FUNCTION™ american physiological society

FUNCTION, 2023, 5(1): zqad061

https://doi.org/10.1093/function/zqad061
Advance Access Publication Date: 23 October 2023
Original Research

RESEARCH ARTICLE

Neutrophil-specific ORAI1 Calcium Channel Inhibition Reduces Pancreatitis-associated Acute Lung Injury

Mengya Niu[1,2,#], Xiuli Zhang[3,4,#], Zengkai Wu[2], Bin Li[2], Jingpiao Bao[2], Juanjuan Dai[2], Zihan Yang[4], Yue Zeng[2], Liang Li[2], Stephen Pandol[5], Robert Sutton[6], Li Wen[3,7,*]

[1]School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou 450001, China, [2]Department of Gastroenterology and Shanghai Key Laboratory of Pancreatic Disease, Institute of Pancreatic Disease, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201600, China, [3]Center for Biomarker Discovery and Validation, National Infrastructures for Translational Medicine (PUMCH), Institute of Clinical Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing 100730, China, [4]Department of Gastroenterology, Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing 100730, China, [5]Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA, [6]Liverpool Pancreatitis Research Group, Liverpool University Hospitals NHS Foundation Trust and Institute of Translational Medicine, University of Liverpool, Liverpool L69 3BX, UK and [7]State Key Laboratory of Complex, Severe, and Rare Diseases, Institute of Clinical Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing 100730, China

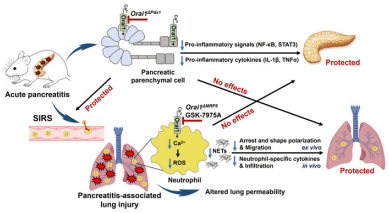
*Address correspondence to L.W. (e-mail: wenli7007@gmail.com)
#Mengya Niu and Xiuli Zhang contributed equally to this work.

Abstract

Acute pancreatitis is initiated within pancreatic exocrine cells and sustained by dysregulated systemic inflammatory responses mediated by neutrophils. Store-operated Ca^{2+} entry (SOCE) through ORAI1 channels in pancreatic acinar cells triggers acute pancreatitis, and ORAI1 inhibitors ameliorate experimental acute pancreatitis, but the role of ORAI1 in pancreatitis-associated acute lung injury has not been determined. Here, we showed mice with pancreas-specific deletion of *Orai1* ($Orai1^{\Delta Pdx1}$, ~70% reduction in the expression of Orai1) are protected against pancreatic tissue damage and immune cell infiltration, but not pancreatitis-associated acute lung injury, suggesting the involvement of unknown cells that may cause such injury through SOCE via ORAI1. Genetic ($Orai1^{\Delta MRP8}$) or pharmacological inhibition of ORAI1 in murine and human neutrophils decreased Ca^{2+} influx and impaired chemotaxis, reactive oxygen species production, and neutrophil extracellular trap formation. Unlike pancreas-specific *Orai1* deletion, mice with neutrophil-specific deletion of *Orai1* ($Orai1^{\Delta MRP8}$) were protected against pancreatitis- and sepsis-associated lung cytokine release and injury, but not pancreatic injury in experimental acute pancreatitis. These results define critical differences between contributions from different cell

Submitted: 20 September 2023; **Revised:** 19 October 2023; **Accepted:** 20 October 2023

types to either pancreatic or systemic organ injury in acute pancreatitis. Our findings suggest that any therapy for acute pancreatitis that targets multiple rather than single cell types is more likely to be effective.



Key words: ORAI1; neutrophils; acute lung injury; early treatment

Introduction

Acute pancreatitis is a painful and life-threatening inflammatory disorder of the exocrine pancreas, the mortality of which approaches 30% among patients with severe acute pancreatitis, but currently there are no targeted therapies available.[1,2] Pancreatitis severity and outcomes are determined not just by the degree of localized pancreatic injury, but more importantly by the presence of persistent organ failure, most commonly pancreatitis-associated acute lung injury. Pancreatitis is initiated within pancreatic exocrine cells and sustained by dysregulated systemic inflammatory responses.[3,4] Activated neutrophils are the first infiltrating cells into the injured pancreas, amplifying localized pancreatic injury and, through further local and systemic activation, contributing to the progression of organ failure, including acute lung injury.[5,6] It is not known; however, the extent to which pancreatitis-associated acute lung injury is dependent on neutrophils alone, nor on which intracellular signaling mechanisms this contribution depends.

We and others have previously shown that ORAI1 inhibitors prevented and/or markedly ameliorated acute pancreatitis in mice, primarily through prevention of Ca^{2+}-associated pancreatic acinar cell death.[7–11] Because of these findings, ORAI1 inhibitors are currently under active clinical development to treat acute pancreatitis.[12] Limited studies also suggest ORAI1 is a principal Ca^{2+} entry channel expressed in neutrophils that may mediate neutrophil functions. ORAI1 deficiency (*Orai1*$^{+/-}$) results in the defects of neutrophil Ca^{2+} signaling, normally associated with neutrophil migration and activation in response to inflammatory stimuli;[13–15] both neutrophil recruitment and phagosomal reactive oxygen species (ROS) production are dependent on ORAI1-mediated store-operated calcium entry (SOCE).[16,17] In this study, we sought to ascertain the role of neutrophil SOCE via ORAI1 in neutrophil Ca^{2+} signal, innate immune functions, and acute pancreatitis to determine the contributions of neutrophil- vs pancreas-specific ORAI1 to pancreatic and lung tissue damage, using cell-specific ORAI1-deficient mice.

Here, we found that deletion or inhibition of ORAI1 in murine and human neutrophils resulted in a decrease in Ca^{2+} influx and impairment of multiple neutrophil functions. Mice with neutrophil-, but not pancreas-specific deletion of *Orai1* were protected against pancreatitis-associated acute lung injury, while those with pancreas-specific, but not neutrophil-specific deletion of *Orai1* were protected against tissue injury in experimental acute pancreatitis. These data show that pancreatic and lung injury are separately dependent on pancreatic parenchymal and activated neutrophils, respectively, demonstrating critical but different roles for these two separate cell populations mediated by SOCE via ORAI1.

Materials and Methods

Animals

Six- to eight-week-old weighing 20–22 g C57BL6/J mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Orai1$^{f/f}$ mice were kindly gifted by Professor Bo Xiao from the State Key Laboratory of Biotherapy, Sichuan University. MRP8-Cre and Pdx1-Cre mice were purchased from the Jackson Laboratories (Bar Harbor, USA). To generate neutrophil-specific Orai1-deficient mice, MRP8-Cre mice were crossed with Orai1$^{f/f}$ mice, respectively. To generate pancreas-specific Orai1-deficient mice, Pdx1-Cre mouse line was crossed with Orai1$^{f/f}$ mice. All the control mice are the littermates of each strain. Mice were housed at 22°C with a 12-h light-dark cycle and maintained on a standard laboratory chow diet with free access to food and water in the Shanghai Model Organisms Center (Shanghai, China). All mice were allocated into each experimental group in a completely randomized manner using a randomized table ($n = 6–8$ per group).

Induction of Experimental Models of Acute Pancreatitis and Sepsis

Caerulein hyperstimulation acute pancreatitis was induced by 10 hourly intraperitoneal injections of caerulein (100 μg/kg). Mice from the littermate controls received equal volume injections of saline. Humane euthanasia was 12 h after the induction. Biliary acute pancreatitis was induced by retrograde biliopancreatic ductal injection with 2% sodium taurocholate (5 μL/min over 10 min by infusion pump). Humane euthanasia was 24 h later. Mice from the sham group received laparotomy only. Sepsis was induced by intraperitoneal injection of 40 mg/kg of lipopolysaccharide. Humane euthanasia was 24 h later to assess disease severity, and the survival rate was monitored up to 80 h.

Isolation of Murine Bone Marrow-Derived Neutrophils

Bone marrow-derived neutrophils (BMDNs) were isolated by using discontinuous Percoll gradients, as previously described.[18] Briefly, following the surgical dissection of the bones, the femur and tibia were flushed with sterile PBS (Sangon, Shanghai, China) and passed through a 70 μm sterile filter (Fisher Scientific, Waltham, USA) to obtain the crude bone marrow cells. After centrifugation at 600 *g* for 5 min at 4°C with the brake off, cells were resuspended in 4 mL PBS and loaded on top of a discontinuous density gradient (62% and 81% Percoll) and centrifuged at 1500 *g* for 20 min at 4°C with the brake off. BMDNs were collected between 62% and 81% Percoll layers and suspended in 4 mL Red Cell Lysis Buffer (eBiosciences, multispecies) for 5

min on a rocker, washed with PBS, and centrifuged at 600 *g* for 5 min before resuspending in RPMI1640 (Hyclone, Logan, USA). Trypan blue dye exclusion indicated that cell viability was 95%–97% after the isolation. The purity of the isolated neutrophils was routinely 90%, as confirmed by flow cytometric analysis.

Isolation of Human Peripheral Blood Polymorphonuclear Neutrophils and in vitro Cultures

Neutrophils from human peripheral blood were isolated by using Polymorphprep (Axis-Shield, Norway) according to the manufacturer's instruction. Briefly, in this one-step centrifugal technique, the polymorphonuclear cells were centrifuged to the bottom of the tube together with the erythrocytes. The neutrophil population was obtained after washing twice with HBSS and resuspension in cell culture medium, consisting of RPMI 1640 medium supplemented with 2% heat-inactivated human serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Calcium Measurement and Quantify of Cell Migratory Phenotype

Freshly isolated BMDNs were resuspended in HEPES buffer containing $CaCl_2$ (1.2 mmol/L) to evaluate cytosolic Ca^{2+} signals as previously. The cells were incubated with Ca^{2+} dye Fluo-4AM (7 μM, Invitrogen, Carlsbad, CA, USA) for 30 min on poly-L-lysine-coated slides. Afterwards, the cells of different groups were imaged while they were mounted on a perfusion chamber (Leica, Germany). At a wavelength of 488 nm, the loaded cells were stimulated, and every 10 s, emission signals longer than 515 nm were recorded. Ca^{2+} signals were recorded for the first 60 s of the experiment using HEPES with 1.2 mM Ca^{2+} (pH 7.4), followed by 5 min of stimulation with N-formyl Met-Leu-Phe (fMLP, 1 nM), then 20 min of recording with GSK-7975A. The recordings were analyzed by ImageJ. Each cell's fluorescence intensity (F) was converted to a ratio of F/F0 after being normalized to the background fluorescence (F0). Then the numbers of arrest and polarized neutrophils were quantified, and cells having a length-to-width ratio more than 1.5 were termed polarized, while those that moved less than 4 μm in 10 s were considered arrested.

Migration Assay

Neutrophil migration was assessed by transwell migration assay. Briefly, BM-derived neutrophils (2×10^5 cells) were resuspended in 200 μL of M medium and seeded in the upper chamber of 24-well transwells of 5-mm pore size (Costar, Corning, NY, USA). Cells were pretreated with GSK-7975A or vehicle for 30 min. Thereafter, media supplemented with C-X-C motif ligand 2 (CXCL2, 10 nmol/L) was added into the bottom chamber, and the cells were incubated at 37°C in 5% CO_2 for 6 h. After the incubation period, the transwell inserts were removed, and the cells from the bottom chamber were collected, centrifuged at 1400 revolutions per minute for 5 min, and counted to assess for migration.

