of SAP in *Lrrk2* Tg mice.

Pancreatic myeloid cells from *Lrrk2*Tg mice produce large amounts of proinflammatory cytokines upon exposure to fungal zymosan

We hypothesized that proinflammatory cytokine responses induced by sensing of fungal components mediate the development of SAP in *Lrrk2* Tg mice. β-glucan, a major cell wall component of fungi, is recognized by cell-surface Dectin-1 expressed in macrophages and DCs to induce proinflammatory cytokine responses through the activation of NF-κB [51]. We examined the expression of Dectin-1 in CD11b$^+$ myeloid cells and CD11c$^+$ DCs in the pancreas and verified Dectin-1 expression in both pancreatic cell types in *Lrrk2* Tg and control C57BL/6 mice with induced SAP. However, Dectin-1 expression tended to be higher in *Lrrk2* Tg mice compared with control C57BL/6 mice (Fig. 7A, mean fluorescence intensity, *Lrrk2* Tg mice vs. control C57BL/6 mice, CD11b$^+$ myeloid cells; 630.85 vs. 313.26, CD11c$^+$ DCs; 1816.99 vs. 1681.41).

We next used PMNCs isolated from control C57BL/6 mice and *Lrrk2* Tg mice with induced SAP and found that PMNCs

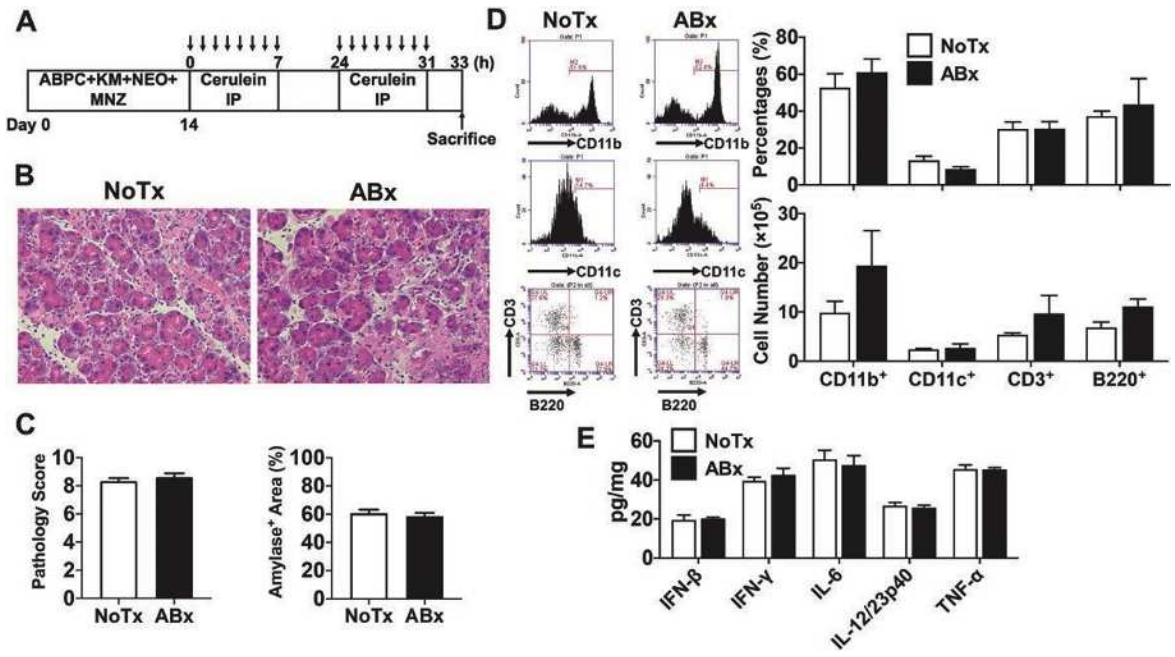

Figure 5. Depletion of gut microbiota does not prevent the development of severe acute pancreatitis in *Lrrk2*Tg mice. (A) Experimental protocol. *Leucine-rich repeat kinase 2* transgenic (*Lrrk2*Tg) mice were untreated ($n = 6$, No Tx) or treated ($n = 4$, ABx) with a broad range of antibiotics (ampicillin, kanamycin, neomycin, and metronidazole). *Lrrk2*Tg mice were subjected to cerulein-induced severe acute pancreatitis protocol. Hematoxylin and eosin staining of the pancreas. The degree of pancreatitis was comparable in *Lrrk2*Tg mice treated with or without antibiotics. Magnification, ×400 (B). Pathological scores for acute pancreatitis and positive areas for amylase staining (C). (D) Pancreatic accumulation of $CD11b^+$ myeloid cells, $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $B220^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (E) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results are combined data obtained from two independent experiments with similar results (No Tx; $n = 4$ and $n = 2$, total $n = 6$, ABx; $n = 3$ and $n = 1$, total $n = 4$). Data are expressed as mean ± SEM.

