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Irisin inhibits neutrophil extracellular traps formation and protects against acute pancreatitis in mice

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ABSTRACT

Introduction: Irisin is a newly discovered myokine which links exercise to inflammation and inflammation-related diseases through macrophage regulation. However, the effect of irisin on the activity of inflammation related immune cells (such as neutrophils) has not been clearly described.
Objectives: The objective of our study was to explore the effect of irisin on the neutrophil extracellular traps (NETs) formation.
Methods: Phorbol-12-myristate-13-acetate (PMA) was used to construct a classic neutrophil inflammation model that was used to observe the formation of NETs *in vitro*. We studied the effect of irisin on NETs formation and its regulation mechanism. Subsequently, acute pancreatitis (AP) was used to verify the protective effect of irisin *in vivo*, which was an acute aseptic inflammatory response disease model closely related to NETs.
Results: Our study found that addition of irisin significantly reduced the formation of NETs via regulation of the P38/MAPK pathway through integrin αVβ5, which might be the one of key pathways in NETs formation, and which could theoretically offset the immunoregulatory effect of irisin. Systemic treatment with irisin reduced the severity of tissue damage common in the disease and inhibited the formation of NETs in pancreatic necrotic tissue of two classical AP mouse models.
Conclusion: The findings confirmed for the first time that irisin could inhibit NETs formation and protect mice from pancreatic injury, which further elucidated the protective effect of exercise on acute inflammatory injury.

1. Introduction

Neutrophils are the most prevalent immune cells present in human blood, and they are preferentially recruited to the inflammatory site during injury or infection [1]. As the primary responders of the immune system, neutrophils are vital to shape the host response to infection and the stabilization of immune function, with the primary role of responding to, and eliminating, invading pathogens [2]. When the infection or tissue damage occurs, neutrophils defend against pathogens through multiple effector mechanisms, such as the production of reactive oxygen species (ROS), and neutrophil extracellular traps (NETs) [1]. NETs are considered to cause a type of cell death independent of

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apoptosis or necrosis. NETs activate neutrophils and extend DNA (deoxyribonucleicacid) fibers composed of the cytoplasmic granule proteins and the histone into the extracellular space, where they perform important biological functions [3]. NETs regulate tissue damage in cases of acute and chronic inflammation, such as in acute respiratory distress syndrome [4], sepsis [5], rheumatoid arthritis [6] and systemic lupus erythematosus [7], et al. Monti et al. [8] found that NETs could trap cancer cells by promoting metastatic proliferation and act as the adhesion matrix of cancer cells; however, the extent of their role was not yet fully known.

Irisin originates from the extracellular portion of a fibronectin domain containing 5 (FNDC5) protein, which is a cleavage fragment found in skeletal muscle; it is the glycosylated protein comprised of 112 amino acid residues that is produced by skeletal muscles and the myocardium during physical exercise [9,10] and is known to be a key regulator of energy and glucose homeostasis. Many reports have shown that irisin has a protective effect on the multiple inflammatory-related diseases such as heart ischemia-reperfusion injury, acute lung injury, and diabetes mellitus [11,12]. Irisin is also involved in immune regulation; recent research has suggested the role of irisin as a potential mediator of the inflammatory processes of macrophages [13,14]. Mazur-Bialy [15] demonstrated that irisin significantly altered the activity of macrophage, and reduced the excessive production of ROS. In addition, Gannon et al. [16] showed that irisin induced the apoptosis of breast cancer cells, suggesting that the anti-tumor properties of irisin could be related to the immune mechanism's activation.

Acute pancreatitis (AP), which is one of the most common causes of emergency hospital admission among digestive system diseases, generally develops from local inflammation of the pancreas that can lead to excessive systemic inflammatory response [17]. Most AP cases are mild and self-resolving; however, some AP diagnoses are characterized by obvious pancreatic necrosis, immune cell activation and release, and large amount of inflammatory mediators' infiltration, which may result in multiple organ failure and even death of the patients [18,19]. The key to the progress of severe AP (SAP) is the excessive inflammatory response mediated by immune cells [20], of which neutrophils are the first to the injury site [21]. Merza et al. [22] fully explored the role of NETs in regulating AP inflammatory response and pancreatic injury and found that obvious NETs formation was observed in the pancreatic tissues of both AP patients and mouse models. NETs aggravated the pancreatic injury, released a large number of inflammatory factors, induced excessive inflammatory response, and ultimately resulted in distant organ injury. Recently, it was reported that treatment with exogenous irisin could protect mice from severe organ damage from experimental AP [23]. However, it is still unclear whether immune cells, such as neutrophils, are involved in this protective effect, especially.

So far as we know, the immune regulation of irisin on the formation of NETs has not been reported; therefore, the present study was designed to explore the potential immunomodulatory role of irisin on the formation of NETs and its immunoregulatory effects on AP.

2. Materials and methods

2.1. Animals

Male wild-type C57BL/6J mice weighing between 20 - 25 g were bought from GemPharmatech Co. Ltd., Nanjing, China. All mice were stored in a specific pathogen-free standard room, with a 12 h/12 h dark/light cycle and a constant temperature regime at 20-25 °C. The mice were fed standard laboratory chow and given water ad libitum until 12 h before the experiment, at which point food was withheld and the mice fasted.

2.2. Ethics statement

All experiments involving animals were conducted in accordance with the ethical policies and procedures approved by the ethics committee of Yangzhou University, China.

2.3. Reagents and antibodies

All reagents and antibodies involved in this study are shown in Supplementary Table 1 and Supplementary Table 2.