Measurement of Intracellular ROS Production

Intracellular ROS production was determined by luminol-enhanced chemiluminescence, as previously described. Briefly, cells (2×10^5) were plated on a 96-well plate and then pre-treated with GSK-7975A (1 or 10 μmol/L) or vehicle for 30 min. Baseline values for the neutrophil suspensions were measured for 10 min with a synergy H1 plate reader (BioTek, Vermont, USA), then the cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and recorded every 1 min for 30 min (Sigma-Aldrich, St. Louis, MO, USA).

Measurement of Neutrophil Extracellular Traps Formation

Neutrophil extracellular traps (NETs) formation was determined by SYTOX Green staining, as previously described.[19,20] Briefly, freshly isolated neutrophils (1×10^5 cells) were cultured in 96-well plates and stimulated with PMA (50 ng/mL) for 3 h, with or without GSK-7975A (0.1–10 μM) pretreatment. After the incubation, SYTOX Green (5 μM), a cell-impermeant DNA binding dye, was added and incubated for 10 min before imaging. 6–8 systematically fields at × 200 magnification were obtained, and 5 selected fields were used by measuring the relative area of fluorescence per high-power field. The degree of the NETs formation was obtained and the area occupied by NETs was analyzed with ImageJ software. Unstimulated neutrophils were used as the control.

Flow Cytometry

Lung immune cells isolated from mice were stained with the following surface marker antibodies: Pacific Blue-conjugated anti-CD45 (Clone S18009F), FITC-conjugated anti-CD11b (Clone M1/70), APC-conjugated anti-CD11c (Clone N418), PerCP-Cyanine5.5-conjugated anti-Ly6C (Clone HK1.4), APC-conjugated anti-Ly6G (Clone 1A8-Ly6g), PE/Cyanine7-conjugated anti-F4/80 (Clone BM8), PE-conjugated anti-SiglecF (Clone 1RNM44N). Flow cytometry was conducted by FACSCanto II (BD Biosciences, Franklin, NJ, USA), and data were analyzed using FlowJo software (FlowJo, Ashland, OR).

Bronchioalveolar Lavage

Mice were euthanized, and their lungs were lavaged three times with phosphate-buffered saline (PBS). The recovered bronchioalveolar lavage (BAL) fluids were centrifuged. BAL cells were subjected to RNA extraction for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) detection.

Confirmation of Orai1 Deletion

To confirm neutrophil- and pancreas-specific deletion of *Orai1*, total RNA was extracted from bone marrow. RT-qPCR was performed to determine the relative expression of Orai1, using the following specific primers: Orai1 forward: 5′-GCTGCTCTGCTGGGTCAAGTTC-3′ and Orai1 reverse: 5′-GCTGCTGTCGCTGTGGTTGG-3′. Samples were normalized to β-actin and expressed as fold change relative to control.

Measurement of Serum Amylase and Lipase

Blood samples were centrifuged at 400 *g* for 20 min at 4°C to obtain serum. The concentrations of amylase and lipase in serum were measured by a Roche Analyzer (Roche Diagnostics, Basel, Switzerland).

Histology and Immunohistochemistry

Pancreatic tissue and lung tissue were fixed in 4% formalin, embedded in paraffin, and were processed for H&E or immunohistochemistry staining. Pancreatic evaluation was performed

on 10 random fields (×200) by 2 blinded independent investigators grading (scale, 0–3) edema, inflammatory infiltration, and necrosis, calculating the means ± SEM (≥6 mice/group). The lung injury score was evaluated on 10 random fields (×200) by two blinded independent investigators grading (scale, 0–3) alveolar thickening and inflammatory infiltration,[21] calculating the means ± SEM (≥6 mice/group). Immunohistochemistry for neutrophil infiltrate marker Ly6G was performed on paraffin-embedded pancreatic tissue sections after xylene deparaffinization, graded ethanol rehydration, and antigen retrieval. Primary antibodies of rabbit anti-mouse Ly6G (1:500) were used and incubated overnight at 4°C, respectively. Then the HRP-labeled secondary antibodies were incubated at room temperature for 50 min. Then visualized by using DAB chromogenic reaction system (Vector Laboratories, Burlingame, CA, USA).

Western Blotting

Pancreatic tissues were lysed in RIPA buffer containing 1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. The protein concentration was quantified by using the BCA Protein Assay Kit (Epizyme ZJ101). Equal proteins were loaded on Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk for 1 h, the membranes were probed with primary antibodies (STAT3 Rabbit mAb, AbClonal, A19566; Phospho-Stat3 (Tyr705) Antibody, 9131S, Cell Signaling Technology; β-Actin (8H10D10) Mouse mAb, 3700S, Cell Signaling Technology; Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb, 3033S, Cell Signaling Technology; NF-κB p65 (D14E12) Rabbit mAb, 8242S, Cell Signaling Technology) overnight at 4°C and then incubated with the secondary antibodies (800 CW anti-Rabbit IgG, LI-COR, 926-32213). The immunoblots were recorded with the Odyssey infrared imaging system (LI-COR, Lincoln, Nebraska).

Lung Epithelial Permeability Assay

To measure permeability changes following acute pancreatitis in *Orai1*$^{\Delta MRP8}$ and *Orai1*$^{f/f}$ mice, fluorescein isothiocyanate (FITC)-dextran leakage from the airways into the circulation was assessed, as previously described. The FITC-Dextran (4 KDa) solution was prepared at a working concentration of 4 mg/mL in sterile PBS. Twenty-three hours after the onset of acute pancreatitis, the FITC-Dextran solution was administered intranasally at a dosage of 10 mg/kg by carefully pipetting 25 μL of the FITC-Dextran solution onto each of the mouse's nostrils at a slow and steady pace. One hour post-inhalation, the mice were euthanized, and blood was collected through cardiac puncture into a EDTA (60 mg/mL, 10 μL)-containing tube. Plasma was obtained by centrifuged at 7000 rpm for 10 min. The fluorescence intensity (FI) of FITC-Dextran in the plasma was measured using a Synergy H1 plate reader (BioTek) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Study Approval

All the experiments involving animals were conducted under the principle for replacement, refinement, and reduction (the 3Rs)[22] and according to the legislation on the protection of animals and were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine (SYXK 2013-0050, Shanghai, China). The collection and use of human samples were approved by the Research Ethics Committee of Shanghai General Hospital (2020-047).

Statistical Analysis

Data were shown as mean ± SEM and analyzed by GraphPad Prism 7.0 software. Comparisons between groups were performed by using analysis of variance. A P-value $< .05$ was considered statistically significant.

Results

Pancreas-Specific Deletion of ORAI1 Protects Against Pancreatic Tissue Damage During Acute Pancreatitis But Does Not Prevent Pancreatitis-Associated Acute Lung Injury

Since acute pancreatitis is initiated within pancreatic acinar and/or ductal cells and we have shown previously that Orai1 inhibitors protected against acute pancreatitis,[7,8] we examined the impact of pancreas-specific deletion of ORAI1 on pancreatic tissue injury. We crossed *Orai1*$^{f/f}$ mice,[23] a gift from Dr Bo Xiao with a pancreas-specific Cre line (Pdx1-Cre) to generate pancreas-specific *Orai1* deficient (*Orai1*$^{\Delta Pdx1}$) mice and RT-qPCR analysis confirmed a successful deletion of *Orai1* in the pancreas (Figure 1A). We examined the impact of pancreas-specific deletion of *Orai1* on localized pancreatic tissue damage. We found that during acute pancreatitis induced by retrograde biliopancreatic infusion of sodium taurocholate, pancreas-specific deletion of *Orai1* resulted in a reduction of overall pancreatic histopathological severity scores, including each component of edema, inflammatory infiltration, and necrosis (Figure 1B and C). Serum amylase and lipase were reduced in *Orai1*$^{\Delta Pdx1}$ mice (Figure 1D). Next, we examined the effect of pancreas-specific deletion of *Orai1* on caerulein-induced pancreatitis and found that this deletion resulted in a marked reduction of pancreatic histological severity, including edema, inflammatory infiltration, and pancreatic necrosis (Supplementary Figure 1A and B). Serum amylase and lipase were similarly reduced in *Orai1*$^{\Delta Pdx1}$ mice (Supplementary Figure 1C).

Macrophages and neutrophils are two major immune cell types infiltrating into the pancreas early during acute pancreatitis, playing an important role in the severe inflammatory response to the primary injury of the acinar cells.[20,24,25] We found that during biliary and caerulein acute pancreatitis, pancreas-specific deletion of *Orai1* caused a marked reduction in pancreatic neutrophil and macrophage infiltration as assessed by Ly6G and F4/80 immunostaining, respectively (Figure 1E and Supplementary Figure 1D). IL-6 trans-signaling via STAT3 phosphorylation at Y705 has been shown to mediate severe acute pancreatitis and pancreatitis-associated lung injury.[26] The transcription factor nuclear factor-κB (NF-κB) is the master regulator of inflammation during acute pancreatitis, activation of which can induce expression of inflammatory genes including *Tnf*, *Il6*, *Il1b*, and *Spi2a*.[27,28] We found that expression of phosphorylated STAT3 at Y705, phosphorylated p65, and total p65 was markedly decreased with pancreas-specific *Orai1* deletion in biliary acute pancreatitis although not in caerulein-induced acute pancreatitis (Figure 1F and Supplementary Figure 1E). The expression of several pro-inflammatory genes, including *Il1b* and *Tnf*, was reduced in both acute pancreatitis models (Figure 1G and Supplementary Figure 1F). Luminex multiplex assays of serum showed that most of pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-17A, TNF-α, G-CSF, MCP-1, MIP-1α, RANTES, Eotaxin, IL-3, and IL-9 were reduced by pancreas-specific *Orai1* deletion (Figure 1H), confirming a role for pancreas-specific SOCE in the