isolated from *Lrrk2* Tg mice produced large amounts of IL-6 and TNF-α upon exposure to the Dectin-1 ligand (Zymosan and ZymD) as compared with the cells from C57BL/6 mice (Fig. 7B). In contrast, comparable levels of proinflammatory cytokine production were observed upon stimulation with TLR2 and TLR4 ligands (PAM, LPS) in PMNCs isolated from C57BL/6 or *Lrrk2* Tg mice. Conventional Zymosan can activate both TLR2 and Dectin-1 whereas ZymD is a Dectin-1-specfic ligand lacking TLR2-stimulating properties [37]. Thus, these data suggest that pancreatic $CD11b^+$ myeloid cells and/or $CD11c^+$ DCs exhibit enhanced sensitivity to Dectin-1 ligands, which are components of fungal cell walls, and that proinflammatory cytokine responses to Dectin-1 ligands might underlie SAP development in *Lrrk2* Tg mice.

Inhibition of the Dectin-1–Syk pathway attenuates SAP development in *Lrrk2*Tg mice

We next addressed whether inhibition of the Dectin-1 pathway would protect against SAP by treating *Lrrk2* Tg mice with piceatannol, an inhibitor of Syk [25]. Ligation of Dectin-1 by β-glucans phospholylates Syk [25]. As shown in Fig. 8A and B, Syk inhibition resulted in the attenuation of SAP in *Lrrk2* Tg mice, as judged by pathological scores. Amylase positive areas were greater in *Lrrk2* Tg mice treated with the Syk inhibitor than in those treated with DMSO (Fig. 8B). Inhibition of Syk significantly reduced the pancreatic accumulation of $CD11b^+$ myeloid cells and $CD3^+$ T cells in *Lrrk2* Tg mice compared to those treated with DMSO (Fig. 8C). Furthermore, the protection of *Lrrk2* Tg mice from SAP was accompanied by reduced proinflammatory cytokine responses, including IL-6, TNF-α, IL-12/23p40, and IFN-γ (Fig. 8D). Thus, activation of the Dectin-1-Syk pathway triggered by the recognition of fungi may be involved in the development of SAP in *Lrrk2* Tg mice. Taken together, these data support the idea that activation of LRRK2 upon sensing of fungal cell wall components mediates the development of SAP through the induction of proinflammatory cytokine responses.

Discussion

In this study, we demonstrate that the activation of LRRK2 in myeloid cells mediates the development of experimental SAP through the induction of proinflammatory cytokine responses triggered by the recognition of fungal cell wall components. Inhibition of LRRK2 by its specific inhibitor efficiently prevented the development of experimental SAP, which was accompanied by reduced proinflammatory cytokine responses, including IL-6, IL-12/23p40, and TNF-α. *Lrrk2* Tg mice were sensitive to an experimental SAP protocol and displayed a more severe form of acute pancreatitis than littermate control mice due to excessive production of proinflammatory cytokines. Moreover, the degree of acute pancreatitis was greater in BM chimeric mice overexpressing LRRK2 in hematopoietic cells than in those expressing endogenous LRRK2 in hematopoietic cells whereas BM chimeric mice overexpressing LRRK2 in non-hematopoietic cells exhibited