2.4. Mild acute pancreatitis (MAP) model preparation and drugs intervention

Firstly, in the experiment of irisin intervention, mice were divided into the control group (control), the caerulein-induced AP group (Cae), and the irisin-treated (1 μg/kg), caerulein-induced AP group (Irisin/Cae). The dose selection of irisin was the best dose selected in the published literature [23,24]. The control group received intraperitoneal injection of the same dose of phosphate buffer saline (PBS) as the other groups. Secondly, in the experiment of cilengitide intervention, cilengitide was dissolved in 100% dimethyl sulfoxide (DMSO) to prepare a stock solution and then diluted it into 5% DMSO solution with PBS before being used. Subsequently, to verify the protective effect of cilengitide on AP, mice were divided into the control group (control), the caerulein-induced AP group (Cae), and the cilengitide-treated (10, 20, and 40 mg/kg), caerulein-induced AP group (Cilengitide/Cae). The control group received intraperitoneal injection of the same dose of 5% DMSO solution with PBS as the other groups. All groups except for the control group received intraperitoneal injections of caerulein (100 μg/kg, 1 h interval, 10 times) to induce the MAP model. Drugs were administered 1 h after the first injection of in the Irisin/Cae and Cilengitide/Cae groups. Mice were anesthetized with sodium pentobarbital (ip, 50 mg/kg) and sacrificed 12 h after the first injection of caerulein.

2.5. SAP model preparation and irisin intervention

As previously reported [25], the model of SAP was established using pancreatic duct ligation (PDL). Mice were divided into control, PDL, and PDL + irisin groups. Irisin (1 μg/kg) was injected intraperitoneally each day for ten days post-operation. The control group only underwent the sham operation of open and closed abdomen. Mice were sacrificed at 48 h post-PDL. The PDL was carried out as follows: One- to two-cm-long longitudinal incisions were made on mice anesthetized by intraperitoneal injection of 50 mg/kg of sodium pentobarbital. The duodenum and pancreas were turned over to uncover the distal side. Then, at 1 cm above the duodenal papilla, blunt dissection of the tissue around the pancreatic duct was performed, subsequently ligated. Finally, closed the abdominal cavity, and all mice were placed on a 37 °C constant temperature heating table for 90 min.

2.6. Sample collection

Orbital venous blood samples were harvested for serum amylase and lipase detection. The analysis was performed according to the kit instructions. Pancreatic tissue samples were collected and immediately fixed in 4% phosphate buffered formaldehyde followed by embedding in paraffin blocks for hematoxylin and eosin staining. The remaining pancreatic tissue samples were stored at −80 °C until use.

2.7. Histological analysis

The pancreatic tissues were fixed with 4% paraformaldehyde embedded in paraffin. The paraffin tissue was dewaxed, dehydrated, stained with hematoxylin-eosin, and examined with a light microscope. Histopathological scoring analysis of the pancreatic tissues was performed blindly by two independent pathologists according to our previously described methods [26].

2.8. Supernatant biochemical analysis

First, the cell supernatant culture medium of each group was collected, and then the floating cell debris in the cell supernatant was removed by low-speed centrifugation, and the required cell supernatant was retained. The levels of supernatant tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), macrophage chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) were detected using enzyme-linked immunosorbent assay kits per the manufacturer's instructions.

2.9. Flow cytometry and determination of ROS

Cells were stained with the following monoclonal antibodies: anti-CD45.2 (clone 104), anti-CD11b (clone M1/70), anti-Ly6G (clone 1A8), and anti-MPO (clone EPR20257). We acquired cells on a Beckman DxFlex and analyzed with CytExpert for DxFlex.

The content of ROS in cells was detected using a DHE fluorescent probe. DHE is one of the most common fluorescent detection probes for superoxide anions. Cells were incubated in the dark with DHE solution for 30 min at 37 °C. Cells were washed with PBS and then stained with the before mentioned monoclonal antibodies for 30 min at 4 °C. Cells were washed with PBS and resuspended with PBS. Finally, we acquired cells on a Beckman DxFlex and analyzed with CytExpert for DxFlex. Detailed methods are outlined in one of our previous studies [27].

2.10. Measurements of NETs formation in vivo

To detect the NETs content of pancreatic tissue *in vivo*, we performed immunofluorescence staining using anti-MPO antibody and anti-CitH3 antibody. Pancreases harvested from the experimental mice were fixed, embedded, and cut into 5 μm-thick sections. The sections were then boiled in ethylene diamine tetra-acetic acid-containing antigen repair buffer. Then, slides were treated with blocking endogenous peroxidase and blocking with normal goat serum. The slides were incubated overnight with anti-MPO (1:100 dilution) and anti-CitH3 (1:300 dilution) antibodies at 4 °C. Then slides were washed three times and subsequently incubated with biotinylated secondary antibody (1:300 dilution) at 37 °C for 2 h. Then, tissue sections were incubated in the DAPI solution at 37 °C. Images were acquired using the confocal microscope (Leica TCS Sp8 sted, Germany) and its corresponding LAS X image software.

2.11. Measurements of NETs formation in vitro

Bone marrow neutrophils were isolated from the femur and tibia of euthanized healthy 6-8-week-old C57BL/6J mice under sterile conditions in strict accordance with the previously described protocol [28]. Flow cytometry confirmed that the cell purity was greater than 95% (Supplementary Fig. 1). Freshly isolated bone marrow neutrophils were seeded in 12 well plates and cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) for 30 min in a humidified atmosphere of 5% CO2 at 37 °C, followed by treatment with 100 nm PMA or PBS for 4 h.

The NETs produced by bone marrow neutrophils were observed by immunofluorescence staining with anti-CitH3 antibody. The main methods are as follows: neutrophils were fixed with 4% paraformaldehyde and incubated in the dark in an anti-CitH3 antibody solution at 4 °C for 12 h. The slides were then placed in PBS and shaken for 5 min three separate times. The slides were then washed three times, and subsequently incubated with biotinylated secondary antibody at 37 °C for 2 h. Cells were then incubated in the DAPI solution at 37 °C for 5 min. Images were acquired using the confocal microscope (Leica TCS Sp8 sted, Germany) and its corresponding LAS X image software.