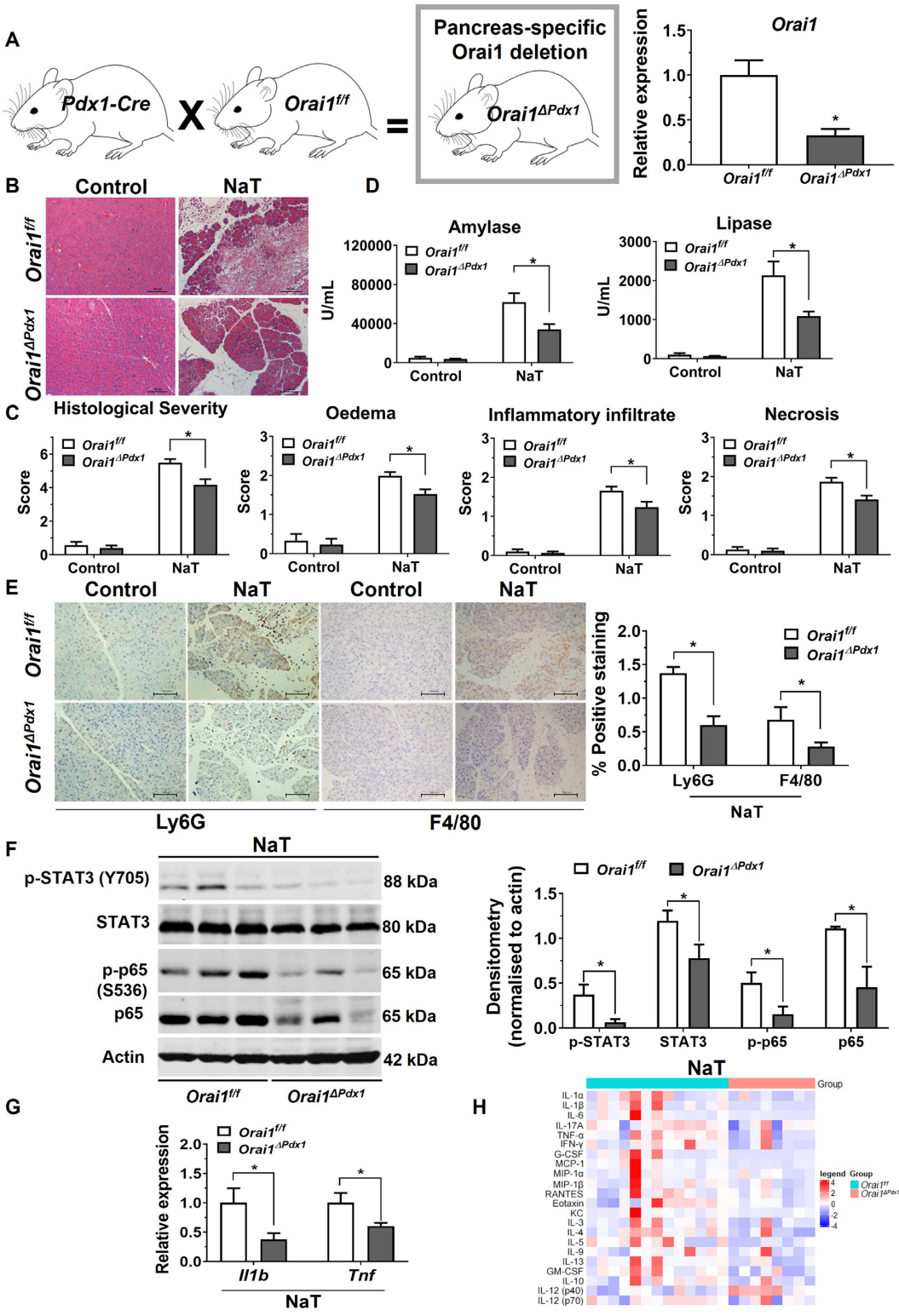


Figure 1. Pancreas-specific deletion of *Orai1* protects against localized pancreatic tissue damage and inflammation. (A) Schematic diagram for generation of pancreas-specific *Orai1*-deficient mice and *Orai1* expression in pancreas tissue from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice. (B) Representative H&E images of the pancreas (original magnification, ×200) from the control and pancreatitis induced by retrograde biliopancreatic ductal infusion of sodium taurocholate (NaT) from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice. (C) Scoring of overall histological severity and sub-scoring for edema, inflammatory infiltrate, and necrosis. (D) Serum amylase and lipase. (E) Representative images of immunohistochemical staining of Ly6G and F4/80 for pancreatic tissue from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice in NaT-induced pancreatitis (left) and positive staining are quantified (right). (F) Immunoblotting for phospho-STAT3 (Y705), phosphor-p65(S536), and p65. (G) Transcript expression of genes (*Il1b* and *Tnf*) from the pancreatic tissues of $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice. (H) Serum cytokines assessed by Luminex multiplex assay from pancreatitis induced by NaT from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice. Data were presented as mean ± SEM (n = 5–10 per group), $*P < .05$, n.s., not significant, compared to $Orai1^{f/f}$. NaT, pancreatitis induced by retrograde biliopancreatic ductal infusion of sodium taurocholate.

initiation of pancreatic tissue damage and of systemic inflammation.

Pancreatitis-associated acute lung injury is a major complication of severe acute pancreatitis and the leading cause of early mortality from acute pancreatitis. Acute lung injury is induced by the release of multiple cytokines and chemokines from the pancreatic parenchyma and infiltrating macrophages and neutrophils, followed by migration of these immune cells through systemic circulation to the lungs.[25,29] We examined the impact of pancreas-specific deletion of *Orai1* on pancreatitis-associated acute lung injury by blinded scoring of alveolar thickening and inflammatory infiltration, and found little change resulting from this deletion in both sodium taurocholate- and cerulein-induced pancreatitis (Figure 2A–D). This may be partly due to the incomplete deletion of *Orai1*, resulting in less profound inhibitory effect on pancreatic injury and limited change of lung injury. To confirm these findings, we examined immune cells infiltrating the lung by multi-channel flow cytometry. The percentages of neutrophils ($CD45^+CD11b^+Ly6g^+$), interstitial macrophages ($CD45^+CD11b^+Ly6g^-F4/80^+CD11c^-SiglecF^-$), and alveolar macrophages ($CD45^+CD11b^-F4/80^+CD11c^+SiglecF^+$) were quantified as shown in Supplementary Figure 2 for the gating strategies. The percentage of neutrophils, alveolar macrophages, and interstitial macrophages in the lung remained unchanged between $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice (Figure 2E and F), and while global diminution of infiltrating immune cell numbers is not ruled out, these data suggest there are other cell sources of *Orai1* that contribute to mediating pancreatitis-associated acute lung injury.

ORAI1 in Neutrophils Mediates Intracellular Ca^{2+} Signals and Cell Migratory Phenotype

Neutrophils are the first immune cells to reach an inflammatory site and contribute to the development of pancreatitis-associated acute lung injury.[30] We previously observed a significant increase in lung infiltration and migration of neutrophils, but not of macrophages.[31] Therefore, we hypothesized that neutrophil ORAI1 has a crucial role in pancreatitis-associated acute lung injury. We firstly examined the role of ORAI1 in the function of neutrophils per se. To delete ORAI1 selectively in neutrophils, we crossed $Orai1^{f/f}$ mouse line with a MRP8-Cre line that was targeted neutrophils, but not other myeloid cells.[32] RT-qPCR confirmed that ORAI1 expression in BMDNs from $Orai1^{\Delta MRP8}$ mice was markedly reduced (Figure 3A). Using these mice, we examined whether neutrophil intracellular Ca^{2+} dynamics are mediated by ORAI1 using fMLP, which elevates neutrophil intracellular Ca^{2+} through G protein-coupled receptor-dependent store release.[33] We found that ORAI1 deletion in neutrophils ($Orai1^{\Delta MRP8}$) inhibited fMLP-induced Ca^{2+} influx (Figure 3B).

The elevation of intracellular Ca^{2+} elicited by fMLP can activate rolling of neutrophils that transit to arrest and subsequent shape-polarized,[13] enabling neutrophil migration. During intracellular time-lapse Ca^{2+} signal imaging, we observed the altered patterns of neutrophil rolling, arrest, and shape polarization. Therefore, we further quantified neutrophil arrest as the cells moving less than 4 μm (approximately half a cell diameter) in 10 s and neutrophil shape polarization as cells with a length/width ratio greater than 1.5. We found that the number of arrested neutrophils from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice remained unaltered until after 380 s, when the number of arrested neutrophils from $Orai1^{\Delta MRP8}$ mice was markedly increased (Figure 3C), while the number of shape-polarized neutrophils was decreased (Figure 3D). These data show that deletion of ORAI1 impaired the efficiency of neutrophil transition from arrest to the migratory phenotype and suggest that this transition is directly dependent on SOCE.

To test this interpretation, we treated BMDNs with GSK-7975A (MCE, USA) to block SOCE through ORAI1 channels and observed that GSK-7975A significantly inhibited fMLP-induced intracellular Ca^{2+} entry at both 1 and 10 μM with a trend toward significance for an increased number of arrested and decreased number of shape-polarized neutrophils (Figure 3E–G). To determine the clinical relevance of our findings, we freshly isolated neutrophils from human peripheral blood and treated these with GSK-7975A. Consistent with our murine data, we found that inhibition of SOCE through ORAI1 in human neutrophils led to a reduction in Ca^{2+} influx induced by fMLP (Figure 3H). The number of arrested cells was increased (Figure 3I), while the number of shape-polarized cells was reduced by decreased SOCE (Figure 3J). Collectively, these data demonstrate that ORAI1 is a key mediator of intracellular Ca^{2+} signals in neutrophils that contribute to their migratory phenotype.

ORAI1 in Neutrophils Modulates Chemotaxis, Intracellular ROS Production, and Neutrophil Extracellular Trap Formation

Ca^{2+} is required for multiple neutrophil functions in response to inflammatory insults or host defense.[34] Therefore, we examined whether ORAI1 is responsible for these neutrophil responses. Firstly, we observed that deletion of Orai1 in neutrophils prevented neutrophil migration stimulated by a potent chemoattractant CXCL2 (Figure 4A). Intracellular ROS production induced by PMA, a well-known activator of neutrophils,[35] was significantly reduced in *Orai1*-deficient neutrophils (Figure 4B). NETs are extracellular web-like structures decorated with citrullinated histones that have been shown to cause cell damage.[36] The formation of NETs during bacterial infection or sterile inflammation is mediated by peptidyl arginine deiminase-4, the activity of which is Ca^{2+}-dependent.[37] Therefore, we examined whether the formation of NETs was mediated by ORAI1. We found that neutrophil-specific deletion of *Orai1* abolished the formation of NETs induced by PMA (Figure 4C). Similar results were also observed with the treatment of GSK-7975A (Figure 4D–F). Specifically, neutrophil migration induced by CXCL2 was prevented by GSK-7975A, and intracellular ROS production stimulated by CXCL2 or PMA was reduced to a lesser extent, while the formation of NETs was significantly reduced with a higher concentration of GSK-7975A (10 μM). Similarly, in human peripheral blood neutrophils, inhibition of ORAI1 prevented cell migration stimulated by CXCL2 (Figure 4G). Intracellular ROS production was decreased by GSK-7975A (Figure 4H), and the formation of NETs was similarly reduced (Figure 4I). These data demonstrate that ORAI1 mediates multiple neutrophil functions, including chemotaxis, intracellular ROS production, and NETs formation in both mouse and human neutrophils.