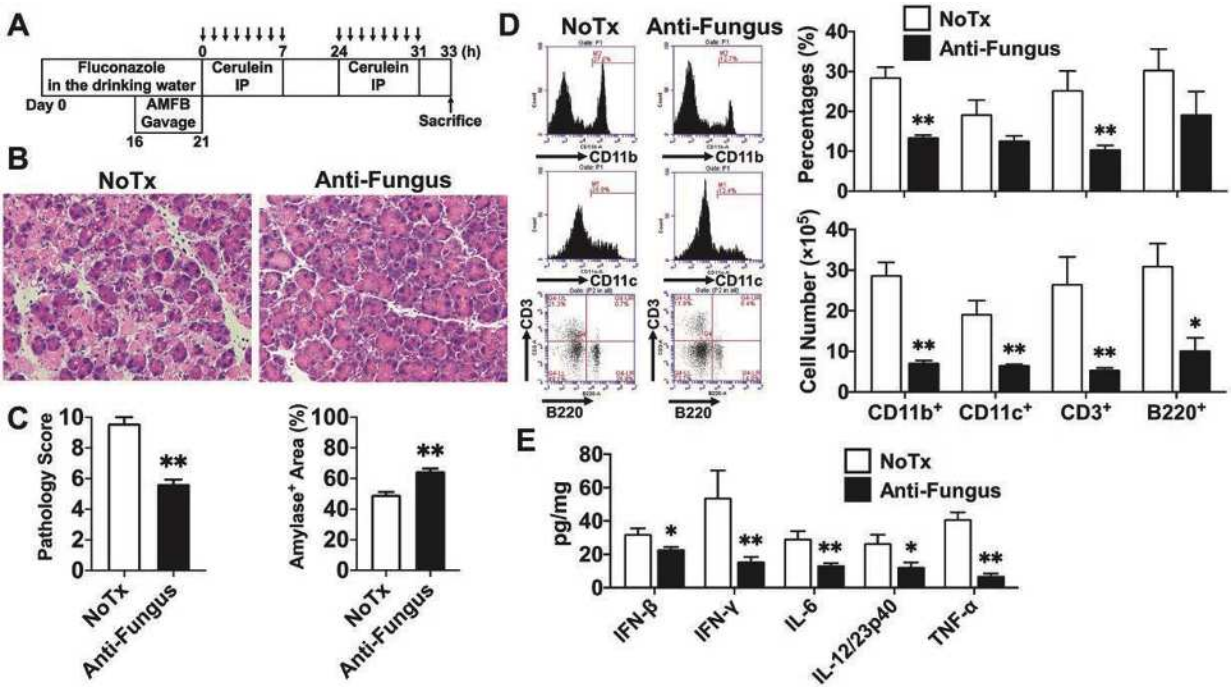

Figure 6. Ablation of fungal mycobiome inhibits the development of severe acute pancreatitis in *Lrrk2* Tg mice. (A) Experimental protocol. *Leucine-rich repeat kinase 2* transgenic (*Lrrk2* Tg) mice were untreated ($n = 5$, No Tx) or treated ($n = 7$, Anti-Fungus) with fluconazole in the drinking water. For the depletion of fungal mycobiome, *Lrrk2* Tg mice were also treated with oral administration of Amphotericin B (AMFB). These mice were subjected to cerulein-induced severe acute pancreatitis protocol. Hematoxylin and eosin staining of the pancreas. The degree of pancreatitis was lower in *Lrrk2* Tg mice treated with anti-fungal agents than untreated mice. Magnification, ×400 (B). Pathological scores for acute pancreatitis and positive areas for amylase staining (C). (D) Pancreatic accumulation of CD11b$^+$ myeloid cells, CD11c$^+$ dendritic cells, CD3$^+$ T cells, and B220$^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (E) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results are combined data obtained from two independent experiments with similar results (No Tx; $n = 3$ and $n = 2$, total $n = 5$, anti-fungal agents-treated mice; $n = 3$ and $n = 4$, total $n = 7$). Data are expressed as mean ± SEM. Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

a similar degree of acute pancreatitis as those expressing endogenous LRRK2 in non-hematopoietic cells in the presence of LRRK2 overexpression in hematopoietic cells. In addition, depletion of the gut fungal mycobiome, but not gut bacteria by anti-microbial agents, protected *Lrrk2* Tg mice from the development of SAP, suggesting the involvement of fungi-driven excessive proinflammatory cytokine responses in *Lrrk2* Tg mice with induced SAP. Pancreatic CD11b$^+$ and CD11c$^+$ cells isolated from *Lrrk2* Tg mice displaying SAP produced large amounts of IL-6 and TNF-α upon stimulation with zymosan and ZymD, both of which are major fungal cell wall components with the ability to stimulate Dectin-1. Finally, blockade of Dectin-1-mediated signaling pathways by a Syk inhibitor attenuated the development of SAP in *Lrrk2* Tg mice. Collectively, these data support the idea that the activation of LRRK2 in myeloid cells mediates the development of SAP through proinflammatory cytokine responses upon exposure to fungal cell wall components (Fig. 9).