2.12. RNA-seq

The RNA-seq technique was used to analyze the mRNA expression in the model of PMA-induced neutrophil NETs formation. The total RNA of the Control group, PMA group, and PMA + irisin group were isolated using the TRNzol Universal Reagent (TIANGEN, China). The mRNA of each of the three groups were extracted for RNA-seq by the Novogene Company (Tianjin, China). Subsequently, volcanic map analysis and functional-enrichment KEGG analysis were performed in R software to identify differentially expressed genes (DEGs) between the three groups.

2.13. Western blot analysis

For immunoblotting analysis, the bone marrow neutrophils proteins were identified using a BCA protein kit (Thermo Fisher Scientific, MA, USA). Protein samples were subjected to 10% SDS- PAGE, then transferred to PVDF membranes. The membranes were blocked with 5% BSA for 2 h, then incubated overnight at 4 °C with the following primary antibodies: anti-PAD4 (1:1000 dilution), anti-p-p38 (1:1000 dilution), anti-p38 (1:1000 dilution), anti-p-Erk (1:1000 dilution), anti-Erk (1:1000 dilution), and anti-β-actin (1:1000 dilution) in blocking buffer. The following day, the membranes were washed with TBST three times for 15 min and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h at 37 °C. After washing, protein bands were detected using the ECL Plus chemiluminescent system. Image J software was used to analyze the image intensity.

2.14. Statistical analysis

Statistical analysis was performed in the GraphPad Prism 7.04 software (GraphPad, San Diego, CA, USA). A student's t-test was used to analyze means between two groups and a one-way analysis of variance (ANOVA) was used to evaluate statistically significant differences among all groups. Survival curves were derived by the Kaplan-Meier method and compared by a log-rank test. Results were presented as mean ± standard error (SE), and $P < 0.05$ was considered statistically significant (two-tailed). Each experiment was conducted three times.

3. Results

3.1. Irisin inhibits NETs formation

We evaluated the effects of irisin on the formation of NETs in an *ex vivo* study. Bone marrow neutrophils were cultured and stimulated with PMA, which was the classic inducing factor for NETs formation. After incubation of the neutrophils with different doses of irisin (5, 10, and 20 ng/ml), neutrophil infiltration (MPO^+ cells represent infiltrating neutrophils) and ROS production were detected using flow cytometry. As shown in Fig. 1A, the inhibitory effect of irisin on NETs formation and ROS production were most obvious in the group treated with 20 ng/ml irisin. As depicted in Fig. 1B–C, PMA-induced neutrophils form greater extensive NETs structures, and irisin treatment significantly inhibited NETs production. Additionally, proinflammatory cytokine levels in the supernatant were also inhibited by irisin in the PMA-induced neutrophils; however, there was no significant difference in TNF-α levels in the supernatant between groups (Fig. 1D).

Of the key enzymes involved in NETs production, including NE, MPO, and PAD4, the expression of PAD4 was found to increase significantly after PMA stimulation, but irisin limited the magnitude of the increase (Fig. 1E–F).

3.2. Irisin restrains NETs formation via P38/MAPK signaling pathway

To identify the molecular mechanism of irisin inhibiting NETs, we extracted RNA from cells from the Control, PMA, and PMA + irisin