Neutrophil-Specific Deletion of ORAI1 Does Not Protect Against Pancreatic Injury in Experimental Models of Acute Pancreatitis

We examined the specific effects of neutrophil ORAI1 on acute pancreatitis. Following the induction of acute pancreatitis by retrograde biliopancreatic duct infusion of sodium taurocholate,[38] we found that mice with neutrophil-specific deletion of *Orai1* developed localized pancreatic injury to the same extent as

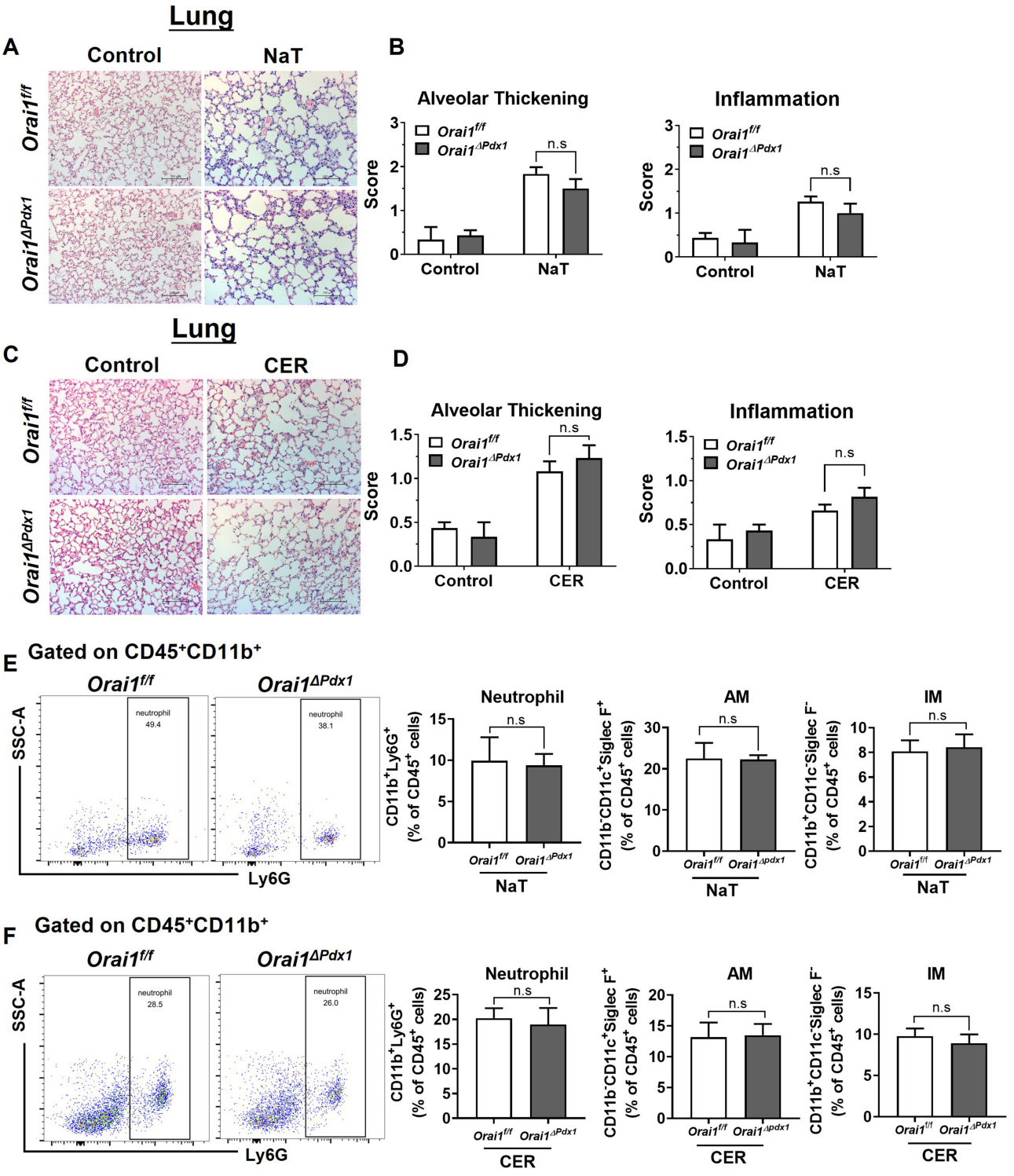


Figure 2. Pancreas-specific deletion of *Orai1* does not limit pancreatitis-associated lung tissue damage and inflammatory infiltration. (A) Representative images of lung H&E from the control and pancreatitis induced by NaT from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice. (B) Scores for alveolar thickening and inflammation. (C) Representative images of lung H&E from the control and pancreatitis induced by caerulein hyperstimulation (CER). (D) Scores for alveolar thickening and inflammation. (E) Lung leukocytes were analyzed by flow cytometry for neutrophil (gated on $CD45^+CD11b^+Ly6G^+$), alveolar macrophages (AM, gated on $CD45^+CD11b^-F4/80^+CD11c^+SiglecF^+$), and interstitial macrophages (IM, gated on $CD45^+CD11b^+Ly6g^-F4/80^+CD11c^-SiglecF^-$), isolated from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice in NaT-induced pancreatitis. (F) Lung leukocytes were analyzed by flow cytometry for neutrophil, AM, and IM, isolated from $Orai1^{f/f}$ and $Orai1^{\Delta Pdx1}$ mice in caerulein hyperstimulation pancreatitis. Data were presented as mean ± SEM ($n = 8$–10 per group), $*P < .05$, n.s., not significant, compared to $Orai1^{f/f}$. CER, caerulein hyperstimulation pancreatitis.

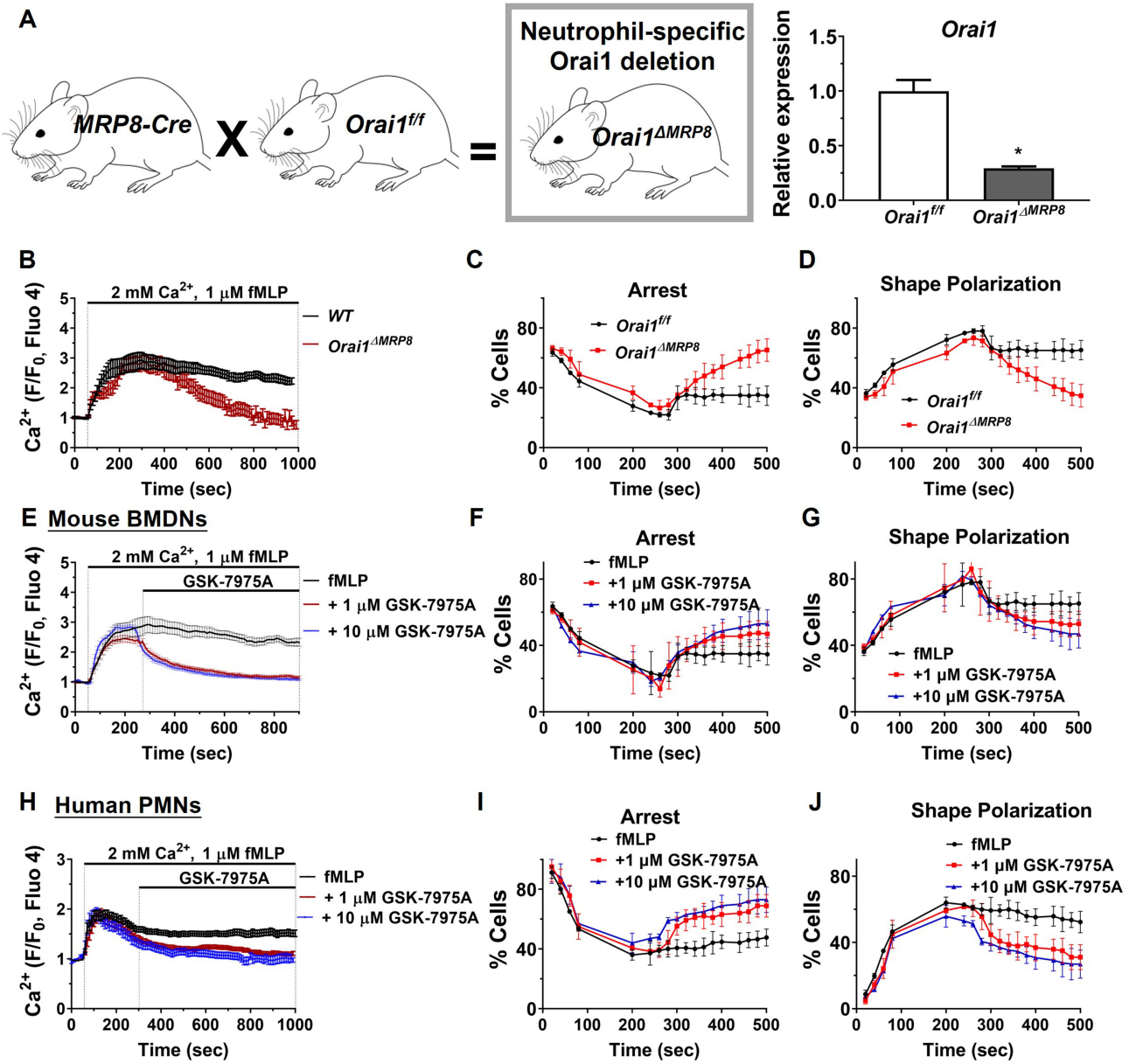


Figure 3. Genetic deletion and pharmacological inhibition of ORAI1 in neutrophils prevent fMLP-induced Ca^{2+} influx and neutrophil migratory phenotype. (A) Schematic diagram for generation of neutrophil-specific *Orai1*-deficient mice and Orai1 expression in mouse BMDNs from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. Mouse BMDNs were freshly isolated, loaded with Fluo 4 (7 μM) for 30 min, and washed with Hepes without Ca^{2+} (pH7.4). (B) Changes of cytosolic Ca^{2+} induced by fMLP in neutrophils from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. The percentage of cells from $Orai1^{f/f}$and $Orai1^{\Delta MRP8}$ mice that underwent (C) arrest and (D) shape polarization. (E) Changes of cytosolic Ca^{2+} induced by fMLP in mouse neutrophils with or without ORAI1 inhibitor, GSK-7975A (1 or 10 μM). The percentage of BMDNs with or without GSK-7975A that underwent (F) arrest and (G) shape polarization. (H) Changes of cytosolic [Ca^{2+}] induced by fMLP in human peripheral blood polymorphonuclear neutrophils (PMNs) with or without ORAI1 inhibitor, GSK-7975A (1 or 10 μM). The percentage of PMNs with or without GSK-7975A that underwent (I) arrest and (J) shape polarization. $N = 3$ independent isolation per condition, $*P < .05$, compared to $Orai1^{f/f}$.

$Orai1^{f/f}$ mice, as assessed by pancreatic histological severity scores and component scores for edema, inflammatory infiltrate, and necrosis (Figure 5A and B). Consistent with this, serum amylase and lipase were unaffected by *Orai1* deletion (Figure 5C). Similarly, during caerulein-induced pancreatitis, we found that mice with neutrophil-specific *Orai1* deficiency developed the same degree of localized pancreatic injury as assessed by pancreatic histological severity scores, including component scores for edema, inflammatory infiltration, and necrosis (Figure 5D and E). Again, serum amylase and lipase were unchanged by *Orai1* deletion (Figure 5F). Luminex analysis, however, showed that there was a downward trend in serum pro-inflammatory cytokines in the circulatory system (Figure 5G). One possible explanation for this last result is during acute pancreatitis, localized pancreatic injury may be more predominantly controlled by macrophages, instead of neutrophils since several studies showed that macrophages play a crucial role in mediating pancreatic tissue damages[39,40] and mice with macrophage depletion induced by clodronate-containing liposomes developed less severe acute pancreatitis.[41] Taken together, these data