Although most cases of acute pancreatitis are self-limiting, approximately20% of patients with this disease develop life-threating SAP [1, 9]. Patients with SAP usually manifest local and systemic complications, such as pancreatic necrosis and sepsis [52, 53]. Translocation of gut bacteria due to impaired intestinal barrier function leads to colonization of bacteria into necrotic pancreatic tissues and subsequent dissemination of the bacteria to systemic organs, leading to endotoxemia and multiple organ failure [52, 53]. In addition, the mortality rate of SAP is much higher in patients with infection than in those without infection [54]. Recent experimental studies also support the idea that proinflammatory cytokine responses caused by pancreatic infection with gut bacteria play critical roles in the development of SAP. Data obtained from experimental acute pancreatitis suggest the involvement of proinflammatory cytokine responses induced by the recognition of intestinal bacteria by NOD1 or TLR4, as shown by the fact that NOD1- or TLR4-deficient mice were resistant to pancreatitis induction due to diminished cytokine responses such as TNF-α and IL-6 [15–17]. In addition to bacterial infection, fungal infection rates in patients with SAP are also high, ranging from 8% to 57% in patients with SAP exhibiting severe pancreatic necrosis [7–9, 55, 56]. Thus, proinflammatory cytokine responses upon pancreatic invasion by gut fungi as well as gut bacteria could drive the development of SAP. Although the administration of antibiotics has been shown to increase the risk of pancreatic colonization of fungi [9], host genetic factors that predispose to fungal infections have not been identified in patients with SAP. Here, we provide evidence that overexpression of LRRK2 in hematopoietic cells increases sensitivity to experimental acute pancreatitis through proinflammatory cytokine responses via sensing of fungal components. Our data suggest the possible involvement of gain-of-function mutations in *Lrrk2* in patients with

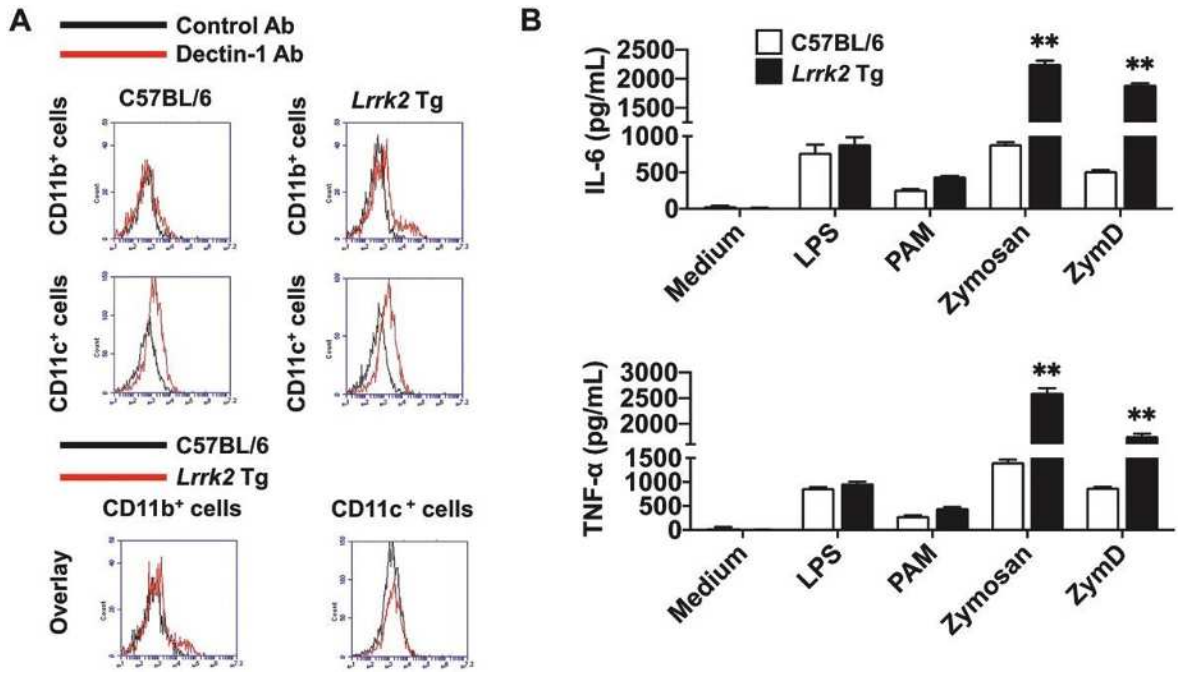

Figure 7. Pancreatic myeloid cells from *Lrrk2* Tg mice produce large amounts of proinflammatory cytokines upon exposure to Dectin-1 ligands. Pancreatic mononuclear cells (PMNCs) were isolated from *leucine-rich repeat kinase 2* transgenic (*Lrrk2* Tg, $n = 4$) and littermate control C57BL/6 mice ($n = 4$) treated with cerulein-induced severe acute pancreatitis. (A) Expression of Dectin-1 in CD11b$^+$ myeloid cells and CD11c$^+$ dendritic cells was determined by flow cytometry. (B) PMNCs were stimulated with Pam_3CSK4 (PAM), lipopolysaccharide (LPS), Zymosan and Zymosan depleted (ZymD) for 24 h. The culture supernatants were subjected to enzyme-linked immunosorbent assay to determine the levels of IL-6 and TNF-α. PMNCs isolated from each mouse were subjected to flow-cytometric and cytokine analyses. Results are expressed as mean ± SEM. $^{**}P < 0.01$.