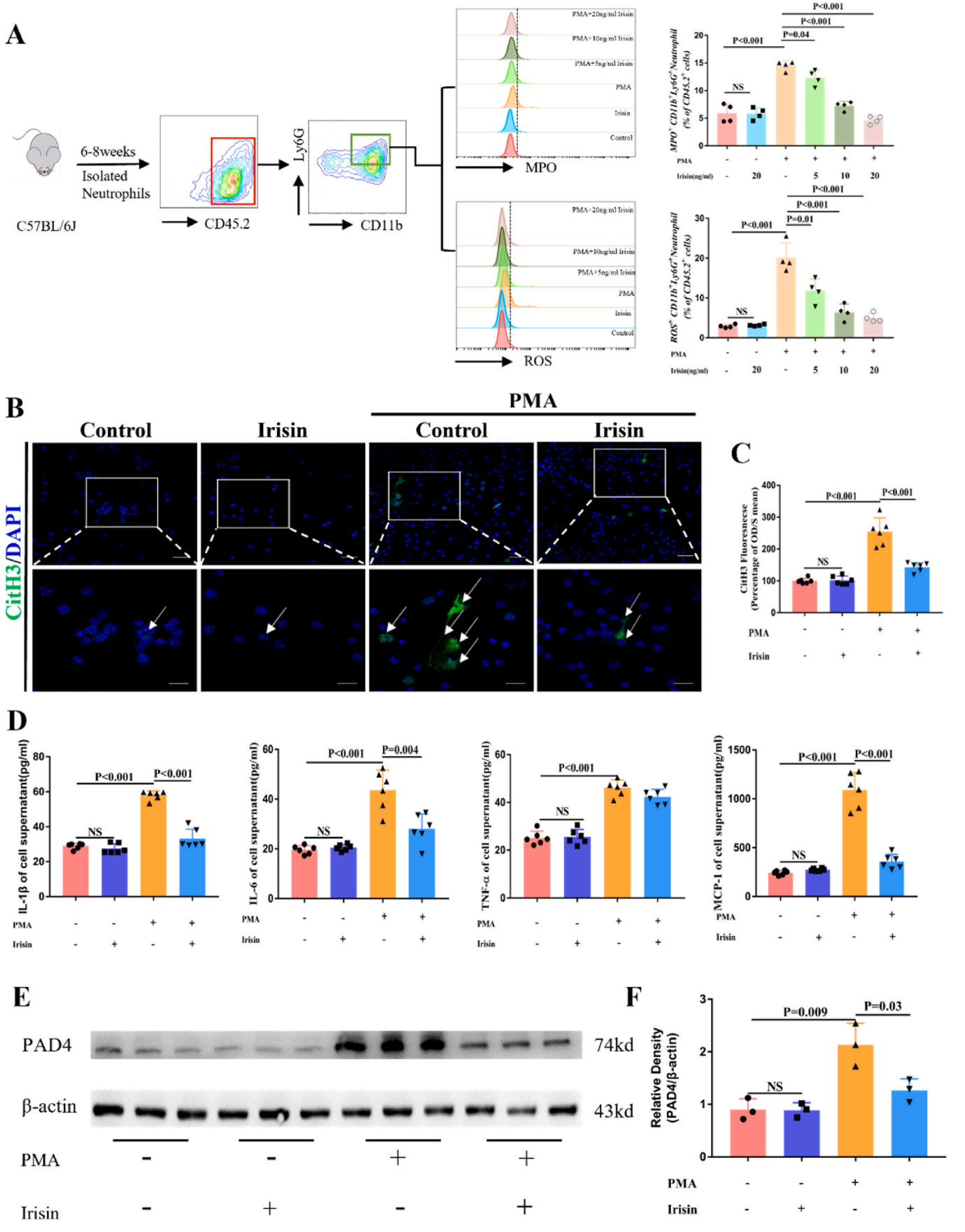

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Fig. 1. Irisin inhibits NETs formation. Bone marrow neutrophils isolated from six to eight-weeks-old C57BL/6J male mice are cultured and stimulated with PMA (100 nM), and incubated with different doses of irisin (5, 10, and 20 ng/ml), then stained for flow cytometry analysis. Representative flow cytometry gating of bone marrow neutrophils of C57BL/6J mice ($CD45.2^{+}CD11b^{+}Ly6G^{+}$). (A) Representative flow cytometry plots and bar graphs depicting the proportion of neutrophils and the expression levels of MPO and ROS. Data is presented as mean ± SE (N = 4 for each group). (B) Representative immunofluorescence image of CitH3 in magnification at 400× and 1000× (N = 6 for each group). Scale Bar = 50 μM. (C) Densitometric analysis of CitH3 fluorescence (N = 6 for each group). (D) The supernatant levels of IL-1β, IL-6, TNF-α, and MCP-1 are detected using ELISA. (N = 6 for each group). (E) Protein levels of PAD4 in neutrophils analyze using Western blotting (N = 3 for each group). (F) Relative protein expression of PAD4 (N = 3 for each group); β-actin is used as control for protein loading.

group to carry out RNA-seq. As shown in Supplementary Fig. 2 A-B, the volcanic maps showed all the differential genes in PMA vs. NC, and PMA + irisin vs. PMA, respectively. Subsequently, we combined the overlapping genes of up-regulated differential genes in PMA vs. NC and down-regulated differential genes in PMA + irisin vs. PMA group, and the overlapping genes of down-regulated differential genes in PMA vs. NC group and up-regulated differential genes in PMA + irisin vs. PMA group, and found that significant changes in MAPK signaling pathway via KEGG enrichment analysis (Supplementary Fig. 2C, Fig. 2A). The effects of irisin on the P38/MAPK pathway, one of the key pathways in the formation of NETs, were verified. High expression of P38 in neutrophils of PMA group was found, and the expression of P38 decreased significantly in PMA + irisin group, as shown in Fig. 2B and Supplementary Fig. 3.

Then, we applied the P38 inhibitor SB203580 to clarify the role of P38 on NETs formation *in vitro*. As shown in Supplementary Fig. 4A, after administration of different doses of SB203580 (2.5, 5, and 10 μM), NETs formed. Additionally, neutrophil infiltration and ROS production were lower in the SB203580-10 μM group compared to the SB203580-2.5 μM and SB203580-5 μM groups after PMA stimulation. We further validated the inhibitory effect of P38 inhibitor on NETs formation using CitH3 staining and subsequent analysis of PAD4 expression, as shown in Supplementary Fig. 4B–E.

To elucidate the importance and specificity of the P38/MAPK pathway, P38 inhibitor (SB203580) and agonist (anisomycin) were utilized to identify the pharmacological mechanism of irisin in the NETs formation. As shown in Fig. 2C and Supplementary Fig. 5, administration of SB203580 inhibited the formation of NETs, however the combine treatment irisin with SB203580 had no further inhibition of NETs formation, which was validated by IF staining and pro-inflammatory cytokines level (Fig. 2D–E). As expected, anisomycin boosted the formation of NETs (Supplementary Figs. 6A–B) and partially abolished the inhibition effects of irisin on NETs formation (Supplementary Figs. 6C–D). Above results further supported the conclusion that exogenous application of irisin inhibited the formation of NETs via potential inhibiting P38/MAPK signaling pathway.

3.3. Integrin αV/β5, a receptor of irisin, participates in the formation of NETs

Previous studies found that integrins αV/β5 may be the direct receptor of irisin, with the high degree of binding. Meanwhile, application of integrin inhibitor could block the interaction between integrin receptors and irisin, restrain downstream signal transduction, and improve inflammatory diseases such as intestinal injury [29,30]. Therefore, we assumed integrin αVβ5, the receptor of irisin, might be involved in the inhibition of irisin on the formation of NETs. We observed that after PMA stimulation induced NETs, the expression of αVβ5 in neutrophils increased significantly, and decreased after irisin intervention, as shown in Fig. 3A–B. We subsequently applied cilengitide, the integrin αVβ5 inhibitor, to explore whether integrin αVβ5 receptor was involved in the formation of NETs. In Fig. 3C, after incubation of the neutrophils with different doses of cilengitide (0.5, 1, and 5 μM), bone marrow neutrophil infiltration and ROS production were detected by flow cytometry. Obviously, neutrophil infiltration and ROS production were lower in the cilengitide −1 μM than that of the other groups after PMA stimulation. Subsequently, we also explored the inhibitory effect of integrin αVβ5 receptor on NETs formation by CitH3 staining and analysis of PAD4 expression (Fig. 3D–F). Above results suggested that integrin αVβ5 inhibitor participated in the formation of NETs, which was consistent with the effect of irisin treatment.

