Fig1.Single-cell RNA sequencing reveals a comprehensive atlas of intestinal cells.

(A) UMAP Visualization of Cells Between Two Groups and Across Different Cell Clusters.The x- and y-axes denote the first and second principal components of the gene-expression matrix across all cells, respectively. Each dot represents a single cell; colors indicate different samples (left panel) or distinct cell clusters (right panel).

(B) Changes in Cell Cluster Proportions Between Different Groups.The x-axis shows the cell clusters; the y-axis shows the log2-transformed ratio of each cluster's relative abundance between the two experimental groups. Bars in the upper-right quadrant indicate increased relative proportion, while those in the lower-left quadrant indicate decreased proportion. Greater fold-changes yield taller bars with darker colors.

(C-E) UMAP of Clustering Trajectories for Two Groups Obtained from Pseudotime Analysis.The cell-annotation results are displayed: each dot represents a single cell, and different colors denote distinct cell types.

(F)Marker Genes for Each Cell Type.The x-axis displays canonical marker genes, and the y-axis shows cell clusters. The color intensity of each dot indicates the average expression level, while the dot size represents the proportion of cells within that cluster expressing the gene.

(G) GO Pathway Analysis of Top 3 Marker Genes in Each Cell Cluster.For each cluster, the three most enriched GO biological-process terms among its marker genes were selected. The corresponding enrichment q-values for these terms were computed per cluster, column-wise z-scored, and visualized as a heatmap; within-column color intensity thus reflects the significance of the enriched pathways for that cluster's markers.

(H) KEGG Pathway Analysis of Top 3 Marker Genes in Each Cell Cluster.For each cell cluster, the three most enriched KEGG pathways among its marker genes were first selected. The enrichment q-values of these pathways were then calculated for every cluster, column-wise z-scored, and displayed as a heatmap. Within-column color intensity allows direct comparison of how significantly the marker genes of that cluster are enriched in each pathway.

(I) Cell Communication Analysis.The network diagram illustrates the number/strength of intercellular communications, where nodes represent cell populations and edge width or color intensity corresponds to the quantity or intensity of signaling interactions between them.

Fig2.Neutrophils contribute to ischemia-reperfusion injury of the small intestinal mucosa in mice.

(A)The expression level of serum diamine oxidase(DAO) in mice was determined using an ELISA kit.A statistically significant difference in serum DAO levels was observed between the IR group and the NC group, and also between the IR group and the neutrophil elimination group(****P<0.0001).

(B)Serum Interleukin-6(IL-6) expression in mice was assessed by ELISA.Serum IL-6 levels in the IR group demonstrated statistically significant differences when compared to both the NC group and the neutrophil elimination group(****P<0.0001).

(C-D) Quantification of small intestinal length in mice.Small intestine length showed a statistically significant difference between the IR group and the NC group, as well as between the IR group and the neutrophil elimination group(***P<0.001).

(E-F) Hematoxylin and eosin (H&E) staining of mouse small intestine and histological evaluation using Chiu's scoring system.The Chiu's score of HE-stained small intestinal sections in the IR group demonstrated a statistically significant difference compared to both the NC group and the neutrophil elimination group(****P<0.0001).

(G-I) Immunofluorescence staining of mouse small intestine for Ly6g and ATF4 expression.A statistically significant difference was observed in the double immunofluorescence staining of Ly6G and ATF4 in the small intestine between the IR group and the NC group, as well as between the IR group and the neutrophil elimination group(**P<0.01,***P<0.001).

(J-K) Expression level of intestinal mucosal barrier protein Occludin in mouse small intestine.The expression level of Occludin protein in the small intestine of the IR group was significantly different compared to that in both the NC group and the neutrophil elimination group(***P<0.001).

Fig3.Neutrophils induce damage to IEC-6 cells.

(A)IL-6 expression in neutrophils was quantified using an ELISA kit.The inflammatory cytokine expression in the IR neu group was significantly elevated compared to that in the NC neu group(****P<0.0001).

(B)Cell viability of IEC-6 cells was assessed using the CCK-8 assay.IEC-6 cells co-cultured with neutrophils from the I/R injury group demonstrated a substantial decline in cell viability compared to those co-cultured with NC neu(***P<0.001).

(C)Transmission electron microscopy (TEM) of IEC-6 cells at magnifications of ×2.0K, ×6.0K, and ×12.0K.Representative electron micrographs showing well-preserved organelles in IEC-6 cells from the NC neu group, in contrast to marked disruption of mitochondria and endoplasmic reticulum in cells co-cultured with IR-injured neutrophils.

(D-E) Apoptosis in IEC-6 cells was detected by TUNEL staining. Quantification of necrotic apoptotic cells reveals a significant increase in the IR neu group relative to the NC neu group(****P<0.0001).

(F-G) Expression of the tight junction protein Occludin in IEC-6 cells was detected by Western blot.Representative analysis shows decreased Occludin expression in the IR neu group versus the NC neu group(***P<0.001).

Fig4.Single-cell RNA sequencing enables in-depth profiling of neutrophil subpopulations.