SAP complicated by fungal infection. It would be intriguing to examine the association between *Lrrk2* polymorphisms and SAP.

As mentioned above, clinicopathological analyses have implicated involvement of fungal infection in SAP [7–9, 55, 56]. However, the molecular mechanisms underlying the relationship between SAP and fungi-induced immune responses are poorly defined. In this study, we found that proinflammatory cytokine responses against fungal components are associated with the development of SAP through activation of LRRK2-mediated signaling pathways. This idea is supported by the finding that PMNCs overexpressing LRRK2 produced large amounts of TNF-α and IL-6 in response to fungal cell wall components (Dectin-1 ligands) and that the development of SAP in *Lrrk2* Tg mice was markedly attenuated upon depletion of the fungal mycobiome. Consistent with our data, siRNA-mediated knockdown of caspase recruitment domain-containing protein 9, a critical downstream signaling molecule for Dectin-1, has been shown to protect rats from experimental acute pancreatitis through downregulation of IL-6 and TNF-α expression [57, 58]. Thus, it is likely that enhanced sensitivity to fungal components predisposes *Lrrk2* Tg mice to pancreatic injury through proinflammatory cytokine responses, even though the pancreatic burden of fungi is comparable between control mice and *Lrrk2* Tg mice. Alternatively, fungal dysbiosis caused by LRRK2 activation may be associated with Dectin-1-mediated proinflammatory cytokine release in *Lrrk2* Tg mice. In this regard, no significant difference was observed in mycobiome diversity between control C57BL/6 mice and *Lrrk2* Tg mice, as assessed by alpha diversity or beta diversity. The relative abundance of several mushroom-forming or plant-derived fungi, including *Claviceps*, *Pseudotomentella*, and *Inocybe* species, was altered in the stool of *Lrrk2* Tg mice compared with those of control C57BL/6 mice [48–50]. Given that differences in relative abundance of fungal species are limited to mushroom-forming and plant-derived fungi, it is likely that consumption of a diet might have affected mycobiome differences in the stool. Thus, we have not identified commensal fungi accounting for the development of SAP in *Lrrk2* Tg mice. Further experiments would help to identify and quantify fungal species translocated into the pancreas that cause proinflammatory cytokine responses. To this end, commensal fungi-colonization model in combination with induction of SAP would be useful [59, 60].

LRRK2 is a multifunctional protein with the ability to induce autophagy and innate immunity [18, 21, 22, 40]. Although mutations in *Lrrk2* are associated with familial cases of Parkinson's disease, Crohn's disease, and tuberculosis, their role in pancreatitis has not been examined. Given that impaired autophagic machinery and/or excessive innate immunity underlie the pathogenesis of pancreatitis, LRRK2 may be involved in pancreatic injury [3]. In this study, we provide evidence that LRRK2 activation is linked to the severity of acute pancreatitis. The development of SAP in *Lrrk2* Tg mice was accompanied by increased production of IL-6 and TNF-α by PMNCs upon stimulation with fungal cell wall components. Thus, proinflammatory cytokine responses against fungi are involved in the aggravation of acute pancreatitis in *Lrrk2* Tg mice. Autophagy is a cellular machinery