3.4. Irisin represses NETs formation via integrin αVβ5-P38/MAPK signaling pathway

Previous results confirmed that the effect of irisin on the P38/MAPK pathway was a key pathway in the formation of NETs. Thus, we explored whether irisin could inhibit the NETs formation via integrin αVβ5-P38/MAPK pathway. As shown in Fig. 4A, the protein expression level of P38 in neutrophils of the PMA + cilengitide group was significantly lower than that of the PMA group. According to the results of neutrophil infiltration and ROS production, it was found that the administration of P38/MAPK pathway inhibitor blocked the inhibitory effect of cilengitide on NETs formation (Fig. 4B). Proceed to the next step, cilengitide was used to identify the role of irisin in the damage of PMA-stimulated neutrophil cells. As shown in Fig. 4C, addition of irisin during cilengitide treatment did not further improve NETs formation. Above results were verified by CitH3 staining (Fig. 4D–E). In brief, these results demonstrated that irisin could inhibit NETs formation via integrin αVβ5-P38/MAPK signaling pathways.

3.5. Irisin represses NETs formation and alleviates disease severity in AP mice model

We first established the classical MAP mice model and verified the role of irisin. Interestingly, the protein expression level of irisin was significantly decreased in the muscle tissue of MAP mice (Supplementary Fig. 7). As shown in Fig. 5A–D, the pancreatic tissue morphology of the control group was basically normal. Intraperitoneal injection of caerulein in mice caused mild acute pancreatic injury, which characterized by edema, inflammatory cell infiltration, acinar cells necrosis, and significantly elevations of serum amylase and lipase activities. After the addition of irisin, the histological damage of the pancreatic tissue was significantly attenuated, with reduced edema, lessened infiltration of inflammatory cells, and decreased necrosis of pancreatic acinar cells. Furthermore, the levels of serum amylase and lipase were also remarkably decreased in the Cae + irisin group ($P < 0.05$).

We found that extensive NETs structures were formed in caeurlein-induced MAP. Neutrophil infiltration in the pancreas, as demonstrated using IF staining of MPO, significantly increased in MAP mice compared to that of the control group (Fig. 5E). CitH3 expression was then merged with MPO expression, showing that CitH3 was released from infiltrating neutrophils. As shown in Fig. 5E–F, according to the density analysis of CitH3/MPO fluorescence, the introduction of irisin remarkably reduced infiltrating neutrophils and NETs formation in pancreatic tissue, as evidenced by the decreased CitH3/MPO colocalization.

Furthermore, we established an additional classic PDL-induced SAP mice model to further explore the protective effect of irisin in SAP (Supplementary Fig. 8A). In SAP model, marked acinar cell necrosis and severe pancreatic tissue damage were more significant than seen in MAP model. As illustrated in Supplementary Fig. 8C–F, all results were consistent with those of the MAP model, which exhibited pancreatic pathological injury and serum enzyme activity changed. Moreover, Kaplan-Meier curves showed the significant difference in survival rate ($P = 0.002$) between PDL and PDL + irisin groups, suggesting that irisin could reduce the long-term mortality of mice in SAP model induced by