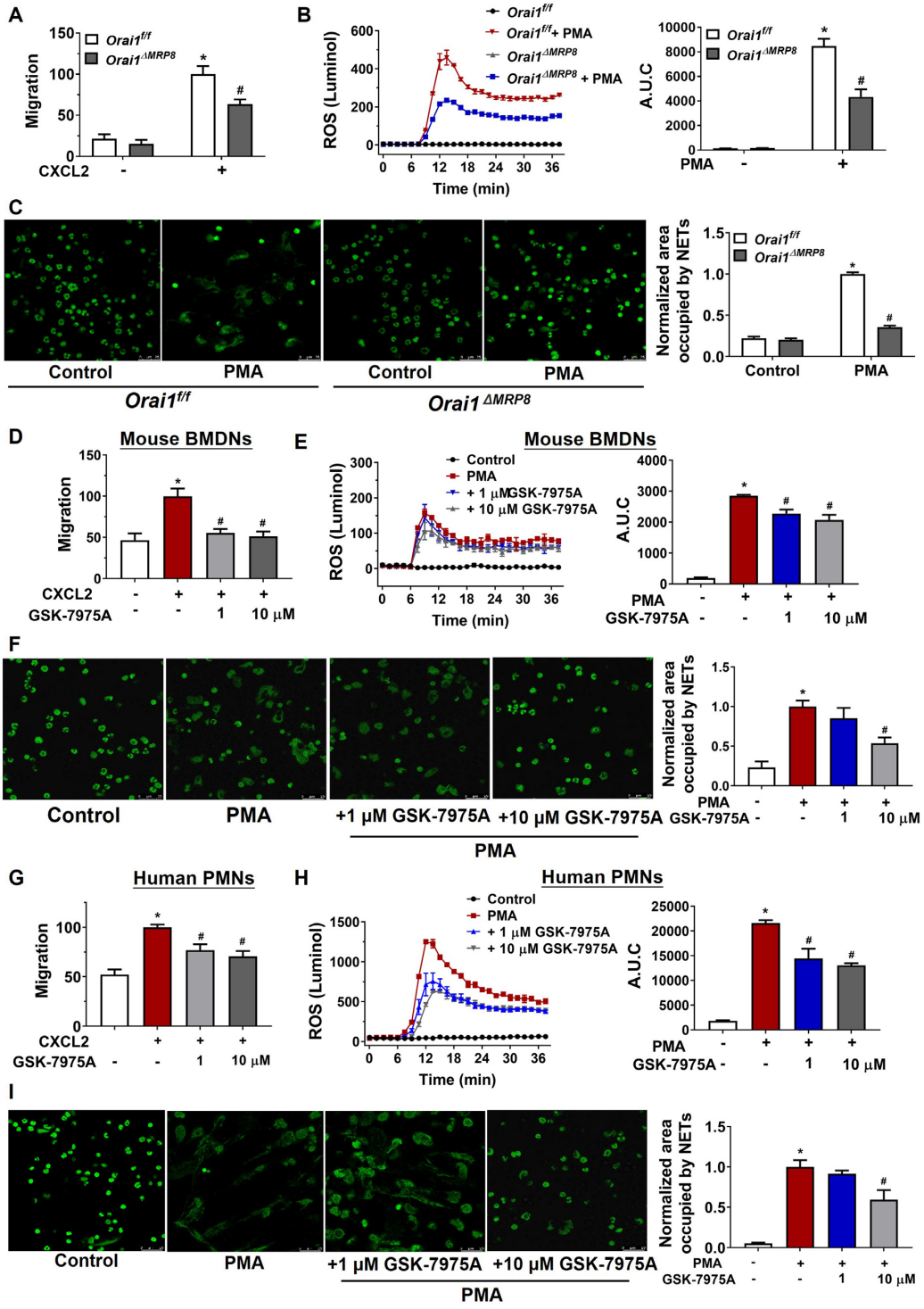


Figure 4. Genetic deletion and pharmacological inhibition of neutrophil ORAI1 inhibit neutrophil chemotaxis, ROS production, and formation of neutrophil extracellular traps. Mouse BMDNs or human peripheral blood polymorphonuclear neutrophils (PMNs) were freshly isolated, cultured in RPMI medium, and seeded in the upper chamber of 24-well transwells of 5-μm pore size. (A) Percentage of migrated mouse BMDNs from *Orai1*$^{f/f}$ and *Orai1*$^{\Delta MRP8}$ mice from transwell migration assay stimulated with CXCL2 in the presence or absence of GSK-7975A pretreatment. (B) Total ROS production of mouse BMDNs in response to PMA was assayed using luminol and further quantified by an area under the curve from *Orai1*$^{f/f}$ and *Orai1*$^{\Delta MRP8}$ mice. NETs were induced by treatment with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) for 3 h, detected by SYTOX Green using fluorescent microscopy (magnification: 63x), and quantified by the occupied area of NETs by Image J. (C) Representative images and quantification of area occupied by NETs from *Orai1*$^{f/f}$ and *Orai1*$^{\Delta MRP8}$ mice. (D) Percentage of migrated mouse BMDNs treated with GSK-7975A. (E) Total ROS production of mouse BMDNs treated with GSK-7975A in response to PMA and further quantified by an area under the curve. (F) Representative images and quantification of area occupied by NETs in BMDNs treated with GSK-7975A. (G) Percentage of migrated neutrophils from human PMNs. (H) Total ROS production of human PMNs treated with GSK-7975A in response to PMA and further quantified by an area under the curve. (I) Representative images and quantification of area occupied by NETs in human PMNs treated with GSK-7975A. $N = 3$ independent isolation per condition, $*P < .05$, compared to the control; $^{\#}P < .05$, compared to the CXCL2- or PMA-stimulated condition.

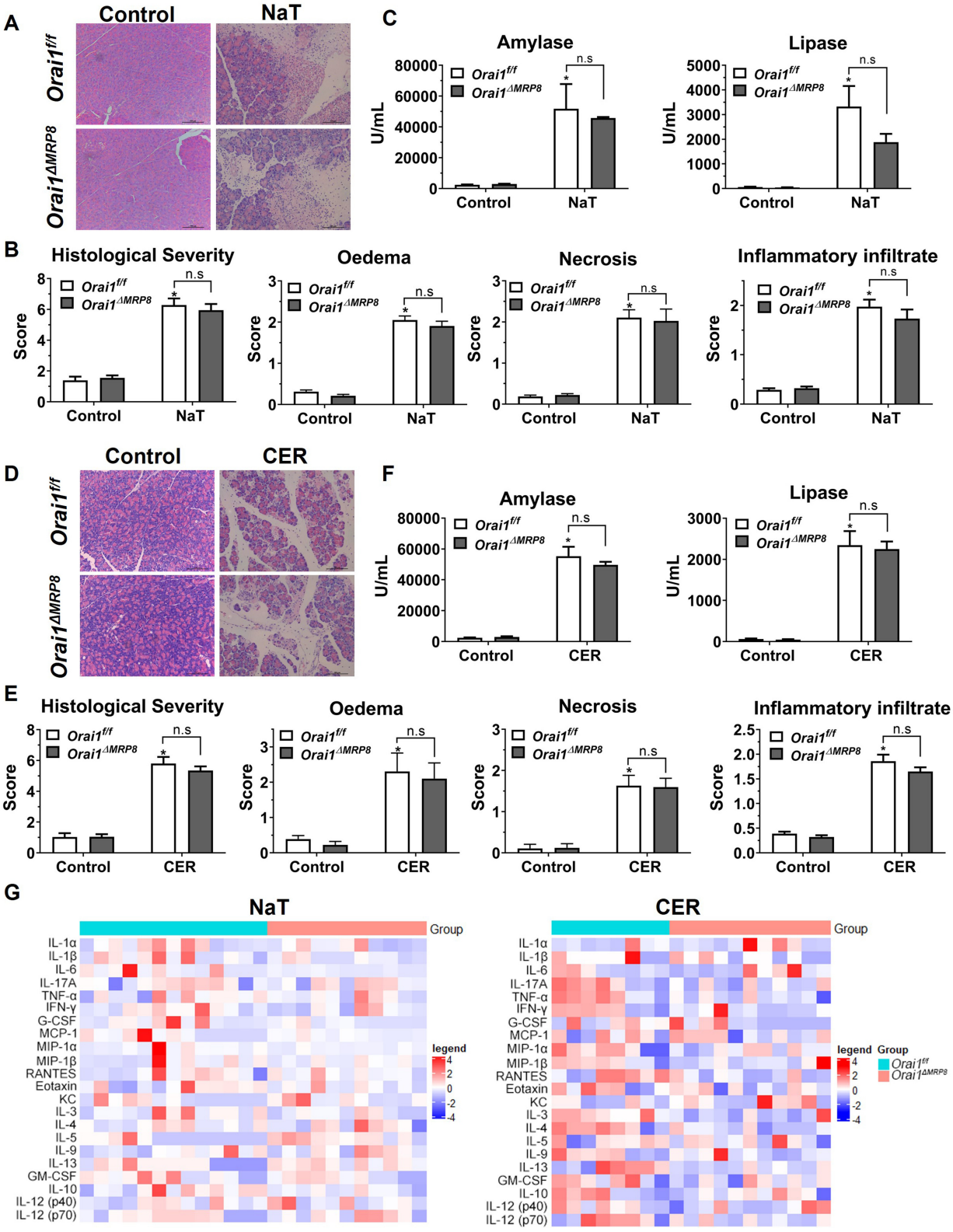


Figure 5. Neutrophil-specific deletion of *Orai1* does not alter disease severity in two models of experimental acute pancreatitis. (A) Representative H&E images of the pancreas (original magnification, ×200) from the control and pancreatitis induced by NaT from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. (B) Scoring of overall histological severity and sub-scoring for edema, inflammatory infiltrate, and necrosis. (C) Serum amylase and lipase. (D) Representative H&E images of the pancreas (original magnification, ×200) from the control and CER-induced pancreatitis from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. (E) Scoring of overall histological severity and sub-scoring for edema, inflammatory infiltrate, and necrosis. (F) Serum amylase and lipase. Data were presented as mean ± SEM ($n = 7$ animals per group). (G) Serum cytokines assessed by Luminex multiplex assay from pancreatitis induced by NaT or CER from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. $*P < .05$, compared to the controls, n.s, not significant compared to $Orai1^{f/f}$.

demonstrated that neutrophil-specific ORAI1 exhibits a limited role in mediating localized pancreatic injury during acute pancreatitis.

Neutrophil-Specific Deletion of Orai1 Protects Against Pancreatitis- or Sepsis-Associated Lung Injury by Inhibiting Neutrophil-Intrinsic Functions

We next assessed the effects of ORAI1 in neutrophils on pancreatitis-associated acute lung injury. During acute pancreatitis induced by retrograde biliopancreatic infusion of sodium taurocholate or caerulein injections, there were obvious pathological changes in the lung, including alveolar wall thickening and massive inflammatory cell infiltration. We found that neutrophil-specific deletion of *Orai1* alleviated alveolar thickening and decreased inflammatory cell infiltration as assessed by histopathological scoring of both experimental models of acute pancreatitis (Figure 6A–D). Compared to $Orai1^{f/f}$, mice with neutrophil-specific deletion of *Orai1* displayed a marked reduction in the percentage of neutrophils in the lung, but not alveolar or interstitial macrophages (Figure 6E and F), demonstrating that ORAI1 mediates neutrophil migration selectively in vivo. Furthermore, we collected BAL cells and found that the expression of pro-inflammatory cytokines, including *Il1b* and *Tnf*, in BAL was markedly decreased in $Orai1^{\Delta MRP8}$ mice (Supplementary Figure 3). These data suggest that neutrophil-specific deletion of *Orai1* protects against pancreatitis-associated acute lung injury by selectively inhibiting lung neutrophil infiltration.

