Interleukin-22 alleviates arginine-induced pancreatic acinar cell injury via the regulation of intracellular vesicle transport system: Evidence from proteomic analysis

QIANQIAN XU[1,2], XINJUAN FU[1,3], ZHIGANG XIU[1,2], HONGLI YANG[2], XIAOXIAO MEN[1,2], MINGYUE LIU[1,2], CHANGQIN XU[2], BIN LI[2], SHULEI ZHAO[2] and HONGWEI XU[1,2]

[1]Department of Gastroenterology, Shandong Provincial Hospital, Shandong University; [2]Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong 250021; [3]Gastroenterology Center, Qingdao Hiser Hospital Affiliated to Qingdao University (Qingdao Traditional Chinese Medicine Hospital), Qingdao, Shandong 266033, P.R. China

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Abstract. Acute pancreatitis (AP) is a severe inflammatory condition characterized by the activation of pancreatic enzymes within acinar cells, leading to tissue damage and inflammation. Interleukin (IL)-22 is a potential therapeutic agent for AP owing to its anti-inflammatory properties and ability to promote tissue repair. The present study evaluated the differentially expressed proteins in arginine-induced pancreatic acinar cell injury following treatment with IL-22, and the possible mechanisms involved in IL-22-mediated alleviation of AP. AR42J cells were stimulated using L-arginine to establish an acinar cell injury model *in vitro* and the damaged cells were subsequently treated with IL-22. The characteristics of the model and the potential therapeutic effects of IL-22 were examined by CCK-8 assay, flow cytometry, TUNEL assay, transmission electron microscopy and ELISA. Differentially expressed proteins in cells induced by arginine and treated with IL-22 were assessed using liquid chromatography-mass spectrometry. The identified proteins were further subjected to Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis to elucidate their functional roles. The present study demonstrated that arginine-stimulated cells showed significant pathological changes resembling those in AP, which were alleviated after IL-22 treatment. Proteomic analysis then demonstrated that in IL-22-treated cells, proteins related to the formation and fusion of autophagosomes with lysosomes were significantly downregulated, whereas endocytosis related proteins were enriched in the upregulated proteins. After IL-22 treatment, western blotting demonstrated reduced expression of autophagy-associated proteins. In conclusion, by inhibiting the formation and fusion of autophagosomes with lysosomes, IL-22 may have mitigated premature trypsinogen activation, subsequently minimizing acinar cell injury induced by L-arginine. This was accompanied by concurrent upregulation of endocytosis, which serves a pivotal role in sustaining regular cellular material transport and signal propagation. This research underscored the potential of IL-22 in mitigating arginine-induced AR42J injury, which could be valuable in refining treatment strategies for AP.

Correspondence to: Professor Hongwei Xu, Department of Gastroenterology, Shandong Provincial Hospital, Shandong University, 324 Jingwuweiqi Road, Jinan, Shandong 250021, P.R. China
E-mail: xhwsdslyy@sina.com

Abbreviations: AP, acute pancreatitis; GO, Gene Ontology; IL-22, interleukin-22; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, liquid chromatography-mass spectrometry; TEM, transmission electron microscopy; Vps, vacuolar protein sorting

Key words: interleukin-22, proteomics, acute pancreatitis, autophagy, endocytosis, intercellular vesicle transport system

Introduction

Acute pancreatitis (AP) is one of the most common acute abdominalgia, manifesting globally in 13 to 45 cases per 100,000 individuals each year. Characterized by its acute onset, rapid progression and unpredictable outcomes, AP can culminate in severe complications such as sepsis, multi-organ failure and systemic inflammatory response syndrome, potentially leading to fatality (1). The inflammatory damage in AP is initiated by premature activation of trypsinogen in pancreatic acinar cells, resulting in a cell-damaging proteolytic cascade (2). The damaged acinar cells and necrotic tissue activate immune cells to release inflammatory mediators, such as tumor necrosis factor-α, interleukin (IL)-1, IL-6 and IL-8 (3), which cause a cascade reaction, resulting in system inflammatory response syndrome and multiple organ dysfunction syndrome (1).

To the best of our knowledge, the mechanism underlying abnormal trypsin activation remains unclear. However, pathological calcium signaling and the colocalization of zymogen granules with lysosomes may be key early steps in the pathogenesis of pancreatitis (4). Cathepsin B, a lysosomal enzyme,

converts trypsinogen to trypsin in these cells upon colocalization. Notably, the colocalization of lysosomal and zymogen fractions has been reported to be an autophagic process (5).

IL-22, a member of the IL-10 family, is produced by T-helper (Th)-17 cells, Th22 cells, γδ T cells, natural killer T cells and innate lymphoid cells (6). Unlike most cytokines, IL-22 acts primarily on non-hematopoietic epithelial cells and fibroblasts, as determined by its idiographic receptor. IL-22 receptor 1 (IL-22R1) is expressed only in the stromal and epithelial cells of specific tissues, with the highest expression observed in pancreatic acinar cells, followed by the intestinal tract and skin (7). The binding of IL-22 to IL-22R1 forms a unique receptor complex (IL-22/IL-22R1 subunit) and enables secondary combination with the IL-10 receptor 2 subunit. This initiates the activation of receptor-associated Janus kinase 1 and leads to the phosphorylation of STAT proteins (mainly STAT3) (8). In addition to STAT signaling, IL-22 binding activates the MAPK and PI3K-Akt signaling pathways (9).

Previous studies have reported that IL-22 mediates the protection and regeneration of epithelial tissues (10,11). Furthermore, it serves an important role in triggering antimicrobial immunity and maintaining the integrity of the mucosal barriers (12). A previous study has reported that serum IL-22 levels are significantly elevated in patients with AP (13). It has also been reported that IL-22 can stimulate the production of proteins such as regenerating islet-derived (Reg)3β, Reg3γ and osteopontin (14), and induce the transcription of anti-apoptotic genes, Bcl-2 and Bcl-Xl (15), thereby serving an important role in the immune response to AP.

Previous studies have reported that the mechanism of IL-22-mediated treatment of AP is mainly related to its anti-inflammatory effect and apoptosis inhibition (16,17). However, the relationship between trypsinogen activation in pancreatic acinar cells and IL-22 treatment requires further study, given it is a key step in the initiation of AP. Proteomics is one of the most effective methods for identifying molecular markers and drug targets. To the best of our knowledge, there has been no proteomic analysis of IL-22-treated pancreatic acinar cells, which indicated that certain possible mechanisms might not have been elucidated. Therefore, the present study assessed the possible molecular mechanisms underlying IL-22-mediated treatment of AP by inducing pancreatic acinar cell injury *in vitro* and performing quantitative proteomics to identify important targets and the clinical potential of IL-22 in the treatment of this condition.

Materials and methods

Cell culture and establishment of AP model. Rat pancreatic cancer AR42J cells (China Center for Type Culture Collection) were cultured at 37˚C in Ham's F-12K basal medium (HyClone; Cytiva) containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified, 5% CO_2 incubator (Thermo Fisher Scientific, Inc.). L-arginine (2.5 or 5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to establish the AP model. Hu *et al* (18) reported that an arginine dose of 2.5 or 5 mg/ml, as used in the present study, induced characteristic changes in pancreatic acinar cells, including an upsurge in apoptosis and autophagy, alongside abnormal activation of tryptic enzymes. After further incubation at 37˚C for 24 h, the cells and the medium supernatant were collected for subsequent experiments.

Assessment of cell viability using Cell Counting Kit-8 (CCK-8) assay. In a 96-well plate (Eppendorf), $5x10^4$ cells per well were seeded. The experimental group was treated with 2.5 or 5 mg/ml arginine, while the control group remained untreated. Subsequently, cells were treated with 10 μl CCK-8 reagent (MedChemExpress) at specified time intervals (0, 6, 24 and 48 h), and incubated at 37˚C for 1.5 h. Optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.) and a proliferation curve was drawn.