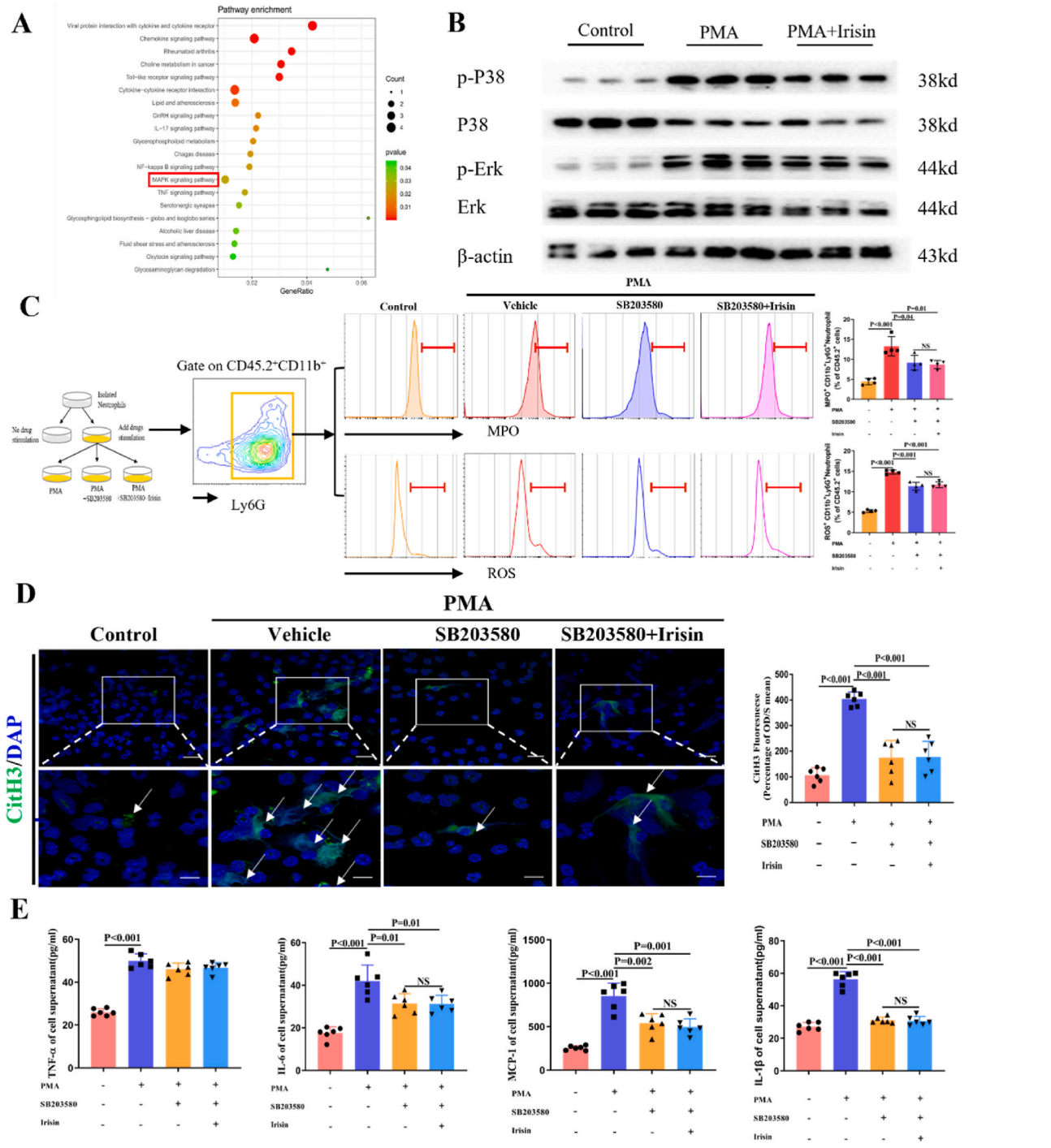

Fig. 2. Irisin restrains NETs formation depending on P38/MAPK signaling pathway. (A) KEGG enrichment analysis of the overlapping genes of the differential genes of PMA vs. NC and PMA + irisin vs. PMA. (B) Western blot analysis of protein levels of p-p38, p-Erk, p38, and Erk in neutrophils (N = 3 for each group). (C) Bone marrow-derived neutrophils were first incubated with SB203580 for 30 min, followed by treatment with irisin for 30 min, and by PMA stimulation for 4 h. Representative flow cytometry gating of neutrophils ($CD45.2^{+}CD11b^{+}Ly6G^{+}$). Representative flow cytometry plots and bar graphs depicting the proportion of neutrophils and the expression levels of MPO and ROS. Data is presented as mean ± SE (N = 4 for each group). (D) Representative immunofluorescence image of CitH3 at magnifications 400× and 1000×, and densitometric analysis of CitH3 fluorescence (N = 6 per group). Scale Bar = 50 μM. (E) The supernatant levels of IL-1β, IL-6, TNF-α and MCP-1 are measured using ELISA (N = 6 per group).

PDL (Supplementary Fig. 8B). We further found that CitH3/MPO colocalization in the pancreas was also significantly reduced after irisin administration (Supplementary Fig. 8G and H). Besides, the protein expression level of irisin was significantly decreased in the muscle tissue of SAP mice (Supplementary Fig. 8I).

3.6. Integrin αVβ5 stifles NETs formation and alleviates disease severity in AP mice model

Whether integrin αVβ5 has the protective effect on caerulein-induced MAP model has not been reported. As shown in Fig. 6A–D and Supplementary Fig. 9, the disease severity of MAP was remarkably relieved after cilengitide administration, accompanied by decreased