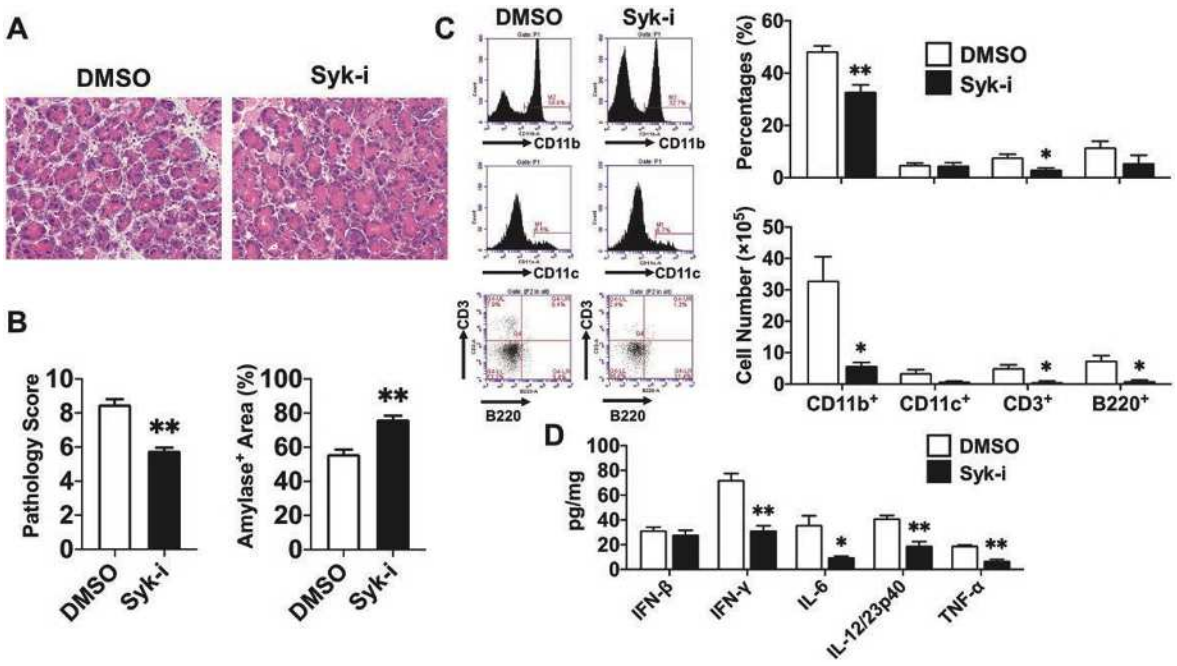

Figure 8. Blockade of Dectin-1-mediated signaling pathways by Syk inhibitor reduces the degree of severe acute pancreatitis in *Lrrk2* Tg mice. *Leucine-rich repeat kinase 2* transgenic (*Lrrk2* Tg) mice were treated with spleen tyrosine kinase (Syk) inhibitor (Syk-i, piceatannol, 1.25 mg, $n = 7$) or DMSO ($n = 7$). These mice were subjected to cerulein-induced severe acute pancreatitis protocol. Hematoxylin and eosin staining of the pancreas. The degree of pancreatitis was smaller in *Lrrk2* Tg mice treated with piceatannol than in those with DMSO. Magnification, ×400 (A). Pathological scores for acute pancreatitis and positive areas for amylase staining (B). (C) Pancreatic accumulation of $CD11b^+$ myeloid cells, $CD11c^+$ dendritic cells, $CD3^+$ T cells, and $B220^+$ B cells was assessed by flow-cytometric analyses. The percentages and numbers of each cell population were calculated. (D) Expression of cytokines in the pancreas was measured by enzyme-linked immunosorbent assays. Results are combined data obtained from two independent experiments with similar results (DMSO-treated mice; $n = 4$ and $n = 3$, total $n = 7$, Syk-i-treated mice; $n = 3$ and $n = 4$, total $n = 7$). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

in which intracellular organelles, proteins, and microbial components are digested and degraded by autophagosomes [61]. A mechanistic link exists between autophagy defects and pancreatitis, as shown by the fact that impaired formation of autophagosomes in the absence of autophagy-related protein 5 or 7 predisposes mice to the development of pancreatitis [3, 62, 63]. Membrane-associated LRRK2 colocalizes with autophagosome membranes and participates in autophagy upon exposure to microbial infection [64]. Takagawa et al. reported that DCs overexpressing LRRK2 produce large amounts of TNF-α upon stimulation with Dectin-1 ligands through the suppression of autophagy [21]. Therefore, it is possible that *Lrrk2* Tg mice develop SAP through impaired autophagy and enhanced proinflammatory cytokine responses upon exposure to Dectin-1 ligands. Whether suppression of autophagy is involved in severe pancreatic injury in *Lrrk2* Tg mice requires further investigation.