Sepsis is a common and potentially fatal systemic illness and is the leading cause of death in hospitals,[42,43] which has similarities with and may complicate acute pancreatitis. Acute pancreatitis and sepsis are similar in that the extent of tissue damage or organ dysfunction is driven by dysregulated systemic activation of immune responses, which often determines disease outcome. Therefore, we also examined the impact of ORAI1 in neutrophils on sepsis-associated lung injury. As in acute pancreatitis, we found that neutrophil-specific deletion of Orai1 led to a significant reduction in alveolar wall thickening and inflammatory infiltration, although without affecting overall survival rate (Supplementary Figure 4A and B). Neutrophil infiltration into the lung was similarly reduced as assessed by immunostaining with Ly6G (Supplementary Figure 4C). Collectively, these data demonstrate that ORAI1 in neutrophils plays a crucial role in mediating acute lung injury in both acute pancreatitis and sepsis.

We then assessed whether neutrophil-specific deletion of *Orai1* affects lung the epithelial barrier and its permeability in acute pancreatitis by evaluating the flux from pulmonary cavity into the circulation of FITC-dextran by nasal inhalation (Figure 7A). Lung epithelial permeability remained the same in $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice (Figure 7B), suggesting deletion of Orai1 in neutrophils did not protect the impairment of lung epithelial integrity. Similarly, tight junction-related genes (*Cldn2*, *Cldn4*, *Cldn7*, *Krt16*, *Gjb4*, and *Itgb4*) remained unchanged in $Orai1^{f/f}$and $Orai1^{\Delta MRP8}$ mice during acute pancreatitis (Figure 7C). However, we checked the expression level of neutrophil-related genes or cytokines and found that those genes, including *Ly6g*, *Mif*, *Cxcl2*, *Mmp8*, and S100a9, were consistently down-regulated in $Orai1^{\Delta MRP8}$ mice (Figure 7D). Collectively, these data suggest that the protective effects of neutrophil-specific *Orai1* deletion on pancreatitis-associated acute lung injury occur primarily through inhibition of neutrophil-intrinsic functions.

Discussion

In this study, we have discovered that ORAI1 in pancreatic parenchymal cells mediates pancreatic but little acute lung injury in murine acute pancreatitis, while ORAI1 in neutrophils mediates neutrophils functions and acute lung injury but little pancreatic injury. The generalizability of these results was confirmed by the consistent changes seen in biochemical, histological, and immunological parameters, both in taurocholate- and caerulein-induced acute pancreatitis, with the role of SOCE through ORAI1 into neutrophils also confirmed in sepsis-induced acute lung injury. These findings advance those made in which SOCE through ORAI1 was shown to be a central mechanism in multiple models of acute pancreatitis and pancreatitis associated acute lung injury.[7,11,44]

Substantial work confirms that abnormal calcium signals causing $[Ca^{2+}]_i$ overload and injury in pancreatic parenchymal cells initiate acute pancreatitis.[45] SOCE through ORAI1 channels in pancreatic parenchymal and immune cells is a key trigger of acute pancreatitis.[7,11] We and others previously showed that intraperitoneal administration of an ORAI1 inhibitor (GSK-7975A or CM_128, also known as CM4620 and as Auxora) protects against Ca^{2+}-associated pancreatic acinar cell injury and experimental acute pancreatitis.[7–11] Here, using $Orai1^{\Delta Pdx1}$ mice, we have demonstrated that this pancreatic injury is primarily dependent on pancreatic parenchymal ORAI1. Deletion of pancreatic parenchymal cell ORAI1 significantly reduced both macrophage and neutrophil infiltration in the pancreas, but infiltration of neither in the lung. This is in keeping with the primary role of the pancreatic parenchyma in the initiation of acute pancreatitis and the major role of macrophages in pancreatic injury.[6,39–41,46,47] Other targeted approaches to prevent pancreatic acinar cell Ca^{2+} overload and pancreatic tissue injury in acute pancreatitis include inhibition of cytosolic Ca^{2+} release from the endoplasmic reticulum via the inositol triphosphate receptor with caffeine[48] or ryanodine receptor with dantrolene.[49–51] Alternatively, plasma membrane calcium ATPase activity can be enhanced with renalase to promote pancreatic acinar cell Ca^{2+} efflux, ameliorating caerulein-induced acute pancreatitis.[52] Blockade of the mechanosensitive, Ca^{2+}-permeable ion channel Piezo1 with spider venom peptide *Grammostola* mechanotoxin #4 reduced the severity of pressure-induced acute pancreatitis,[53] which with sufficient pressure activates further Ca^{2+} entry and overload via transient receptor potential (TRP) vanilloid subfamily 4 (TRPV4) channels, knockout of which also reduces pressure-induced acute pancreatitis severity.[54] Nevertheless, if, how, and to what extent these alternative approaches inhibit the specific contribution of neutrophils to the severity of acute pancreatitis and to pancreatitis-associated acute lung injury are undetermined.

Acute lung injury is the most common systemic complication of acute pancreatitis. The neutrophil is the major infiltrating immune cell in the lung central to acute lung injury in acute pancreatitis.[31,55] Here, extensive examination of the responses of mouse and human neutrophils confirmed and extended findings that identify SOCE through ORAI1 as a major Ca^{2+} signaling mechanism integral to many neutrophil functions.[34,56,57] Grimes et al. found that ORA1 and ORAI2 isoforms are the primary components of the neutrophil CRAC channel.[31] Derayvia showed that decreased SOCE in ORAI1-, ORAI2-, and ORAI1/2-deficient neutrophils impaired neutrophil phagocytosis, degranulation, leukotriene synthesis, and ROS production.[57] Our results, using genetic or pharmacological inhibition confirmed that neutrophil ORAI1 inhibition significantly

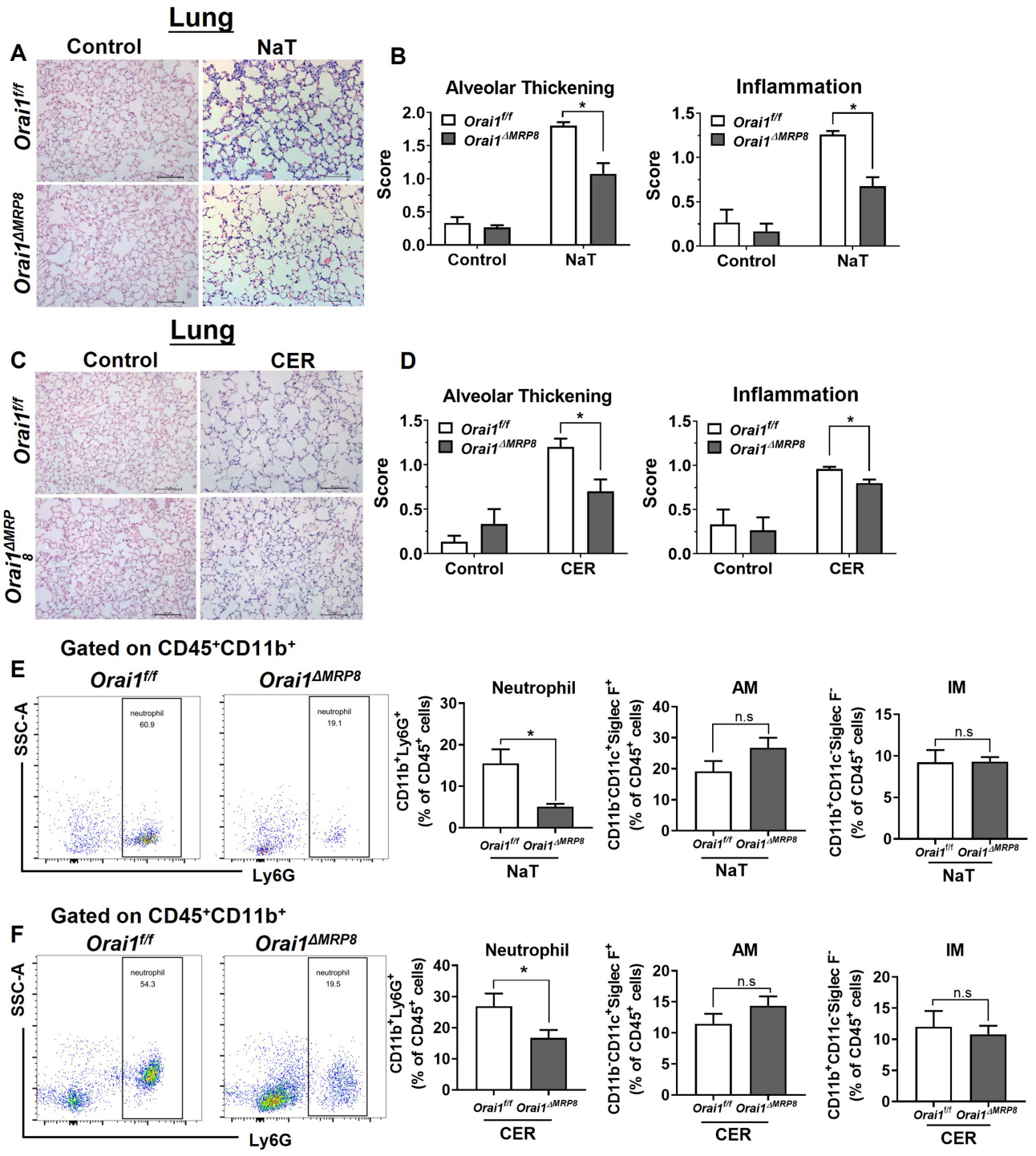


Figure 6. Neutrophil-specific deletion of *Orai1* protects against pancreatitis-associated lung tissue damage and inflammatory infiltration. (A) Representative images of lung H&E from the control and pancreatitis induced by NaT from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. (B) Scores for alveolar thickening and inflammation. (C) Representative images of lung H&E from the control and pancreatitis induced by CER from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice. (D) Scores for alveolar thickening and inflammation. (E) Lung leukocytes were analyzed by flow cytometry for neutrophil (gated on $CD45^+CD11b^+Ly6G^+$), alveolar macrophages (AM, gated on $CD45^+CD11b^-F4/80^+CD11c^+SiglecF^+$), and interstitial macrophages (IM, gated on $CD45^+CD11b^+Ly6g^-F4/80^+CD11c^-SiglecF^-$), isolated from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice in NaT-induced pancreatitis. (F) Lung leukocytes were analyzed by flow cytometry for neutrophil, AM, and IM, isolated from $Orai1^{f/f}$ and $Orai1^{\Delta MRP8}$ mice in caerulein hyperstimulation pancreatitis. Data were presented as mean ± SEM ($n = 5$–6 per group), $*P < .05$, n.s, not significant compared to $Orai1^{f/f}$.