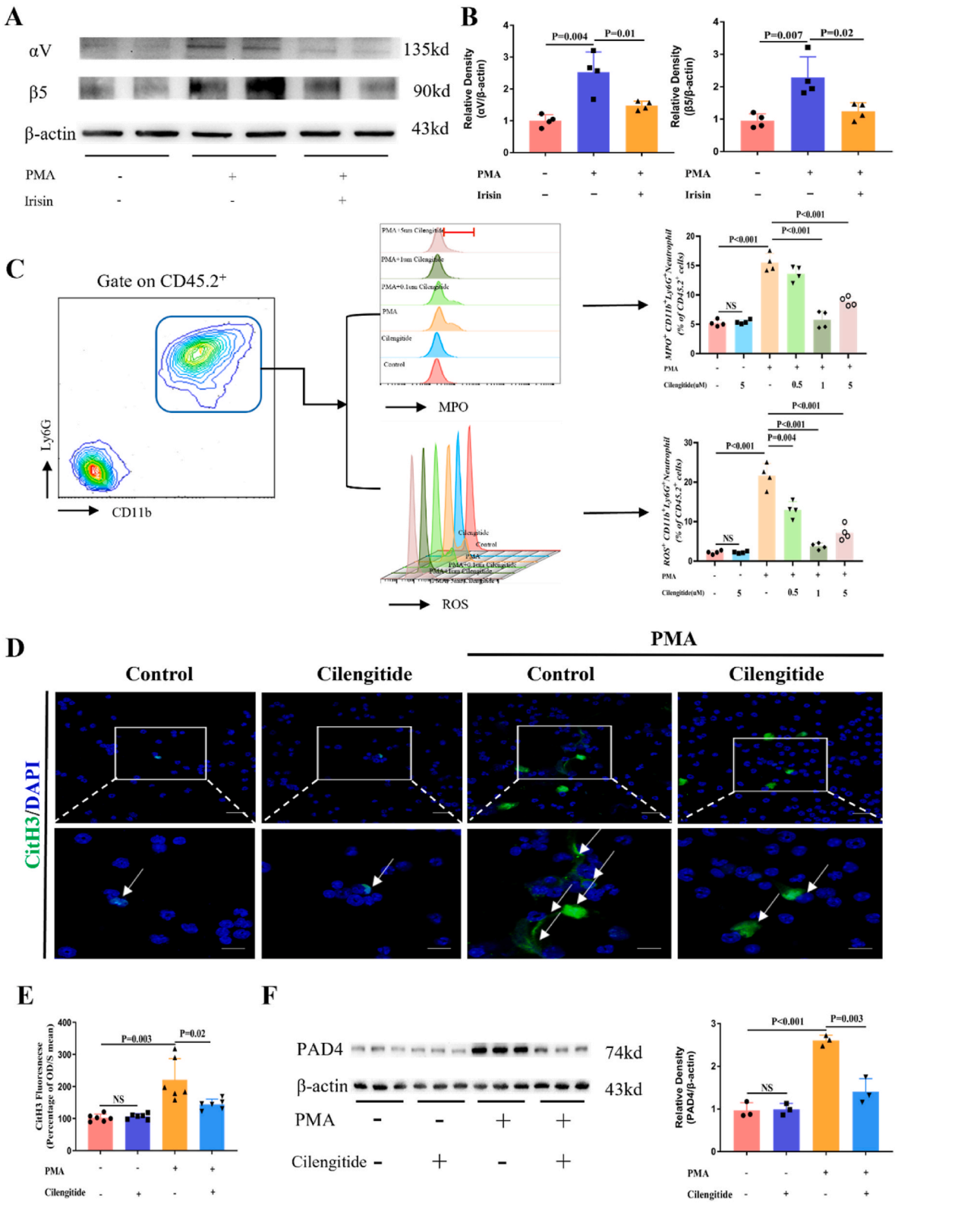

Fig. 3. Integrin αVβ5 suppresses NETs formation. (A) Western blot analysis of integrin αVβ5 level in neutrophils (N = 4 per group). (B) Relative protein expression of integrin αVβ5; β-actin is used as a control for protein loading (N = 4 per group). (C) Representative flow cytometry gating of bone marrow neutrophils of C57BL/6J mice ($CD45.2^{+}CD11b^{+}Ly6G^{+}$). Representative flow cytometry plots and bar graphs depicting the proportion of neutrophils and the expression levels of MPO and ROS. Data is presented as mean ± SE (N = 4 for each group). (D) Representative immunofluorescence image of CitH3 in magnification 400× and 1000× (N = 6 for each group). Scar Bar = 50 μM. (E) Densitometric analysis of CitH3 fluorescence (N = 6 for each group). (F) Western blot analysis of PAD4 levels in neutrophils. Relative protein expression of PAD4; β-actin is used as a control for protein loading (N = 3 each group).