(A) Distribution Proportions of Different Cell Populations.In accordance with the findings of previous studies, significant disparities were identified in the proportions of various cell populations, with inflammatory and neutrophil cell populations showing a marked increase in the IR group.

(B) GO Analysis of Differentially Expressed Genes in Neutrophils.The differentially expressed genes in the neutrophil population were enriched in pathways related to inflammation, immunity, and neutrophil chemotaxis.

(C) Clustering Analysis of Neutrophil Subpopulations.Subpopulation and pseudotime analyses were conducted on neutrophils to identify neutrophil subpopulations with distinct functions and characteristics.

(D) Top 5 Marker Genes of Neutrophil Subpopulations

(E) Immune Pathway Analysis of Neutrophil Subpopulations.The expression levels of the top five marker genes and inflammatory pathway expression within these six neutrophil subpopulations were analyzed.

(F) Pseudotime Analysis of Neutrophils and Distribution of Neutrophil Subpopulations in Pseudotime.

(G) Differentially Expressed Transcription Factors in C5 Subgroup.

(H) GO Analysis of Differentially Expressed Genes in C5 Subgroup Cells.

(I) ATF4-Target Gene Co-expression Network.The results revealed that the target genes regulated by Atf4 were also enriched in inflammation and immune-related pathways.

Fig5.Neutrophils mediate intestinal mucosal ischemia-reperfusion injury through endoplasmic reticulum stress.

(A)The expression level of serum DAO in mice was determined using an ELISA kit.Serum DAO levels are elevated in the IR group versus NC, further increased by Tunicamycin (IR+Tun), but attenuated by 4-PBA (IR+4PBA)(***P<0.001,****P<0.0001)

(B)Serum IL-6 expression in mice was assessed by ELISA.Serum IL-6 and inflammatory cytokine levels are highest in the IR+Tun group, moderate in the IR group, and lowest in the IR+4PBA group(***P<0.001,****P<0.0001)

(C-D) Quantification of small intestinal length in mice.Small intestine length is shortened in the IR group versus NC, further reduced by Tunicamycin (IR+Tun), but rescued by 4-PBA (IR+4PBA)(*P<0.05,**P<0.01).

(E-F) H&E staining of mouse small intestine and histological evaluation using Chiu's scoring system.Intestinal morphological damage (Chiu's score) is increased in the IR group versus NC, exacerbated by Tunicamycin (IR+Tun), but alleviated by 4-PBA (IR+4PBA)(*P<0.05,****P<0.0001).

(G-I)Immunofluorescence staining of mouse small intestine for Ly6g and ATF4 expression.The relative fluorescence area of Ly6G+ neutrophils is significantly increased in the IR group compared to the NC group(***P<0.001,****P<0.0001).

(J-M) Expression levels of the intestinal mucosal barrier protein Occludin and endoplasmic reticulum stress markers ATF4 and GRP78 in mouse intestinal tissue.Occludin expression is decreased by I/R injury and further modulated by

treatments, showing an inverse correlation with the expression of ER stress markers (ATF4/GRP78)(**P<0.01,***P<0.001,****P<0.0001).

Fig6.Neutrophils induce IEC-6 cell injury through endoplasmic reticulum stress.

(A)IL-6 expression in neutrophils was quantified using an ELISA kit.Inflammatory cytokine levels are elevated in the IR neu group versus NC neu, further increased in the IR neu+Tun group, but reduced in the IR neu+4PBA group.(***P<0.001,****P<0.0001).

(B)Cell viability of IEC-6 cells was assessed using the CCK-8 assay.Viability of IEC-6 cells is reduced in the IR neu group versus NC neu, further decreased by Tunicamycin (IR neu+Tun), but rescued by 4-PBA (IR neu+4PBA).(***P<0.001).

(C)Transmission electron microscopy (TEM) of IEC-6 cells at magnifications of ×2.0K, ×6.0K, and ×12.0K.Representative electron micrographs reveal that 4-PBA alleviates, while Tunicamycin exacerbates, the mitochondrial damage induced by I/R neutrophils.

(D-E) Apoptosis in IEC-6 cells was detected by TUNEL staining.Apoptotic cell count is increased in the IR neu group versus NC neu, further elevated in the IR neu+Tun group, but not substantially increased in the IR neu+4PBA group.(**P<0.01,***P<0.001).

(F-I) Expression levels of the tight junction protein Occludin and endoplasmic reticulum stress markers ATF4 and GRP78 in IEC-6 cells.Representative blots showing that Tunicamycin exacerbates, while 4-PBA attenuates, I/R neutrophil-induced Occludin downregulation and ER stress activation.(***P<0.001， ****P<0.0001).

Fig7.Neutrophils mediate intestinal mucosal injury through the endoplasmic reticulum stress–ATF4 pathway.