Assessment of apoptosis using flow cytometry. In a 6-well plate (Eppendorf), $2x10^6$ cells per well were seeded. The experimental group cells were treated with either 2.5 mg/ml arginine or a combination of 2.5 mg/ml arginine and 10 ng/ml recombinant (r)IL-22, incubated at 37˚C for 24 h. Untreated cells served as a negative control group. The choice of IL-22 concentration was based on a previous study (19), and a preliminary experiment was performed to ascertain the optimal concentration for use in the present study (Fig. S1). Briefly, $5x10^4$ cells per well were seeded in a 96-well plate. After 24 h of co-administration with varying concentrations (0, 0.1, 1, 5, 10, 15 and 20 ng/ml) of rIL-22 and 2.5 mg/ml arginine, cell survival was assessed using a CCK-8 assay (MedChemExpress) and incubated for 1.5 h at 37˚C. Post-treatment, cells from each group were digested with EDTA-free trypsin (Gibco; Thermo Fisher Scientific, Inc.) and centrifuged at 300 x g for 5 min at 4˚C for collection. Binding buffer (1X) (BD Biosciences) was added to resuspend the cells and adjust the cell density to $1x10^6$-$5x10^6$ cells/ml. Subsequently, 100 μl of cell suspension was mixed with 5 μl annexin V-FITC and the mixture was incubated at room temperature in the dark for 5 min. PI staining solution (10 μl) and 400 μl binding buffer (1X) were added to the samples, and flow cytometry was performed using the Attune NxT Flow Cytometer (Thermo Fisher Scientific, Inc.). The results were analyzed and processed using FlowJo software (version 10.8.1; FlowJo LLC).

Assessment of cell death using terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. Briefly, a total of $2.5x10^5$ cells were seeded in a 24-well plate (Eppendorf) and incubated for 24 h with arginine and IL-22 as aforementioned. Post-incubation, cells were fixed in 4% formaldehyde for 30 min at room temperature and permeabilized using 0.3% Triton X-100 for 5 min at room temperature. Subsequently, the cells were incubated with terminal deoxynucleotidyl transferase enzyme and fluorescein-dUTP for 2 h at 37˚C, allowing the enzyme to incorporate fluorescein-labeled dUTPs to the 3'-OH ends of fragmented DNA. The reaction was terminated by washing the cells with PBS (Wuhan Servicebio Technology Co., Ltd.), which were then counterstained with DAPI (5 μg/ml) (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. The stained cells were then visualized under an EVOS M7000 microscope (Invitrogen; Thermo Fisher Scientific, Inc.) in fluorescence mode.

Observation of cell ultrastructure using transmission electron microscopy (TEM). A total of $2.5x10^5$ cells were seeded on

glass coverslips (Corning Inc.) in a 24-well plate and then incubated for 24 h with arginine and IL-22, as aforementioned. Post-incubation, the culture was discarded and cells were fixed using a 2.5% glutaraldehyde electron microscope fixative solution (Wuhan Servicebio Technology Co., Ltd.) at 4˚C for 2-4 h. Samples were rinsed in isotonic cacodylate buffer (Shanghai Xinyu Biotechnology Co., Ltd.) and post-fixed with 1% OsO_4 in isotonic cacodylate buffer at 4˚C for 30 min. After dehydrating the cells in 50, 75, 95 and 100% ethanol (2x15 min for each concentration), the cells were infiltrated and embedded at 37˚C overnight using a gradient of acetone and 812 embedding agent (Structure Probe, Inc.). Thin sections of 70 nm were cut using an ultramicrotome (Leica Microsystems GmbH) and stained with uranyl acetate at room temperature for 20 min and then with lead citrate at room temperature for 5 min. The specimens were visualized using TEM (Hitachi, Ltd.) and images were captured for analysis.

Assessment of rat α-amylase using ELISA. The activity of α-amylase in the cell culture supernatant was evaluated employing a commercial ELISA kit (cat. no. SP12818; Wuhan Saipei Biotechnology Co., Ltd.), following the manufacturer's protocol. Optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Analysis of autophagy-related proteins using western blotting. RIPA lysis solution (Beijing Solarbio Science & Technology Co., Ltd.) with protease inhibitor was added to arginine- and IL-22-treated cells to extract the proteins. Protein concentration was determined by the BCA assay (Beyotime Institute of Technology). Protein samples (20 μg/lane) were separated on a 10% gel (w/v) by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The nonspecific sites were blocked with a solution containing 5% skimmed milk powder for 1 h at room temperature and the membrane was then incubated with primary anti-ATG5 (1:1,000; cat. no. A0203; ABclonal Biotech Co., Ltd.), anti-ATG7 (1:30,000; cat. no. ab133528; Abcam), anti-beclin-1 (1:1,000; cat. no. ab210498; Abcam), anti-LC3B (1:2,000; cat. no. ab192890; Abcam) and anti-β-actin (1:2,000; cat. no. gb15003; Beijing Solarbio Science & Technology Co., Ltd.) antibodies, diluted in 0.05% TBST (1X) (Wuhan Servicebio Technology Co., Ltd.) containing 5% bovine serum albumin overnight at 4˚C with gentle shaking. After the membrane was washed with TBST (1X), it was incubated with HRP Goat Anti-Rabbit IgG (H+L) (1:10,000; cat. no. AS014; ABclonal Biotech Co., Ltd.) at room temperature for 1 h. Protein bands were visualized using ECL (Wuhan Servicebio Technology Co., Ltd.). β-actin was used as the internal reference. Quantity One® 1-D software (version 4.6.6; Bio-Rad Laboratories, Inc.) was used to analyze the gray-scale values and the target protein/internal reference was used to assess the relative expression level of each protein.

Proteomic analysis

Protein extraction. Two groups, each containing $1x10^7$ cells, were prepared. The negative control group was treated with 2.5 mg/ml arginine at 37˚C for 24 h. Simultaneously, the experimental group was treated with 2.5 mg/ml arginine and 10 ng/ml IL-22 under identical conditions for 24 h. The cell samples were then sonicated three times on ice using a lysis buffer composed of 8 M urea and 1% protease inhibitor cocktail (cat. no. HY-K0010; MedChemExpress), with a high-intensity ultrasonic processor (Ningbo Scientz Biotechnology Co., Ltd.). The remaining debris was removed by centrifugation at 12,000 x g at 4˚C for 10 min. Finally, the supernatant was collected and the protein concentration was determined using the BCA kit (Beyotime Institute of Technology), performed according to the manufacturer's instructions.

Trypsin digestion. The protein solution was reduced with 5 mM dithiothreitol (Sigma-Aldrich; Merck KGaA) for 30 min at 56˚C and then alkylated with 11 mM iodoacetamide (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature in darkness. The protein sample was then diluted, by adding 100 mM tetraethylammonium borohydride (Sigma-Aldrich; Merck KGaA) until a urea concentration of <2 M was reached. Finally, trypsin (Promega Corporation) was added at a ratio of 1:50 (trypsin-to-protein mass) for the first digestion overnight at 37˚C and a ratio of 1:100 (trypsin-to-protein mass) for the second 4-h digestion at the same temperature.