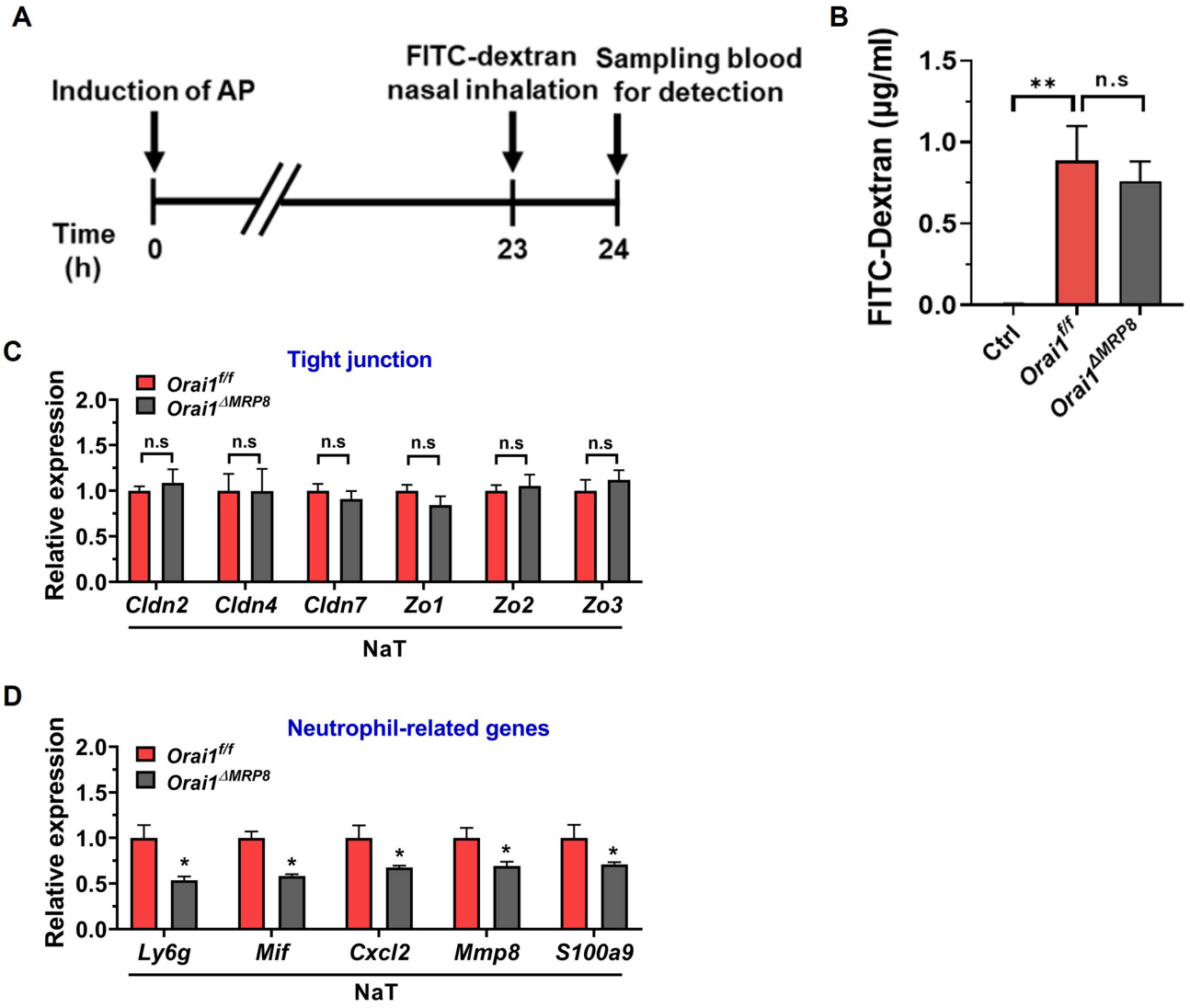


Figure 7. Neutrophil-specific deletion of *Orai1* does not alter lung tight junction integrity but reduces neutrophil-intrinsic gene expression. (A) Schematic diagram of lung epithelial permeability measurement. (B) The change of lung permeability in $Orai1^{\Delta MRP8}$ and $Orai1^{f/f}$ mice in NaT-induced pancreatitis. Genes related to (C) tight junction and (D) neutrophils validated by qRT-PCR from the lung of $Orai1^{\Delta MRP8}$ and $Orai1^{f/f}$ mice in NaT-induced pancreatitis. Data were presented as mean ± SEM ($n = 5$–6 per group), $*P < .05$, n.s, not significant compared to $Orai1^{f/f}$.

reduced neutrophil SOCE and ROS production, now identifying diminished shape change, migration, and NET formation of both murine and human neutrophils with such inhibition. Reducing neutrophil migration into the lung tissue has been proposed as a means of attenuating acute lung injury.[58] Liu showed that inhibiting neutrophil NETosis protects against lipopolysaccharide-induced acute lung injury.[59] In line with this, we found that deletion of neutrophil *Orai1* resulted in significantly reduced neutrophil infiltration and alveolar membrane thickening in the lung, and although this did not affect our measure of lung permeability, lung cytokine expression was also reduced by RT-qPCR analysis of the lung tissue. The role of SOCE through ORAI1 into neutrophils was also confirmed in sepsis-induced acute lung injury.

Marked reductions, but not complete suppression, of neutrophil functions were obtained by deletion of *Orai1*, which being incomplete could have limited the impact of neutrophil *Orai1* deletion on pancreatic injury. ORAI1 may be the principal SOCE channel in neutrophils, but there are others, notably ORAI2 and ORAI3, as well as TRP channels that may also allow receptor-operated Ca^{2+} entry.[56] Equivalent suppression of the sustained Ca^{2+} plateau elicited by fMLP was obtained using GSK7975A, however, despite the known inhibitory effect of this compound on ORAI2.[60] Deletion of neutrophil *Orai1* did not abolish the early peak of $[Ca^{2+}]_i$ from intracellular Ca^{2+} release, an initial activator of neutrophil functions,[34] and we did not examine Ca^{2+} independent controls on these, for example, in neutrophil exocytosis.[56] Previously, inhibition of nicotinamide adenine dinucleotide phosphate oxidase by deletion of neutrophil cytosolic factor 1 (*Ncrf1*, also known as *p47phox*) significantly reduced pancreatic injury in caerulein-induced acute pancreatitis,[61] but this deletion also inhibits pro-inflammatory M1 macrophages.[62] Deletion of *Orai1* from other cell types to inhibit SOCE may provide further detail on the insights gained in our work, for example, in specific monocyte-macrophage lineages, or stellate cells.

In clinical acute pancreatitis, pancreatic necrosis is significantly more frequent and more extensive in patients who develop organ failure, most frequently respiratory,[63–65] indicative of a loose correlation in severity between pancreatic and systemic organ injury. As acute pancreatitis is initiated in the pancreas, this correlation could be attributed either to changes in the pancreas as the driver of progression or responses out the pancreas having their own determinants, or amplification loops between the pancreas and other organs.[66] Our new work reported here demonstrates the importance of cell-specific intracellular networks with a common Ca^{2+} signaling toolkit that utilizes SOCE via ORAI1, each with a distinct and important role in acute pancreatitis. While caffeine, dantrolene, GsMTx4, and TRPV4 inhibitors ameliorate pancreatic injury in experimental acute pancreatitis, the impact of these compounds on systemic injury has not been adequately described, or there appears to be none.[48] These data contrast with those found here by separate deletion of pancreatic and neutrophil Orai1, demonstrating separate requirements for reduction of both pancreatic and acute lung injury in acute pancreatitis. Development of a drug for acute pancreatitis that inhibits a signaling mechanism common to multiple cell types, which each have different, deleterious actions in different organs, is thus far bore out by a Phase 2a clinical trial that found better tolerated solid food, less persistent systemic inflammatory response syndrome, and reduced hospitalization in acute pancreatitis from ORAI1 inhibition with Auxora,[12] further trials of which are currently ongoing.

Acknowledgment

We thank Dr Bo Xiao from the State Key Laboratory of Biotherapy, West China Hospital Sichuan University for kindly providing *Orai1*$^{f/f}$ mouse line.

Author Contributions

LW designed and supervised the study and interpreted the data. MN, XZ, ZW, BL, ZY, and LL acquired, analyzed, and interpreted the data. JB, JD, YZ, and LL provided technical support. LW, MN, and XZ drafted the manuscript. LW and LL revised the manuscript and obtained the funding. RS, SP, YZ, and LL participated in the intellectual discussions. All the authors approved the final edited version. The degree of contribution to the design, to performance of the experiments, and to manuscript writing were used to assign authorship order among co-first authors.

Supplementary material

Supplementary material is available at the *APS Function* online.

Funding

This work is supported by the National Natural Science Foundation of China (82122010, 82070659, and 81900585 to Li Wen, 32100635 to Liang Li), a Shanghai Pujiang Program (19PJ1408400 to Li Wen), the National High Level Hospital Clinical Research Funding (2022-PUMCH-E-003 to Li Wen), the CAMS Innovation Fund for Medical Science (2023-I2M-3-001 to Li Wen and 2022-I2M-1-004), and the UK National Institute for Health and Care Research Senior Investigator Award (to Robert Sutton). The funders did not have any role in study design, data collection, data analyses, interpretation, or writing of the report.

Conflict of Interest

None declared

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Gardner TB. Acute pancreatitis. *Ann Intern Med*. 2021;**174**(2):ITC17–ITC32.
2. Forsmark CE, Vege SS, Wilcox CM. Acute pancreatitis. *N Engl J Med*. 2016;**375**(20):1972–1981.
3. Lee PJ, Papachristou GI. New insights into acute pancreatitis. *Nat Rev Gastroenterol Hepatol*. 2019;**16**(8):479–496.
4. Cavaillon JM, Singer M, Skirecki T. Sepsis therapies: learning from 30 years of failure of translational research to propose new leads. *EMBO Mol Med*. 2020;**12**(4):e10128. doi: 10.15252/emmm.201810128.
5. Zheng L, Xue J, Jaffee EM, Habtezion A. Role of immune cells and immune-based therapies in pancreatitis and pancreatic ductal adenocarcinoma. *Gastroenterology*. 2013;**144**(6):1230–1240.
6. Habtezion A, Gukovskaya AS, Pandol SJ. Acute pancreatitis: a multi-faceted set of organellar and cellular interactions. *Gastroenterology*. 2019;**156**(7):1941–1950.
7. Wen L, Voronina S, Javed MA, et al. Inhibitors of ORAI1 prevent cytosolic calcium-associated Iinjury of human pancreatic acinar cells and acute pancreatitis in 3 mouse models. *Gastroenterology*. 2015;**149**(2):481–492.
8. Gerasimenko JV, Gryshchenko O, Ferdek PE, et al. Ca^{2+} release-activated Ca^{2+} channel blockade as a potential tool in antipancreatitis therapy. *Proc Nat Acad Sci USA*. 2013;**110**(32):13186–13191.
9. Peng S, Gerasimenko JV, Tsugorka T, et al. Calcium and adenosine triphosphate control of cellular pathology: asparaginase-induced pancreatitis elicited via protease-activated receptor 2. *Philos Trans R Soc Lond B Biol Sci*. 2016;**371**(1700):20150423. doi: 10.1098/rstb.2015.0423.
10. Voronina S, Collier D, Chvanov M, et al. The role of Ca^{2+} influx in endocytic vacuole formation in pancreatic acinar cells. *Biochem J*. 2015;**465**(3):405–412.
11. Waldron RT, Chen Y, Pham H, et al. The Orai Ca(2+) channel inhibitor CM4620 targets both parenchymal and immune cells to reduce inflammation in experimental acute pancreatitis. *J Physiol*. 2019;**597**(12):3085–3105.
12. Bruen C, Miller J, Wilburn J, et al. Auxora for the treatment of patients with acute pancreatitis and accompanying systemic inflammatory response syndrome: clinical development of a calcium release-activated calcium channel inhibitor. *Pancreas*. 2021;**50**(4):537–543.
13. Schaff UY, Dixit N, Procyk E, Yamayoshi I, Tse T, Simon SI. Orai1 regulates intracellular calcium, arrest, and shape polarization during neutrophil recruitment in shear flow. *Blood*. 2010;**115**(3):657–666.
14. Dixit N, Kim MH, Rossaint J, Yamayoshi I, Zarbock A, Simon SI. Leukocyte function antigen-1, kindlin-3, and calcium flux orchestrate neutrophil recruitment during inflammation. *J Immunol*. 2012;**189**(12):5954–5964.