(A)The expression level of serum DAO in mice was determined using an ELISA kit.Serum DAO levels were significantly higher in the NC+IR+Tun group than in the NC+IR group, but were reduced in the KO+IR+Tun group compared to the KO+IR group.（***P<0.001）

(B)Serum IL-6 expression in mice was assessed by ELISA.Serum IL-6 levels were significantly higher in the NC+IR+Tun group than in the NC+IR group, but were reduced in the KO+IR+Tun group compared to the KO+IR group.（***P<0.001）

(C-D) Quantification of small intestinal length in mice.Small intestine length was significantly reduced in the NC+IR+Tun group versus NC+IR, but showed no appreciable difference between the KO+IR and KO+IR+Tun groups.(**P<0.01).

(E-F) H&E staining of mouse small intestine and histological evaluation using Chiu's scoring system.The NC+IR+Tun group showed the most severe intestinal damage (highest Chiu's score), while both KO+IR and KO+IR+Tun groups exhibited intact morphology with no significant difference.(*P<0.05).

(G-I) Immunofluorescence staining of mouse small intestine for Ly6g and ATF4 expression.Neutrophil infiltration (Ly6G+ area) was significantly increased in the NC+IR+Tun group versus NC+IR, but remained low with no significant difference between the KO+IR and KO+IR+Tun groups.(**P<0.01).

(J-K) Expression level of intestinal mucosal barrier protein Occludin in mouse small intestine.Occludin expression was significantly reduced in the NC+IR+Tun group versus NC+IR, but showed no significant difference between the KO+IR and KO+IR+Tun groups.(**P<0.01)

Fig8.Neutrophils induce IEC-6 cell injury through the endoplasmic reticulum stress–ATF4 pathway.

(A) IL-6 expression in neutrophils was quantified using an ELISA kit.Inflammatory cytokine expression was significantly higher in the IR Neu+Tun group versus IR Neu, but remained low with no significant difference between the [(KO+IR) neu] and [(KO+IR) neu+Tun] groups.(***P<0.001)

(B)Cell viability of IEC-6 cells was assessed using the CCK-8 assay.Cell viability was significantly reduced in both IR Neu and IR Neu+Tun groups (with IR Neu+Tun being the lowest), while both (KO+IR) neu and (KO+IR) neu+Tun groups maintained higher viability with no significant difference.(***P<0.001)

(C)Transmission electron microscopy (TEM) of IEC-6 cells at magnifications of ×2.0K, ×6.0K, and ×12.0K.Mitochondrial and ER membrane damage was observed in IR Neu groups (more severe in +Tun), while (KO+IR) Neu groups showed preserved mitochondrial outer membrane and ER structure.

(D-E) Apoptosis in IEC-6 cells was detected by TUNEL staining.
The number of apoptotic cells was higher in the IR Neu and IR Neu+Tun groups (greatest in +Tun), while no substantial apoptosis was observed in the [(KO+IR) neu] and [(KO+IR) neu+Tun] groups.(***P<0.001).

(F-G) Expression of the tight junction protein Occludin in IEC-6 cells was detected by Western blot.Occludin expression was significantly reduced in IR Neu groups (lowest in +Tun), while (KO+IR) Neu groups showed higher expression with no significant difference.(***P<0.001).

FigS1

Representative immunofluorescence staining of CD3, CD45, and CD11b in the small intestine of mice from the first animal experiment.IR group showed increased fluorescent intensity of CD45/CD3/CD11b compared to control.
(***P<0.001,****P<0.0001).

FigS2

Heatmap of expression changes in the top 50 genes across neutrophil subpopulations during pseudotime analysis.The heatmap illustrates the changes in the expression levels of the top 50 genes across neutrophil subpopulations during pseudotime.

FigS3

Representative immunofluorescence staining of CD3 in the small intestine of mice from the second animal experiment.The relative fluorescence area of CD3 cells was significantly higher in the IR group than in the NC group.(***P<0.001).

FigS4

Representative immunofluorescence staining of CD45 in the small intestine of mice from the second animal experiment.The relative fluorescence area of CD45 was significantly higher in the IR group than in the NC group.(*P<0.05， ****P<0.0001) .

FigS5

Representative immunofluorescence staining CD11b in the small intestine of mice from the second animal experiment.CD11b fluorescence area was significantly higher in NC+IR+Tun versus NC+IR, but remained low with no significant difference between KO+IR and KO+IR+Tun groups.(***P<0.001,****P<0.0001).

FigS6

Representative immunofluorescence staining of CD3 in the small intestine of mice from the third animal experiment.CD3 fluorescence area was significantly higher in NC+IR+Tun versus NC+IR, but remained low with no significant difference between KO+IR and KO+IR+Tun groups.(**P<0.01).

FigS7

Representative immunofluorescence staining of CD45 in the small intestine of mice from the third animal experiment.CD45 fluorescence area was significantly higher in NC+IR+Tun versus NC+IR, but remained low with no significant difference between KO+IR and KO+IR+Tun groups.(***P<0.001).

FigS8

Representative immunofluorescence staining of CD11b in the small intestine of mice from the third animal experiment.CD11b fluorescence area was significantly increased in NC+IR+Tun group compared to NC+IR group, while both KO+IR and KO+IR+Tun groups showed lower levels with no significant difference.(**P<0.01).