High-performance liquid chromatography (HPLC) fractionation. The sample (20 μl) was fractionated using high pH reverse-phase HPLC on an Agilent 1260 Infinity II (Agilent Technologies, Inc.), utilizing an Agilent Zorbax 300Extend-C18 column (Agilent Technologies, Inc.; particles, 5 μm; internal diameter, 4.6 mm; length, 250 mm). Wavelength was set to 214 nm, column oven temperature was set to 35˚C, and 95% buffer A (2% acetonitrile, pH 9.0 adjusted by ammonia) and 5% buffer B (98% acetonitrile, pH 9.0 adjusted by ammonia) were used to equilibrate the column for ≥30 min. A stepwise gradient method was used after the baseline was flattened. Subsequently, 1 ml buffer A was added to the peptide sample and vortexed to dissolve it. The mixture was centrifuged at 12,000 x g for 5 min at 4˚C and transferred to a new tube. The mixture was centrifuged again under the same conditions to remove the supernatant and the sample (20 μl) was loaded for HPLC analysis. The sample was separated and simultaneously collected in the automatic collector. The sample was collected at 1 min/tube from tubes 11-46, a total of 36 tubes. The HPLC was conducted at a flow rate of 1 ml/min. Finally, tubes 11-20, 21-30, 31-40 and 41-46 were combined to form four separate fractions, and dried using vacuum centrifuging at 12,000 x g for 30 min at 4˚C.

Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis. Tryptic peptides were dissolved in 0.1% formic acid (solvent A) and directly loaded onto a home-made reverse-phase analytical column (length, 15 cm; internal diameter, 75 μm). The gradient comprised a solvent increasing from 6 to 23% (solvent B; 0.1% formic acid in 98% acetonitrile) in 26 min, 23 to 35% in 8 min and increasing to 80% in 3 min, then holding at 80% for the last 3 min. This was performed at a constant flow rate of 400 nl/min on an EASY-nLC 1000 ultra-performance (UP)LC (Thermo Fisher Scientific, Inc.) system. The LC-MS/MS was operated in positive ion mode, with a nitrogen gas temperature at 320˚C and nebulizer pressure optimized at 55 psi for the instrument used. The peptides were then analyzed using the Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ MS (Thermo Scientific™; Thermo Fisher Scientific, Inc.), coupled online to the UPLC system. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350-1,800 for a full scan and intact peptides were

assessed in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using a normalized collision energy setting of 28 and the fragments were assessed using the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with a 15.0 s dynamic exclusion. The automatic gain control was set to 5E4. The first fixed mass was set to 100 m/z.

Database search. Resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8; http://www.maxquant.org/). Tandem mass spectra were searched against the rat UniProt database (UniProt Consortium, 2019; https://www.uniprot.org/) and concatenated with a reverse decoy database. Promega trypsin was specified as the cleavage enzyme, allowing for ≤4 missing cleavages. The mass tolerance for the precursor ions was set to 20 ppm in the first search and 5 ppm in the main search, and the mass tolerance for the fragment ions was set to 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, whilst acetylation modification and oxidation on Met were specified as variable modifications. False discovery rate was adjusted to <1% and the minimum score for the modified peptides was set at >40.

Functional enrichment analysis. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the GO database (http://geneontology.org) and KEGG database (http://www.genome.jp/kegg/), respectively. Statistical significance was assessed using Fisher's exact test. Genes with an enrichment factor >1.5 and $P<0.05$ were deemed statistically significant, defining enriched GO and KEGG terms accordingly.

Statistical analysis. Statistical analysis was performed using SPSS software (version 18.0; SPSS, Inc.). Data are expressed as mean ± standard deviation. Unpaired Student's t-test was used to compare two groups and one-way analysis of variance (ANOVA) was used to compare multiple groups, followed by the Tukey-Kramer post hoc test. Each experiment was performed independently in triplicate. All reported P-values were two-tailed and $P<0.05$ was considered to indicate a statistically significant difference.

Results

IL-22 treatment ameliorates arginine-induced acinar cell injury in vitro. Characteristic changes of the cell ultrastructure in the arginine-induced group, as observed by TEM, included a notable reduction of zymogen granules, cytoplasmic vacuolization, nuclear chromatin margination and marked appearance of autophagic bilayer membranes containing organelles (Fig. 1). These changes are commonly observed in cells undergoing autophagy or apoptosis (20).

The CCK-8 assay demonstrated that exposure of AR42J cells to arginine was associated with a notable reduction in cell viability in a concentration-dependent manner (Fig. 2A), with a significant difference between the control and arginine-induced groups. A concentration of 2.5 mg/ml arginine was chosen for subsequent studies. This concentration effectively elicited the observed effects while minimizing potential toxicity, aligning with the study's objectives and ensuring the reliability and relevance of subsequent investigations.

To determine the effects of IL-22 treatment on arginine-induced acinar cell injury, cell viability, apoptosis and acinar cell inflammation were assessed. A notable increase in necrotic (Q1) and apoptotic (Q2 and Q3) cells was demonstrated in the arginine-induced group compared with the control group. However, following IL-22 treatment, there was no marked difference in the number of apoptotic cells compared with the control group, presenting a decrease compared with that in the arginine-induced group (Fig 2B). Notably, a slight increase in the number of necrotic (Q1) cells was observed. Despite this, the enhancement in the overall percentage of live cells was marked. This is further demonstrated in Fig. 2D, where the relative number of apoptotic cells is illustrated for clarity. The arginine-induced group had a significant surge in apoptotic cells (17.46±4.42%) compared with the control group (5.82±3.03%). By contrast, in the IL-22-treated group, the relative number of apoptotic cells (6.16±2.11%) was comparatively lower, aligning closely with the control group. The protective effect of IL-22 and the impact on arginine-induced cell death were further evaluated by comparing the proportion of TUNEL-positive cells between the treatment groups (Fig. 2C and E). The arginine-induced group displayed high levels of apoptosis (42.4±1.52%), whereas the IL-22-treated group exhibited markedly diminished apoptosis levels (15.3±5.3%). This significant reduction in cell death in the IL-22-treated group compared with the arginine-induced group underscores the effective therapeutic potential of IL-22.

Amylase is an important serological marker for the clinical diagnosis of AP (21), therefore, acinar cell inflammatory damage was evaluated by measuring amylase levels in the culture medium supernatant. The amylase concentration in the arginine-induced group (17.28±0.15 ng/ml) was significantly increased compared with the control group (12.52±1.02 ng/ml), however in the IL-22-treated group, the amylase concentration (13.20±0.79 ng/ml) was lower and not markedly different from the control group (Fig. 2F).

The aforementioned changes in cell survival and functional status demonstrated that an *in vitro* model of pancreatic acinar cell injury was successfully established by adding L-arginine to the culture medium, and that IL-22 was able to ameliorate arginine-induced apoptosis and inflammation of the acinar cells.

Proteomic analysis demonstrates the possible mechanism of IL-22-mediated alleviation of arginine-induced pancreatic acinar cell injury

Differentially expressed proteins in IL-22-treated acinar cells compared with arginine-induced cells. LC-MS/MS analysis identified a total of 48,298 specific peptides. Among these, quantitative information was garnered for 4,108 distinct proteins. With fold-changes of ≥1.5-fold or ≤0.67-fold included, the IL-22-treated group demonstrated marked changes in 321 proteins compared with the arginine-induced group, of which 174 proteins were upregulated and 147 proteins were downregulated.