15. Dixit N, Yamayoshi I, Nazarian A, Simon SI. Migrational guidance of neutrophils is mechanotransduced via high-affinity LFA-1 and calcium flux. *J Immunol* 2011;**187**(1):472–481.
16. Sogkas G, Vogtle T, Rau E, et al. Orai1 controls C5a-induced neutrophil recruitment in inflammation. *Eur J Immunol*. 2015;**45**(7):2143–2153.
17. Steinckwich N, Schenten V, Melchior C, Brechard S, Tschirhart EJ. An essential role of STIM1, Orai1, and S100A8-A9 proteins for Ca2+ signaling and FcγR-mediated phagosomal oxidative activity. *J Immunol*. 2011;**186**(4):2182–2191.
18. Zhu QS, Xia L, Mills GB, Lowell CA, Touw IP, Corey SJ. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. *Blood*. 2006;**107**(5):1847–1856.
19. Gupta AK, Giaglis S, Hasler P, Hahn S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One*. 2014;**9**(5):e97088.
20. Merza M, Hartman H, Rahman M, et al. Neutrophil extracellular traps induce trypsin activation, inflammation, and tissue damage in mice with severe acute pancreatitis. *Gastroenterology*. 2015;**149**(7):1920–1931.e8.
21. Bhatia M, Brady M, Zagorski J, et al. Treatment with neutralising antibody against cytokine induced neutrophil chemoattractant (CINC) protects rats against acute pancreatitis associated lung injury. *Gut*. 2000;**47**(6):838–844.
22. Guhad F. Introduction to the 3Rs (refinement, reduction, and replacement). *Contemp Top Lab Anim Sci*. 2005;**44**(2):58–59.
23. Ahuja M, Schwartz DM, Tandon M, et al. Orai1-mediated antimicrobial secretion from pancreatic acini shapes the gut microbiome and regulates gut innate immunity. *Cell Metab*. 2017;**25**(3):635–646.
24. Mikami Y, Takeda K, Shibuya K, et al. Peritoneal inflammatory cells in acute pancreatitis: relationship of infiltration dynamics and cytokine production with severity of illness. *Surgery*. 2002;**132**(1):86–92.
25. Gryshchenko O, Gerasimenko JV, Petersen OH, Gerasimenko OV. Calcium signaling in pancreatic immune cells. *Function*. 2021;**2**(1):zqaa026. doi: 10.1093/function/zqaa026.
26. Zhang H, Neuhofer P, Song L, et al. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. *J Clin Invest*. 2013;**123**(3):1019–1031.
27. Neuhofer P, Liang S, Einwachter H, et al. Deletion of IkappaBalpha activates RelA to reduce acute pancreatitis in mice through up-regulation of Spi2A. *Gastroenterology*. 2013;**144**(1):192–201.
28. Huang H, Liu Y, Daniluk J, et al. Activation of nuclear factor-kappaB in acinar cells increases the severity of pancreatitis in mice. *Gastroenterology*. 2013;**144**(1):202–210.
29. Kandikattu HK, Upparahalli Venkateshaiah S, Kumar S, Yadavalli CS, Mishra A. IL-18-mediated neutrophil recruitment promotes acute lung injury in inflammation-mediated chronic pancreatitis. *Mol Immunol*. 2023;**155**:100–109.
30. Singhal A, Dhankani P, Gupta KL, et al. Rho signaling inhibition mitigates lung injury via targeting neutrophil recruitment and selectin-AKT signaling. *Biochim Biophys Acta Mol Cell Res*. 2021;**1868**(12):119122.
31. Wen L, Javed TA, Dobbs AK, et al. The protective effects of calcineurin on pancreatitis in mice depend on the cellular source. *Gastroenterology*. 2020;**159**(3):1036–1050.e8.
32. Zhu B, Zhang R, Li C, et al. BCL6 modulates tissue neutrophil survival and exacerbates pulmonary inflammation following influenza virus infection. *Proc Natl Acad Sci USA*. 2019;**116**(24):11888–11893.
33. Cai C, Tang S, Wang X, et al. Requirement for both receptor-operated and store-operated calcium entry in N-formyl-methionine-leucine-phenylalanine-induced neutrophil polarization. *Biochem Biophys Res Commun*. 2013;**430**(2):816–821.
34. Immler R, Simon SI, Sperandio M. Calcium signalling and related ion channels in neutrophil recruitment and function. *Eur J Clin Invest*. 2018;**48**(Suppl 2):e12964.
35. Chen MS, Lin WC, Yeh HT, Hu CL, Sheu SM. Propofol specifically reduces PMA-induced neutrophil extracellular trap formation through inhibition of p-ERK and HOCl. *Life Sci*. 2019;**221**:178–186.
36. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. 2018;**18**(2):134–147.
37. Arita K, Hashimoto H, Shimizu T, Nakashima K, Yamada M, Sato M. Structural basis for Ca(2+)-induced activation of human PAD4. *Nat Struct Mol Biol*. 2004;**11**(8):777–783.
38. Perides G, van Acker GJ, Laukkarinen JM, Steer ML. Experimental acute biliary pancreatitis induced by retrograde infusion of bile acids into the mouse pancreatic duct. *Nat protoc*. 2010;**5**(2):335–341.
39. Wu J, Zhang L, Shi J, et al. Macrophage phenotypic switch orchestrates the inflammation and repair/regeneration following acute pancreatitis injury. *EBioMedicine*. 2020;**58**:102920.
40. Manohar M, Jones EK, Rubin SJS, et al. Novel circulating and tissue monocytes as well as macrophages in pancreatitis and recovery. *Gastroenterology*. 2021;**161**(6):2014–2029.
41. Saeki K, Kanai T, Nakano M, et al. CCL2-induced migration and SOCS3-mediated activation of macrophages are involved in cerulein-induced pancreatitis in mice. *Gastroenterology*. 2012;**142**(4):1010–1020e9.
42. Fink MP, Warren HS. Strategies to improve drug development for sepsis. *Nat Rev Drug Discovery*. 2014;**13**(10):741–758.
43. Rudd KE, Kissoon N, Limmathurotsakul D, et al. The global burden of sepsis: barriers and potential solutions. *Critical care*. 2018;**22**(1):232.
44. Pallagi P, Gorog M, Papp N, et al. Bile acid- and ethanol-mediated activation of Orai1 damages pancreatic ductal secretion in acute pancreatitis. *J Physiol*. 2022;**600**(7):1631–1650.
45. Petersen OH, Gerasimenko JV, Gerasimenko OV, Gryshchenko O, Peng S. The roles of calcium and ATP in the physiology and pathology of the exocrine pancreas. *Physiol Rev*. 2021;**101**(4):1691–1744.
46. Zhao Q, Wei Y, Pandol SJ, Li L, Habtezion A. STING signaling promotes inflammation in experimental acute pancreatitis. *Gastroenterology*. 2018;**154**(6):1822–1835e2.
47. Sendler M, Weiss FU, Golchert J, et al. Cathepsin B-mediated activation of trypsinogen in endocytosing macrophages increases severity of pancreatitis in mice. *Gastroenterology*. 2018;**154**(3):704–718e10.
48. Huang W, Cane MC, Mukherjee R, et al. Caffeine protects against experimental acute pancreatitis by inhibition of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ release. *Gut*. 2015;**66**(2):301–313.
49. Husain SZ, Prasad P, Grant WM, Kolodecik TR, Nathanson MH, Gorelick FS. The ryanodine receptor mediates early zymogen activation in pancreatitis. *Proc Nat Acad Sci USA*. 2005;**102**(40):14386–14391.

50. Orabi AI, Shah AU, Ahmad MU, et al. Dantrolene mitigates caerulein-induced pancreatitis in vivo in mice. *Am J Physiol Gastrointest Liver Physiol*. 2010;**299**(1):G196–G204.
51. Husain SZ, Orabi AI, Muili KA, et al. Ryanodine receptors contribute to bile acid-induced pathological calcium signaling and pancreatitis in mice. *Am J Physiology-Gastrointestinal and Liver Physiol*. 2012;**302**(12):G1423–G1433.
52. Kolodecik TR, Reed AM, Date K, et al. The serum protein renalase reduces injury in experimental pancreatitis. *J Biol Chem*. 2017;**292**(51):21047–21059.
53. Romac JM, Shahid RA, Swain SM, Vigna SR, Liddle RA. Piezo1 is a mechanically activated ion channel and mediates pressure induced pancreatitis. *Nat Commun*. 2018;**9**(1):1715.
54. Swain SM, Romac JM, Shahid RA, et al. TRPV4 channel opening mediates pressure-induced pancreatitis initiated by Piezo1 activation. *J Clin Invest*. 2020;**130**(5):2527–2541.
55. Yang SC, Tsai YF, Pan YL, Hwang TL. Understanding the role of neutrophils in acute respiratory distress syndrome. *Biomed J*. 2021;**44**(4):439–446.
56. Hann J, Bueb JL, Tolle F, Bréchard S. Calcium signaling and regulation of neutrophil functions: still a long way to go. *J Leukoc Biol*. 2020;**107**(2):285–297.
57. Grimes D, Johnson R, Pashos M, et al. ORAI1 and ORAI2 modulate murine neutrophil calcium signaling, cellular activation, and host defense. *Proc Natl Acad Sci USA*. 2020;**117**(39):24403–24414.
58. Hu Q, Yao J, Wu X, et al. Emodin attenuates severe acute pancreatitis-associated acute lung injury by suppressing pancreatic exosome-mediated alveolar macrophage activation. *Acta Pharm Sin B*. 2022;**12**(10):3986–4003.
59. Liu Q, Zhu CL, Li HR, et al. Salvianolic acid a protects against lipopolysaccharide-induced acute lung injury by inhibiting neutrophil NETosis. *Oxid Med Cell Longev*. 2022;**2022**:7411824.
60. Tiffner A, Derler I. Isoform-specific properties of orai homologues in activation, downstream signaling, physiology and pathophysiology. *Int J Mol Sci* 2021;**22**(15):8020. doi: 10.3390/ijms22158020.
61. Gukovskaya AS, Vaquero E, Zaninovic V, et al. Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. *Gastroenterology*. 2002;**122**(4):974–984.
62. Yi L, Liu Q, Orandle MS, et al. p47(phox) directs murine macrophage cell fate decisions. *Am J Pathol*. 2012;**180**(3):1049–1058.
63. Schepers NJ, Bakker OJ, Besselink MG, et al. Impact of characteristics of organ failure and infected necrosis on mortality in necrotising pancreatitis. *Gut*. 2019;**68**(6):1044–1051.
64. Garg PK, Singh VP. Organ failure due to systemic injury in acute pancreatitis. *Gastroenterology*. 2019;**156**(7):2008–2023.
65. Akbarshahi H, Rosendahl AH, Westergren-Thorsson G, Andersson R. Acute lung injury in acute pancreatitis—awaiting the big leap. *Respir Med* 2012;**106**(9):1199–1210.
66. Barreto SG, Habtezion A, Gukovskaya A, et al. Critical thresholds: key to unlocking the door to the prevention and specific treatments for acute pancreatitis. *Gut* 2021;**70**(1):194–203.