Retrograde migration of duodenal fungi into the pancreas via the papilla of Vater has been implicated in the pathogenesis of pancreatic cancer [27, 65]. Aykut et al. showed that *Malassezia* species preferentially colonized pancreatic tumors and promoted pancreatic cancer growth [27]. Such migration of gut fungi to pancreatic tumors promotes the generation of the tumor microenvironment through the activation of Dectin-1, followed by enhanced production of IL-33 [65]. Thus, alterations in the intratumoral pancreatic mycobiome may be a possible trigger for pancreatic cancer growth. It remains unknown whether such retrograde migration of fungi into the pancreas is involved in SAP development in *Lrrk2* Tg mice. In this regard, relative abundance of *Malassezia* species in the stool was comparable between wild-type mice and *Lrrk2* Tg mice. To identify pathogenic or protective fungi associated with SAP in *Lrrk2* Tg mice, sequencing studies utilizing pancreatic samples may be required. It should be noted, however, that pancreatic macrophages and DCs isolated from *Lrrk2* Tg mice displaying SAP produced large amounts of IL-6 and TNF-α upon stimulation with fungal cell wall components (Dectin-1 ligands), but not bacterial cell wall components (TLR2 and TLR4 ligands), compared with wild-type mice subjected to the same SAP protocol. Thus, sensitivity to Dectin-1 ligands rather than the fungal mycobiome composition is likely to underlie the immunopathogenesis of SAP in *Lrrk2* Tg mice. Collectively, our data support the idea that the presence of LRRK2-overexpressing macrophages and DCs with the ability to produce proinflammatory cytokines upon sensing of fungal components is indispensable for the development of SAP.

Pancreatitis is unique in that inflammation increases the permeability of the bowel wall and allows translocation of gut bacteria into the circulation, followed by colonization into the pancreas [3]. In experimental cerulein-induced acute and chronic pancreatitis models, intestinal barrier dysfunction leads to translocation of commensal gut bacteria into the pancreas, and NOD1 expressed in pancreatic acinar cells recognizes translocated bacteria to induce proinflammatory cytokine responses [15, 16]. Moreover, the