Proteomics analysis demonstrated that proteins involved in DNA repair, such as histone H2A.V and 26S proteasome non-ATPase regulatory subunit 7, were notably upregulated. By contrast, acyl-CoA-binding domain-containing protein

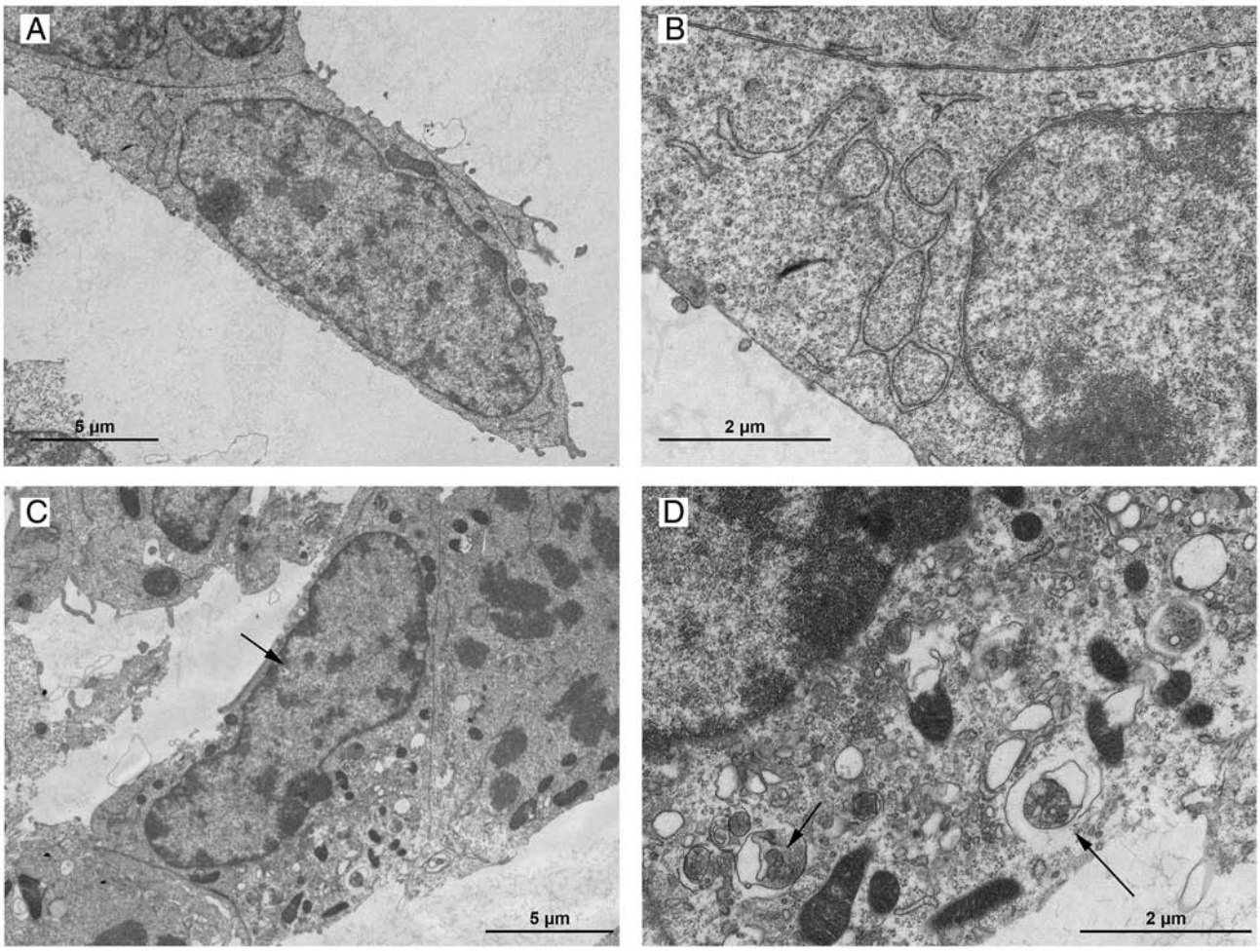

Figure 1. Ultrastructure of AR42J cells visualized using transmission electron microscopy. Normal control group at a magnification of (A) x1,500 and (B) x5,000. Arginine-induced group at a magnification of (C) x1,500, demonstrating nuclear chromatin margination (arrow) and (D) x5,000, demonstrating the autophagosome composed of a bilayer membrane (arrows).

5, which acts as the peroxisome receptor for pexophagy, and the regulator complex protein late endosomal/lysosomal adaptor, MAPK and MTOR activator 5, which is involved in lysosomal enzyme activity and transport, were downregulated after IL-22 treatment compared to the arginine-induced group.

GO analysis of biological processes, cellular compartments and molecular functions was performed using notably altered proteins, and independent functional annotation classifications were performed for upregulated and downregulated proteins (Fig. 3). The results demonstrated that proteins with marked changes were mainly in the cellular biological process (46.8%), such as 'cellular process', 'single-organism process', 'biological regulation', 'metabolic process' and 'cellular component organization or biogenesis'. Most proteins classified under component category (37.1%) were involved in the 'cell', 'organelle', 'membrane', 'macromolecular complex' and 'membrane-enclosed lumen'. In addition, the proteins assessed were also associated in eight molecular functional GO terms, mainly 'binding' and 'catalytic activity'.

These results collectively highlighted the substantial impact of IL-22 on various protein expression levels, underscoring its significant role in modulating a multitude of cellular functions and activities.

Regulated proteins involved in cellular composition and biological process are related to the intracellular vesicle transport system. Analyses of GO terms and KEGG pathways in which the differentially expressed proteins were enriched were performed to determine whether these proteins were significantly enriched in various functional types (Fig. 4). In total, GO enrichment analysis identified five significantly enriched biological processes based on 174 upregulated proteins, four of which were related to actin organization and function. These included 'membrane lipid metabolic process', 'actin filament organization', 'nuclear chromosome segregation', 'response to IL-1' and 'establishment of chromosome localization' (Fig. 4A). Important proteins involved in these biological processes included Abl interactor 2 (Abi2), vasodilator-stimulated phosphoprotein (Vasp1), epidermal growth factor receptor kinase substrate 8, monooxygenase MICAL1 (Mical-1) and sororin. KEGG pathway enrichment analysis demonstrated only one statistically significant enriched pathway, 'mmu05206 microRNAs in cancer' (Fig. 4B). Out of the 147 downregulated proteins, GO enrichment analysis identified 10 significantly enriched biological processes (Fig. 4C), including 'hormone catabolic process', 'alcohol catabolic process', 'lytic vacuole organization', 'lysosome organization' and 'autophagy'. These downregulated proteins,