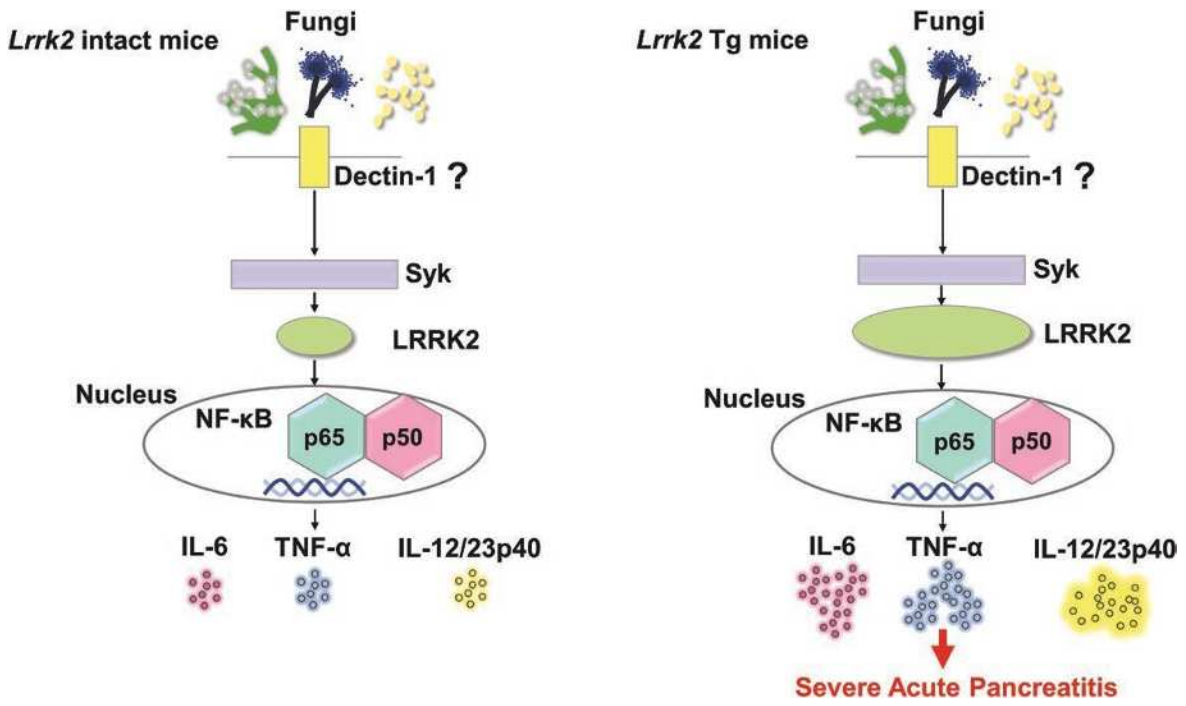

Figure 9. Activation of LRRK2 mediates severe acute pancreatitis through sensing of fungal cell wall components. *Leucine-rich repeat kinase 2* transgenic (*Lrrk2*Tg) mice were sensitive to cerulein-induced severe acute pancreatitis. Myeloid cells and dendritic cells overexpressing LRRK2 produce large amounts of proinflammatory cytokines (IL-6, TNF-α, IL-12/23p40) upon sensing of fungal cell wall components. Dectin-1-spleen tyrosine kinase (Syk)-LRRK2-nuclear factor-kappa B (NF-κB) axis might be involved in the robust production of proinflammatory cytokines in *Lrrk2*Tg mice (right panel). Proinflammatory cytokine responses upon sensing of fungal components are smaller in *Lrrk2*-intact mice than *Lrrk2*Tg mice.

association between the presence of bacteremia and the severity of acute pancreatitis has been demonstrated in recent studies employing sequencing analyses targeting bacterial 16S ribosomal RNA [66]. In line with these human and experimental SAP cases, we assume that pancreatic colonization of fungi is facilitated by increased intestinal permeability associated with the initiation of acute pancreatitis, as in the case of gut bacterial translocation [3]. Such pancreatic translocation of gut fungi may activate myeloid cells and DCs to induce proinflammatory cytokine responses. However, we cannot exclude the possibility of a retrograde pathway via the papilla Vater [27, 65]

NF-κB activation could be involved in the generation of proinflammatory cytokine responses in LRRK2 pathway-induced SAP development. Sensing of β-glucans by Dectin-1 induces proinflammatory cytokine production by myeloid cells through activation of NF-κB [51]. Nuclear translocation of the NF-κB subunits p65, p50, and c-Rel was observed in the pancreas of *Lrrk2* Tg mice displaying SAP. Thus, the activation of NF-κB is associated with increased production of TNF-α, IL-12/IL-23p40, and IL-6 in the pancreas, since the expression of these cytokines requires NF-κB transactivation [3]. Therefore, Dectin-1-mediated activation of NF-κB might be linked to proinflammatory cytokine responses, leading to the development of SAP. Consistent with this idea, the depletion of the fungal mycobiome and Dectin-1 ligands by antifungal agents attenuated the severity of acute pancreatitis, which was accompanied by reduced proinflammatory cytokine responses in the pancreas. Collectively, the data presented in this study suggest that the activation of the LRRK2-NF-κB axis upon exposure to fungi plays pathogenic roles in severe pancreatic injury.

Pancreatic myeloid cells isolated from *Lrrk2* Tg mice produced large amounts of proinflammatory cytokines upon stimulation with fungal cell wall components, i.e. Dectin-1 ligands. In addition, the blockade of Dectin-1-mediated signaling pathway by Syk inhibitor attenuated the development of SAP in *Lrrk2* Tg mice. These findings together with the preventive effects of anti-fungal agents support the idea that enhanced sensitivity of *Lrrk2* Tg mice to SAP depends on the activation of the Dectin-1-Syk pathway. Of note, however, Syk is a downstream signaling molecule not only for Dectin-1 but also Dectin-2 and macrophage-inducible C type lectin [67, 68]. In addition to C-type lectins, integrins activates Syk [67, 68]. Therefore, it remains uncertain whether activation of the Dectin-1-Syk axis triggered by fungal sensing predisposes *Lrrk2* Tg mice to SAP (Fig. 9). Dectin-1-deficient *Lrrk2* Tg mice would be useful to verify this hypothesis.

In conclusion, activation of LRRK2 upon fungal sensing mediates the development of SAP. The inhibition of the LRRK2 pathway may be a new therapeutic approach for patients with SAP. Further human studies addressing the relationship between the activation status of this pathway and the severity of acute pancreatitis would be useful to verify this idea.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

All animal experiments were approved by the Review Board of the Kindai University Faculty of Medicine and adhered to the ARRIVE guidelines.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Data availability

The data obtained in this study may be shared with other researchers upon reasonable request from the corresponding author.

Author contributions

Yasuo Otsuka: Conceptualization, Methodology, Investigation, Formal Analysis, Resources, Writing—Original Draft; Akane Hara, Kosuke Minaga, Ikue Sekai, Masayuki Kurimoto, Yasuhiro Masuta, Ryutaro Takada, Tomoe Yoshikawa, Ken Kamata; Investigation, Formal Analysis; Masatoshi Kudo: Study Supervision; Tomohiro Watanabe: Conceptualization, Methodology, Investigation, Formal Analysis; Resources, Writing—Original Draft; Writing—Review & Editing.

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