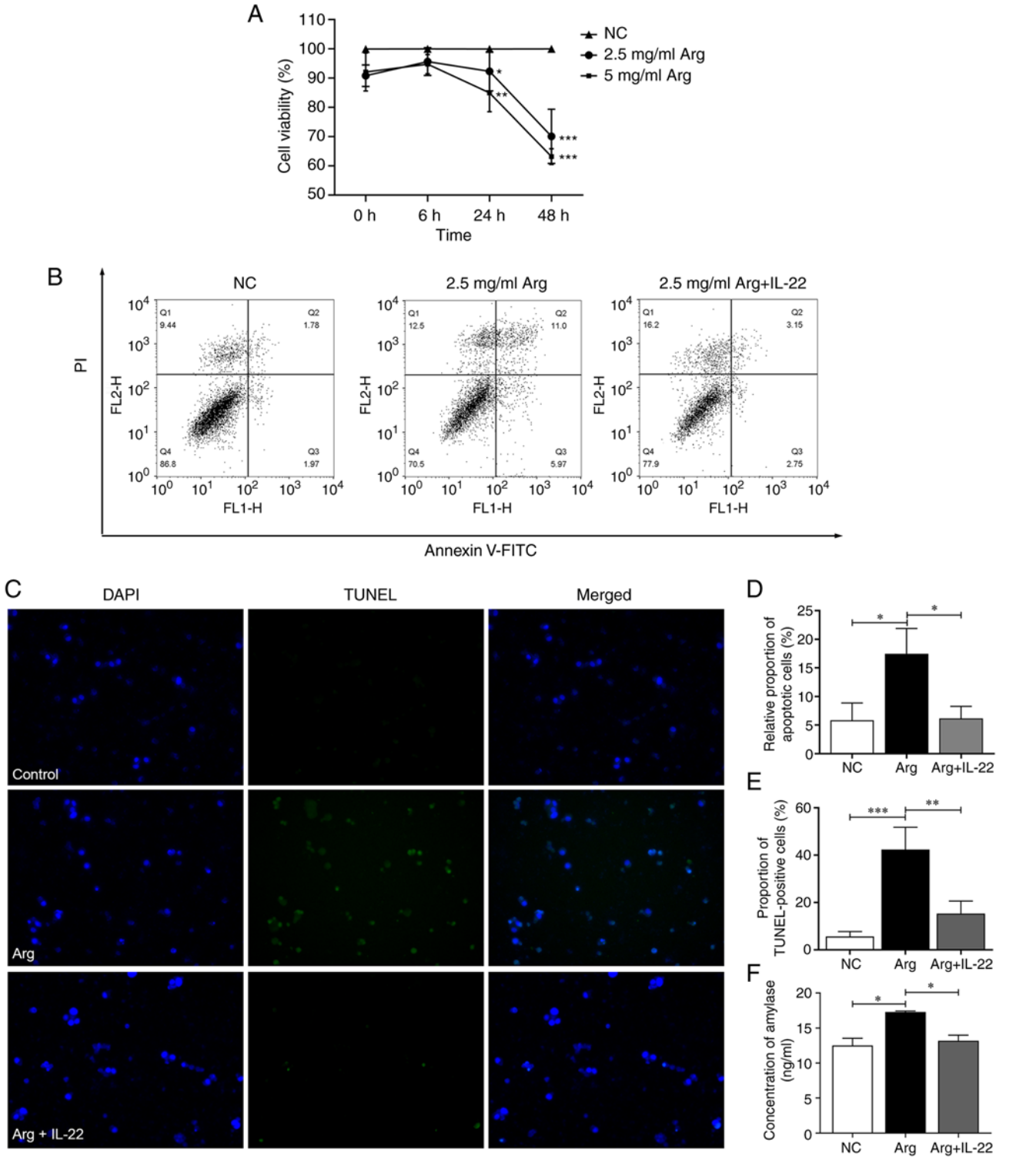

Figure 2. Effect of IL-22 treatment on arginine-induced acinar cell injury. (A) Cell viability assessed using the Cell Counting Kit-8 assay under two concentrations of arginine stimulation. (B) Flow cytometry analysis of cells. (C) TUNEL assay demonstrating apoptotic cells (magnification, x100), with TUNEL labeling (green), and cell nuclei counterstained with DAPI (blue). (D) Relative proportion of apoptotic cells, determined using flow cytometry. (E) Proportion of TUNEL-positive cells determined using the TUNEL assay. (F) Concentration of amylase in the supernatant of each group, quantified using ELISA. *P<0.05, **P<0.01 and ***P<0.001. NC, negative control; Arg, arginine; IL, interleukin.

mainly vacuolar protein sorting (Vps)18, Vps11 and beclin-1, are associated with autophagy, lysosome organization and lytic vacuole organization, in addition to their enrichment in the catabolic processes of multiple substances (such as hormone, steroid and alcohol). Furthermore, eight cellular components were significantly enriched, namely 'endosome', 'cytoplasmic vesicle', 'intracellular vesicle', 'late endosome', 'endosome membrane', 'endosomal part', 'Golgi apparatus part' and 'autophagosome' (Fig. 4D). The related proteins included beclin-1, Eps15 homology domain-containing protein 4, neuropilin-1 and multiple vacuolar protein sorting-associated proteins (Vps18 and Vps11). These cellular components were

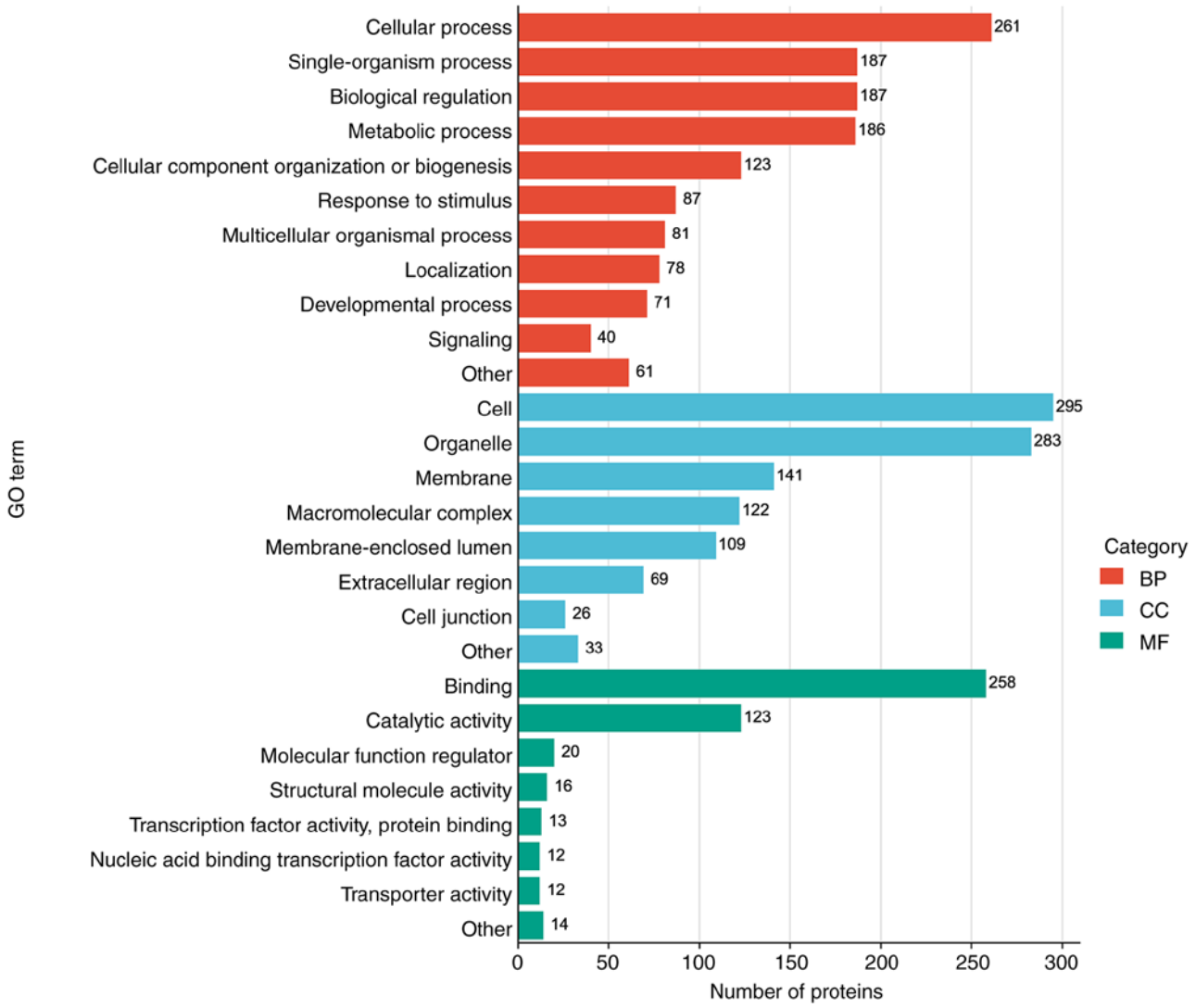

Figure 3. GO analysis of regulated proteins. GO, Gene Ontology; BP, biological process; CC, cell compartment; MF, molecular function.

associated with intracellular vesicle to lysosomes transport system (endocytosis and autophagy), which serve a key role in the premature activation of trypsinogen in pancreatic acinar cells (22).

This comprehensive analysis elucidated the significant roles of regulated proteins in cellular composition and related biological processes, particularly within the intracellular vesicle transport system. These findings underscore the potential inhibitory role of IL-22 in the premature activation of trypsinogen in pancreatic acinar cells.

Autophagy is significantly inhibited after IL-22 treatment, whilst endocytosis is activated. The differentially expressed proteins were divided into four groups (Q1-4) based on differential expression levels to assess the association between protein function and differential fold expression (Fig. 5A). Compared with the arginine-induced group, the Q1 group included proteins with fold expression <0.667 and the Q4 group included proteins with fold expression >1.5. For each group, GO terms and KEGG enrichment analysis were performed separately, and cluster analyses were performed (Fig. 5B-E). The results demonstrated that proteins in Q1, including Vps18, Vps11 and beclin-1, were associated with cellular components such as 'autophagosome', 'endosome', 'late endosome' and 'intracellular vesicle' (Fig. 5C). Specifically, these proteins were crucially involved in biological processes such as 'autophagy', 'lysosome organization' and 'lytic vacuole organization' (Fig. 5D). In addition, the regulation of biological process such as 'vesicle-mediated transport', 'negative regulation of protein metabolic process', 'regulation of endocytosis' and 'peptide biosynthetic process' were enriched in proteins in Q2 (Fig. 5D), which demonstrated that these processes were subtly inhibited after IL-22 treatment. Proteins such as 60S ribosomal proteins, 39S ribosomal proteins, tuberin and ubiquilin-2 were involved in these processes. However, 'endocytosis', a KEGG signaling pathway, was notably enriched in proteins in Q3 (Fig. 5B). The proteins involved included Ras-related protein Rab-5A, Vps36, Vps37b, Rab35, Sorting nexin-4 and Vps37b. Fig. 5E reveals a majority of proteins impacting molecular function within the Q2 group, suggesting that IL-22 plays a role in regulating molecular function to some extent.

The effect of IL-22 on the expression of autophagy-related proteins, including beclin-1, autophagy-related gene (ATG)5, ATG7 and microtubule-associated protein 1 light chain 3 (LC3)-I/II, were assessed to determine whether IL-22

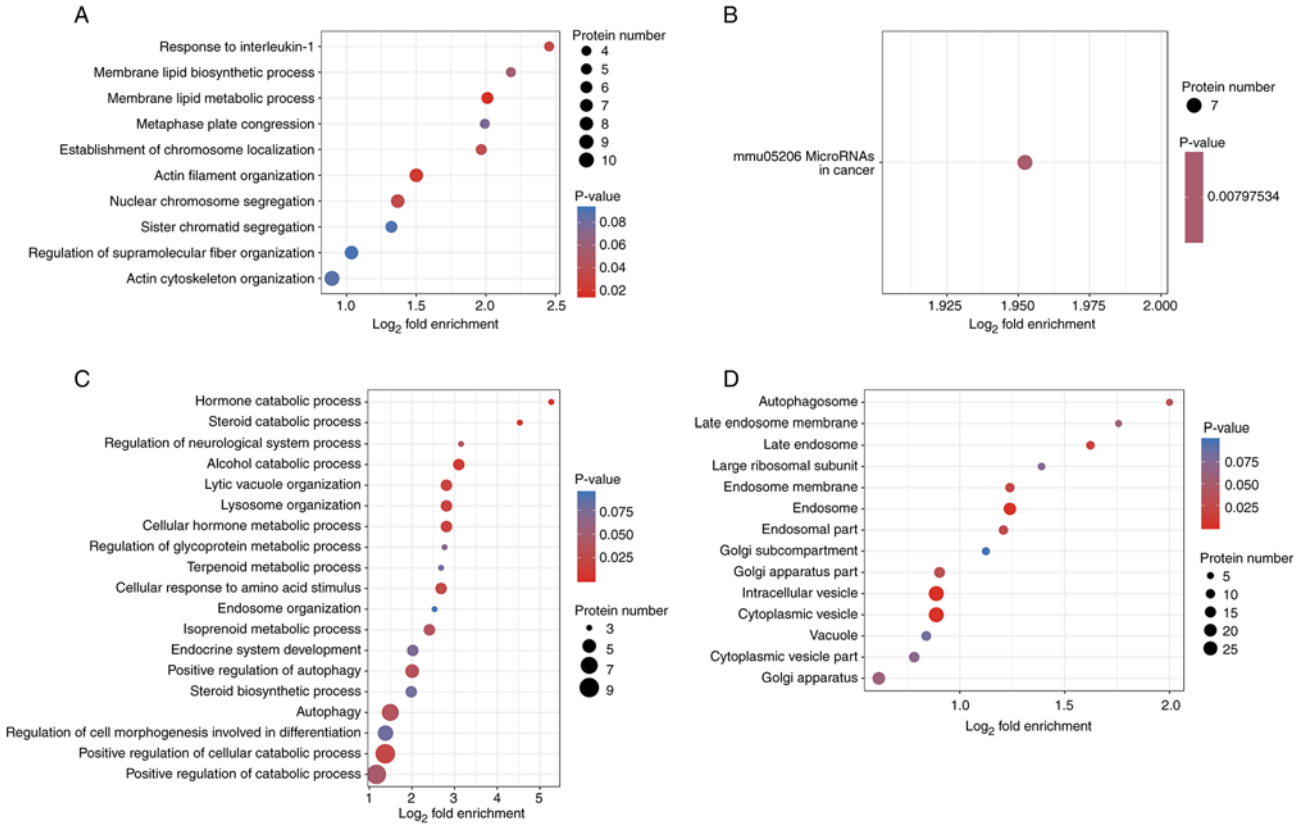

Figure 4. Enrichment analysis of GO terms and KEGG pathways for regulated proteins. Upregulated proteins enriched in (A) biological processes and (B) KEGG pathways. Downregulated proteins enriched in (C) biological processes and (D) cellular components. Vertical axes indicate the GO term or KEGG signaling pathway, and horizontal axes indicate the $\log_2$ converted value of the fold change of the proportion of the different proteins in the function type, compared with the proportion of the identified protein. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

treatment regulated autophagy. Western blotting results revealed a significant reduction in the expression of beclin-1, ATG5, ATG7 and LC3-I/II following IL-22 treatment (Fig. 6).

These findings enhance the understanding of the impact of IL-22 on acute pancreatitis treatment. Specifically, IL-22 inhibits the intracellular vesicular transport systems, particularly autophagy, while sustaining endocytosis and other molecular functions. Through this mechanism, IL-22 effectively prevents the activation of trypsinogen, helping to preserve cellular functionality during the initial stages of arginine-induced stimulation in pancreatic acinar cells.

Discussion

AP is an inflammatory condition of the pancreas that can be caused by bile stones and excessive alcohol consumption. It is characterized by acute pancreatic inflammation and histological acinar cell destruction (23). The present study demonstrated that exposure of AR42J cells to arginine led to changes commonly observed in cells undergoing apoptosis or autophagy and which resemble the morphological changes seen in AP (24). Arginine also decreased the viability of AR42J cells in a concentration-dependent manner, as demonstrated by results from the CCK-8 assay, and flow cytometry and TUNEL assays demonstrated that the change was mainly manifested as a significant increase in apoptosis and necrosis. In the arginine-induced group, the concentration of amylase in the cell culture supernatant was significantly increased, compared with that in the negative control, suggesting that pancreatin in the acinar cells was abnormally activated and released from the cytoplasm following arginine exposure. The results indicated that arginine stimulation triggered apoptosis of the acinar cells, cytoplasmic vacuole formation and zymogen granules fusion with the lysosomes. Combined with previous studies, we hypothesized that under this mechanism, trypsinogen would subsequently be activated in pancreatic acinar cells, thereby initiating pancreatitis (25,26).

A clinical study by Vasseur *et al* (13) reported high plasma IL-22 levels in patients with AP, regardless of the disease severity. In our previous studies, the therapeutic potential of exogenous IL-22 in attenuating the severity of arginine-induced severe AP and associated lung and kidney injury was identified (16,27). To further evaluate the role of IL-22 in acinar cell damage associated with AP, the present study established an *in vitro* pancreatic acinar cell injury model using an arginine concentration of 2.5 mg/ml, due to the relatively stable mortality and survival rate exhibited by cells at this concentration. After treatment with rIL-22, the amylase concentration in the supernatant and the number of apoptotic cells were both significantly reduced compared with those in the untreated group. Proteomic analysis then demonstrated that the expression of protective proteins, such as those involved in DNA damage repair and apoptosis regulation, were notably upregulated after IL-22 treatment. These changes indicated that IL-22 can effectively ameliorate arginine-induced pancreatic acinar cell injury.

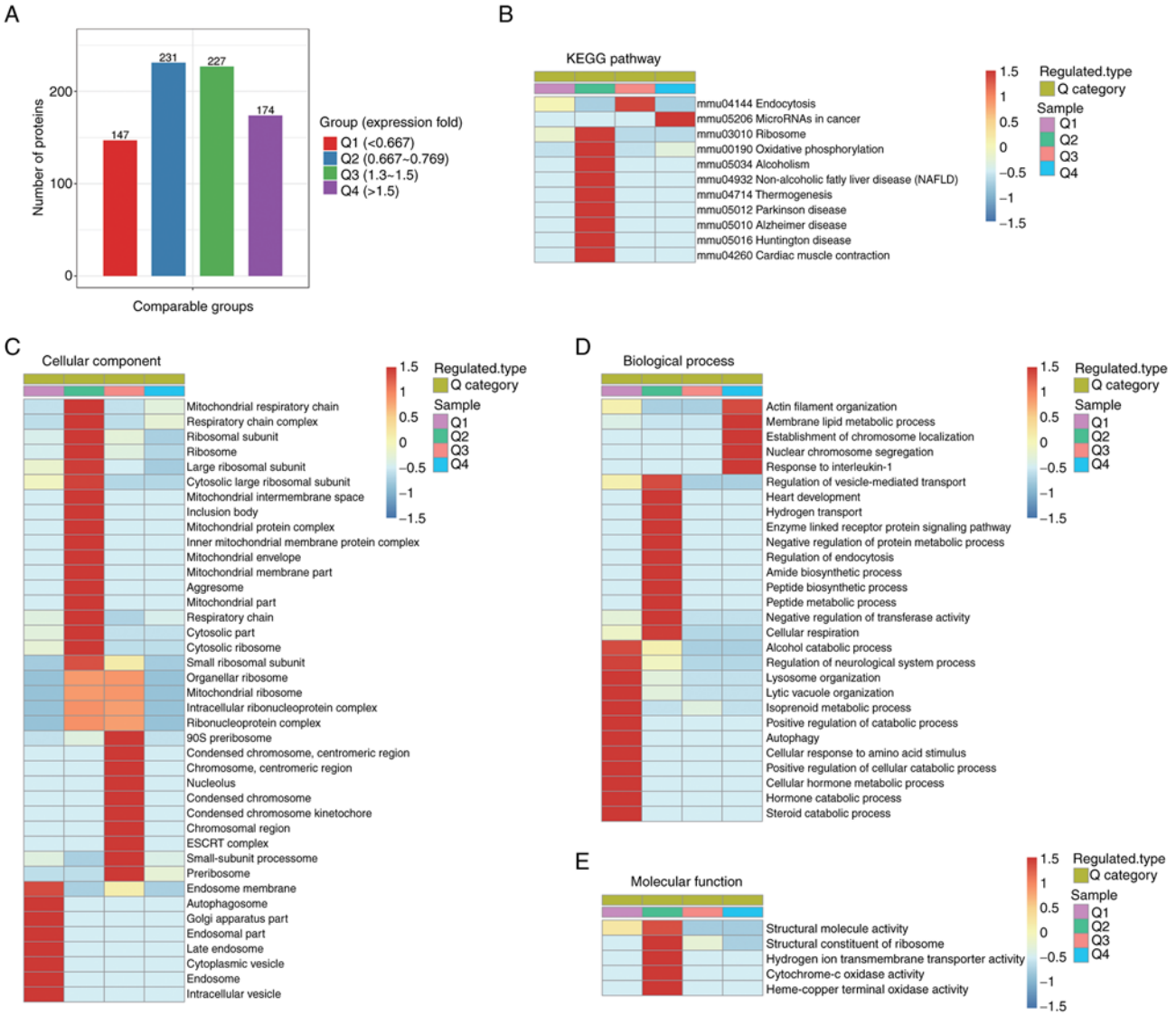

Figure 5. Cluster analysis of differentially expressed proteins. (A) Classification of proteins into four groups based on differential expression levels. (B-E) Heatmap (red, strong enrichment; blue, strong reduction) generated by hierarchical clustering for (B) KEGG pathways, and Gene Ontology terms associated with (C) cellular component, (D) biological process and (E) molecular function. KEGG, Kyoto Encyclopedia of Genes and Genomes.

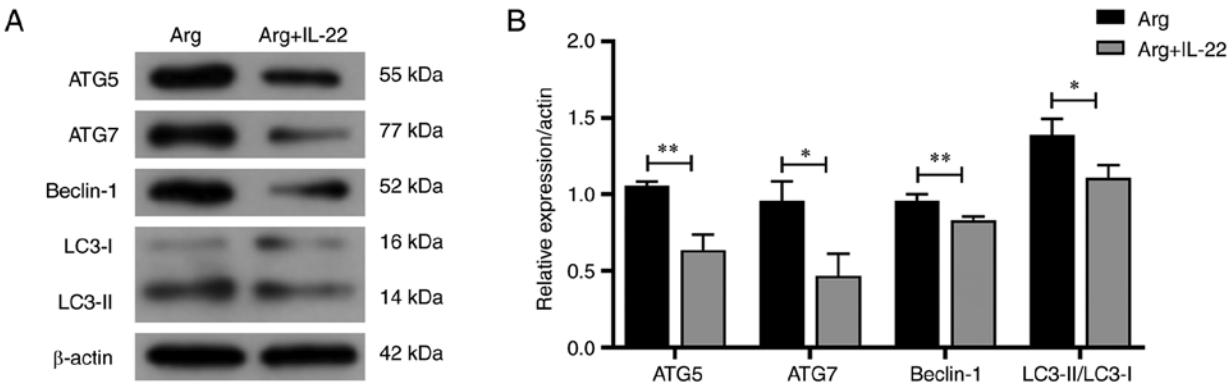

Figure 6. Downregulation of autophagy-related proteins following IL-22 treatment. (A) of Protein expression of ATG5, ATG7, beclin-1, LC3-I and LC3-II, demonstrating using western blotting. (B) Relative protein expression of ATG5, ATG7, beclin-1 and LC3-II/LC3-I. *P<0.05 and **P<0.01 (n=3). IL, interleukin; ATG, autophagy-related gene; LC3, microtubule-associated protein 1 light chain 3.

Moreover, IL-22 treatment might serve a role in maintaining the cytoskeleton, thereby contributing to the maintenance of cell morphology, material transportation and organelle movement. Actin is a component of the cytoskeleton (28), and its

organization and function were significantly increased by upregulated proteins (Vasp1, Mical-1, Eps and Abi2) after IL-22 treatment in the present study. In addition to participating in redox regulation of the actin cytoskeleton, Mical-1 acts as a negative regulator of apoptosis (29), which may explain the reduced apoptosis demonstrated in the present study after IL-22 treatment.

The expression levels of proteins related to biological processes, such as 'autophagy', 'lysosome organization' and 'lytic vacuole organization', were notably reduced after IL-22 treatment, according to GO analysis. Among the downregulated proteins, Vps18, Vps11 and beclin-1 garnered particular attention due to their involvement in the composition of the endosome, as well as their association with autophagosomes and lysosomes. Cluster analysis of the notably regulated proteins identified was used to assess the change in expression levels and associations of these proteins. Proteins with a fold change of <0.667 were significantly enriched in the biological processes of 'autophagy', 'lysosome organization' and 'lytic vacuole organization'. Furthermore, western blotting demonstrated that the protein expression of beclin-1, ATG5, ATG7 and LC3-II/LC3-I was significantly decreased in the IL-22-treated group, compared with the arginine-induced only group.

In the arginine-induced pancreatic acinar cell injury model in the present study, autophagy and lysosomal organization were significantly inhibited following IL-22 treatment. This inhibitory effect may have contributed to the alleviation of premature activation of pancreatic enzymes that originated from the colocalization of organelles. Autophagy, a type of programmed cell death other than apoptosis that is used to degrade organelles, proteins and other components (30), is essential for maintaining cellular homeostasis, and its impairment is related to the pathogenesis of a number of diseases, such as cancer, neurodegenerative diseases, and infectious diseases (31). The system responsible for transporting intracellular vesicles to lysosomes, primarily involving autophagy, plays a role in the early activation of trypsinogen during the initial stages of AP (30). Abnormal autophagic responses have been observed in experimental models of AP and studies have reported that impaired autophagy results in mitochondrial dysfunction that leads to ER stress, dysregulated lipid metabolism and increased severity of pancreatitis (4,32). However, inhibition of autophagy can suppress trypsinogen activation and reduced pancreatic damage (33).

Autophagosome maturation is a complex process involving several vesicle trafficking components. The key step in autophagic degradation of components is vesicle fusion, for which vesicle tethering is a prerequisite. The tethering events in the yeast endosome/vacuole (equivalent to mammalian lysosomes) have been studied and reported to require the class C Vps complex (34). Vps11 and Vps18 regulate the fusion between endosomes, lysosomes and autophagosomes by forming the core class C Vps complex. Beclin-1, an important marker to monitor autophagy levels, is involved in autophagic vesicle enucleation and autophagolysosome maturation (35). LC3 is a ubiquitin-like modifier involved in autophagosomes formation, and the ratio of LC3-II and LC3-I is commonly used to determine autophagy flux (36). ATG5 and ATG7 are proteins involved in autophagy-vesicle formation (37). The decreased protein expression levels of beclin-1, ATG5, ATG7 and LC3-II/LC3-I in the present study indicated that IL-22 inhibited the accumulation of autophagosomes at the initial step, whereas the reduced expression of LC3-II demonstrated that IL-22 treatment alleviated the blockage of autophagy flux. Beclin-1 is also involved in other pathways besides autophagy, including endocytic trafficking and LC3-associated phagocytosis (38). ATG5 also serves an important role in augmenting susceptibility to apoptosis. It likely interacts with mitochondrial components and participates in the activation of key apoptotic molecules, such as Bcl-xL (39). The present study demonstrated that IL-22 alleviated pancreatic acinar cell injury in the early stages of AP by inhibiting the intracellular lysosomal degradative system, based on the reduced expression of these proteins in the IL-22-treated cells.

Similar to autophagy, endocytosis is the internalization of macromolecules and surface proteins by cells. Endocytosis serves a pivotal role in the uptake of signaling molecules, which in turn activates cascades that can result in pathophysiological conditions (40). Studies have reported that endocytosis affects the degradation of trypsin in pancreatic acinar cells (41,42), and colocalization of organelles is hypothesized to result from the endocytosis of damaged acinar cells or their components (4). Endocytosis shares many effector proteins with autophagy, including Ras-like GTPases (Rabs) and Vps-associated proteins (43). These common effectors indicate an association between budding and fusion of membrane-bound vesicles. The crosstalk between autophagy and endocytosis implicates a novel endocytic regulatory pathway of autophagy. In the present study, hierarchical clustering of the KEGG pathway demonstrated that 'endocytosis' was enriched in the Q3 proteins. Some of these proteins were involved in both endocytosis and autophagy. For example, Rab5, a component of the Vps34-beclin-1 complex, is an essential component for early endosome biogenesis and endosomal fusion (44). Moreover, it serves a role in membrane elongation during macroautophagy (45). The adjustment of these co-effector proteins suggests that the crosstalk between autophagy and endocytosis may serve a role in IL-22-mediated treatment of arginine-induced pancreatic acinar cell injury.

Various methods are currently available to induce experimental pancreatitis, including the use of agents such as arginine, cerulein and taurolithocholic acid 3-sulfate. Despite the diversity in drugs and induction techniques, several other proteomic studies offer comprehensive insights into the development of this disease (46-48). Findings from the present study align with these findings, particularly with regard to modifying elements like Vps, Rabs, and ubiquitinated proteins. Collectively, these studies underscore the pivotal role of intricate processes such as apoptosis, autophagy, endocytosis, mitochondrial function and cytoskeletal dynamics in the activation of pancreatic enzymes, induction of acinar cell damage and promotion of cellular repair mechanisms. The present study demonstrated that following IL-22 treatment, endocytosis and autophagy showed opposite regulation. This differential regulation was further evaluated by analyzing the roles of the two aforementioned processes in acinar cell injury in AP. In the early stages of AP, impaired autophagy serves an important role

in the premature activation of trypsinogen, which is manifested by impaired fusion of autophagosomes and lysosomes, leading to excessive accumulation of autophagolysosomes in the cytoplasm and incomplete substrate degradation (30). In addition, impaired autophagy causes mitochondrial dysfunction, resulting in endoplasmic reticulum stress, dyslipidemia and increased severity of pancreatitis (32). Therefore, after IL-22 treatment in the present study, autophagy-related proteins may have been downregulated to reduce cell and tissue damage caused by premature activation of trypsin. However, despite the co-effector proteins shared by endocytosis and autophagy, maintaining normal endocytosis is necessary to alleviate the disease because of its role in the uptake of signaling molecules and transport of necessary substances (49). Therefore, certain proteins (Rab-5A, Vps36, Vps37b, Rab35, Sorting nexin-4 and Vps37b) involved in endocytosis were demonstrated to have been upregulated to maintain cellular physiological functions.

The present study has several limitations. First, proteomic analysis was not performed on untreated control cells. This omission restricts the comprehensive understanding of baseline protein expression and potential changes unrelated to specific treatments. This may have hindered the ability of the study to definitively determine the impact of IL-22 treatment on protein expression. Additionally, comparative experiments involving similar cell lines were not performed to ascertain the reproducibility of the outcomes across various cellular models. Whilst the AR42J cell line is commonly used to model AP (50-52), excluding other cell lines with similar traits restricts its broader applicability. Moreover, limitations arise from the complex interplay between endocytosis and autophagy in pancreatitis. Although the present study suggests a connection between these processes, complete understanding of how they interact during AP progression remains a prospect for future investigation.

In conclusion, the present study demonstrated that the transport system from intracellular vesicles to lysosomes, with a particular emphasis on autophagy, serves an important role in the pathogenesis and treatment of pancreatitis. IL-22 treatment downregulated the expression of autophagy-related proteins (Vps18, Vps11, beclin-1, ATG5, ATG7 and LC3-II/LC3-I), further inhibiting autophagosome maturation and fusion with lysosomes. Inhibition of autophagy helps to reduce the premature activation of trypsin and protects acinar cells from arginine-induced damage (53). Relative activation of the endocytic pathway occurs during this process, and has been reported to be related to the maintenance of cell signal transduction and physiological needs (54). Further research into the transport system from intracellular vesicles to lysosomes and the crosstalk between endocytosis and autophagy will help to elucidate the regulatory mechanism of IL-22. In summary, the present study demonstrates the potential of IL-22 as a new therapeutic molecule for treating AP by repairing impaired autophagy.

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Availability of data and materials

The proteomic datasets generated and analyzed during the current study are available in the 'figshare' repository (https://doi.org/10.6084/m9.figshare.21077194.v1) and the other datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QX conceived the study and wrote the manuscript. QX, XF and ZX performed the experiments and analyzed the data. HY and XM collected and processed the samples. ML constructed figures and interpreted the data. CX, BL and SZ contributed to the design of the study, interpretation of the data and the writing of the manuscript. HX designed and supervised the study, and critically revised the manuscript. QX and XF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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