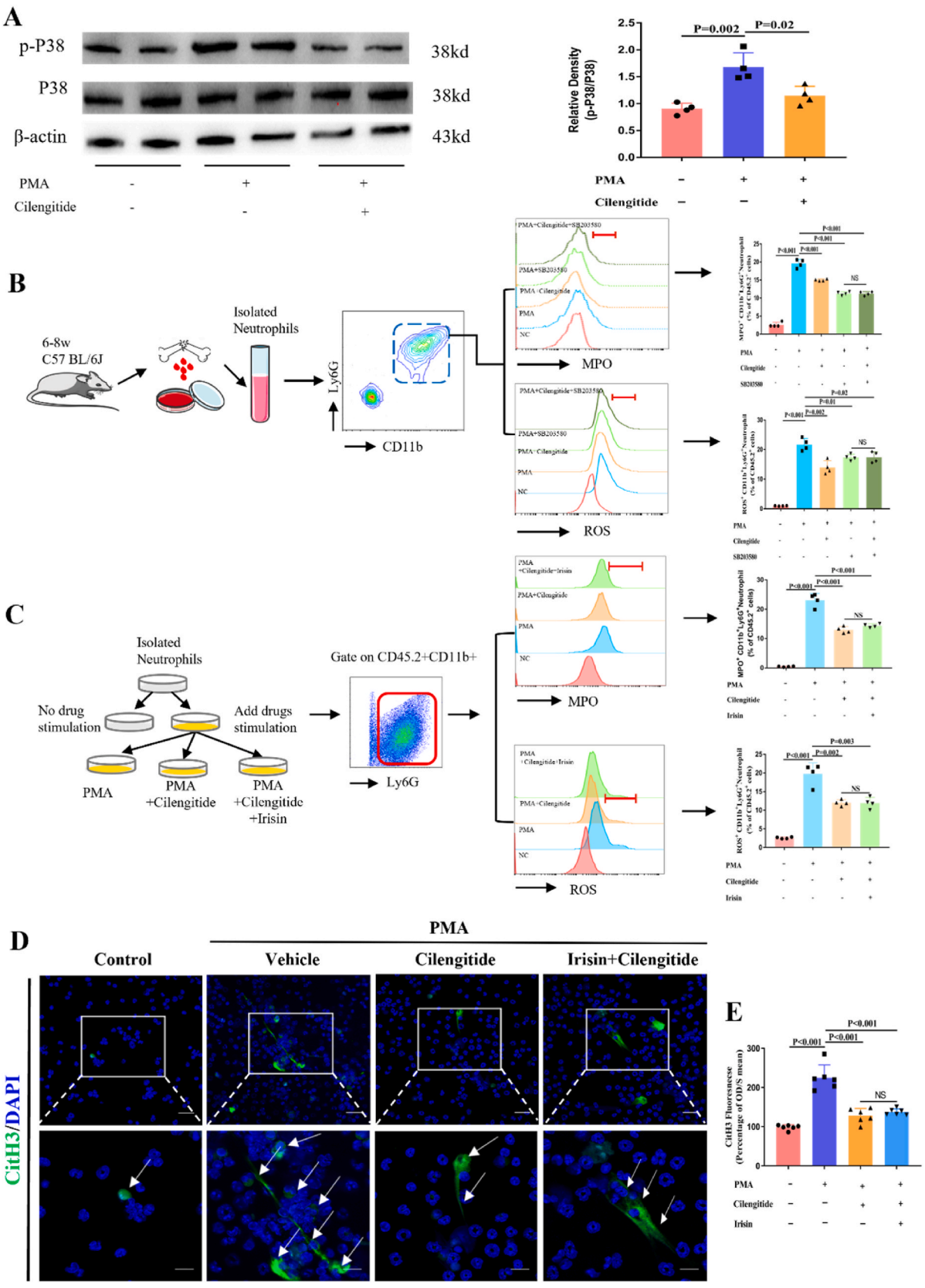

Fig. 4. Irisin represses NETs formation via the integrin αVβ5-p38/MAPK signaling pathway. (A) Western blot analysis of protein levels of p-p38 and p38 in neutrophils. Relative protein expression of p-P38, p38 is used as controls for protein loading (N = 4 per group). (B–C) Representative flow cytometry gating of bone marrow neutrophils of C57BL/6J mice ($CD45.2^{+}CD11b^{+}Ly6G^{+}$). Representative flow cytometry plots and bar graphs depicting the proportion of neutrophils and the expression levels of MPO and ROS. Data is presented as mean ± SE (N = 4 for each group). (D) Representative immunofluorescence image of CitH3 in magnification 400× and 1000× (N = 6 for each group). Scar Bar = 50 μM. (E) Densitometric analysis of CitH3 fluorescence (N = 6 for each group).

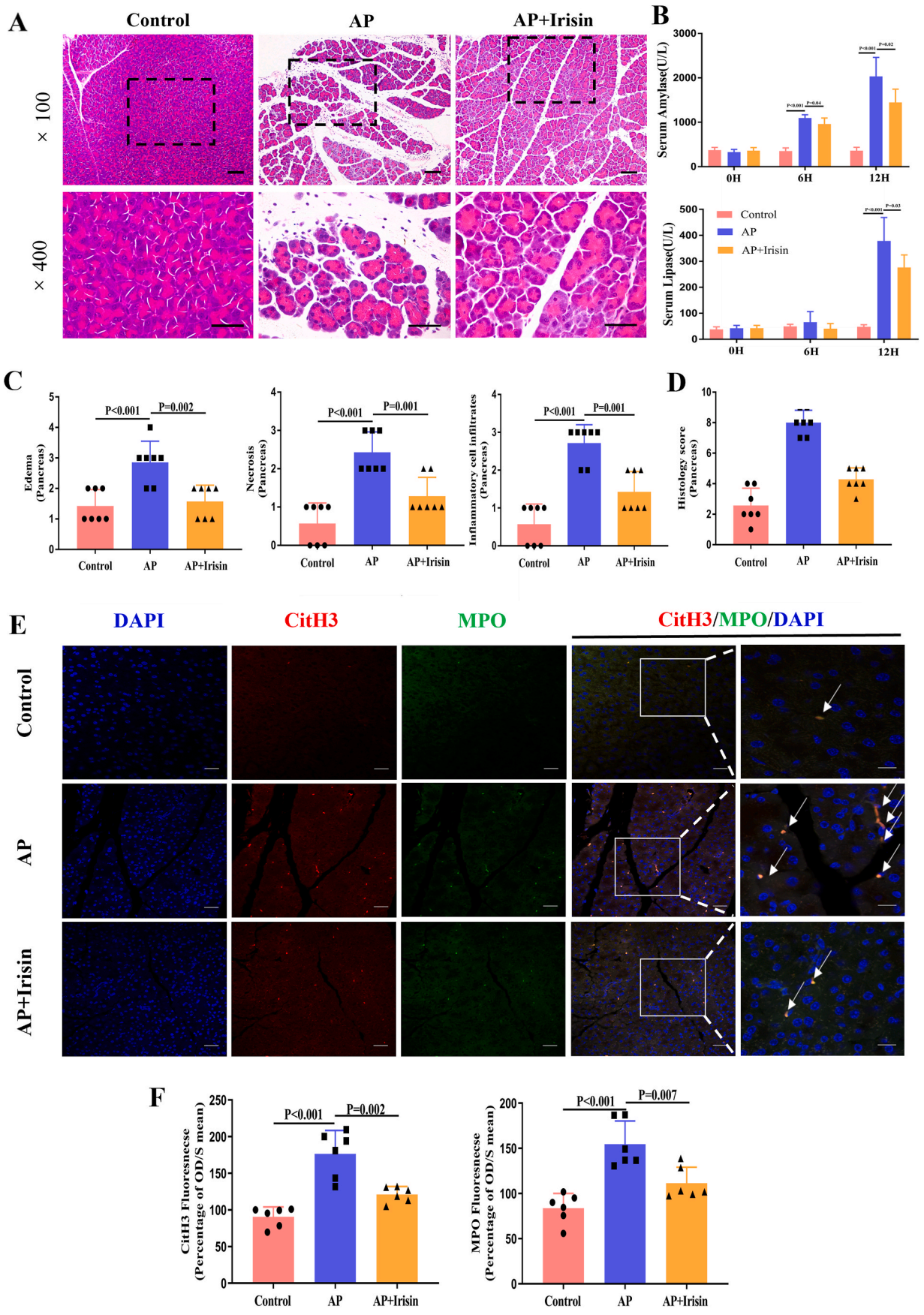

Fig. 5. Irisin curbs NETs formation and alleviates disease severity in MAP mice model. (A) Representative HE staining of pancreatic tissues at magnifications 100× and 400×. Scale Bar = 50 μM. (B) Serum levels of amylase and lipase. (C–D) The pathological scores of pancreatic tissues. (E) Representative immunofluorescence image of CitH3 and MPO at magnifications 400× and 1000×. Scale Bar = 50 μM. (F) Densitometric analysis of CitH3 and MPO fluorescence. (N = 7 per group).

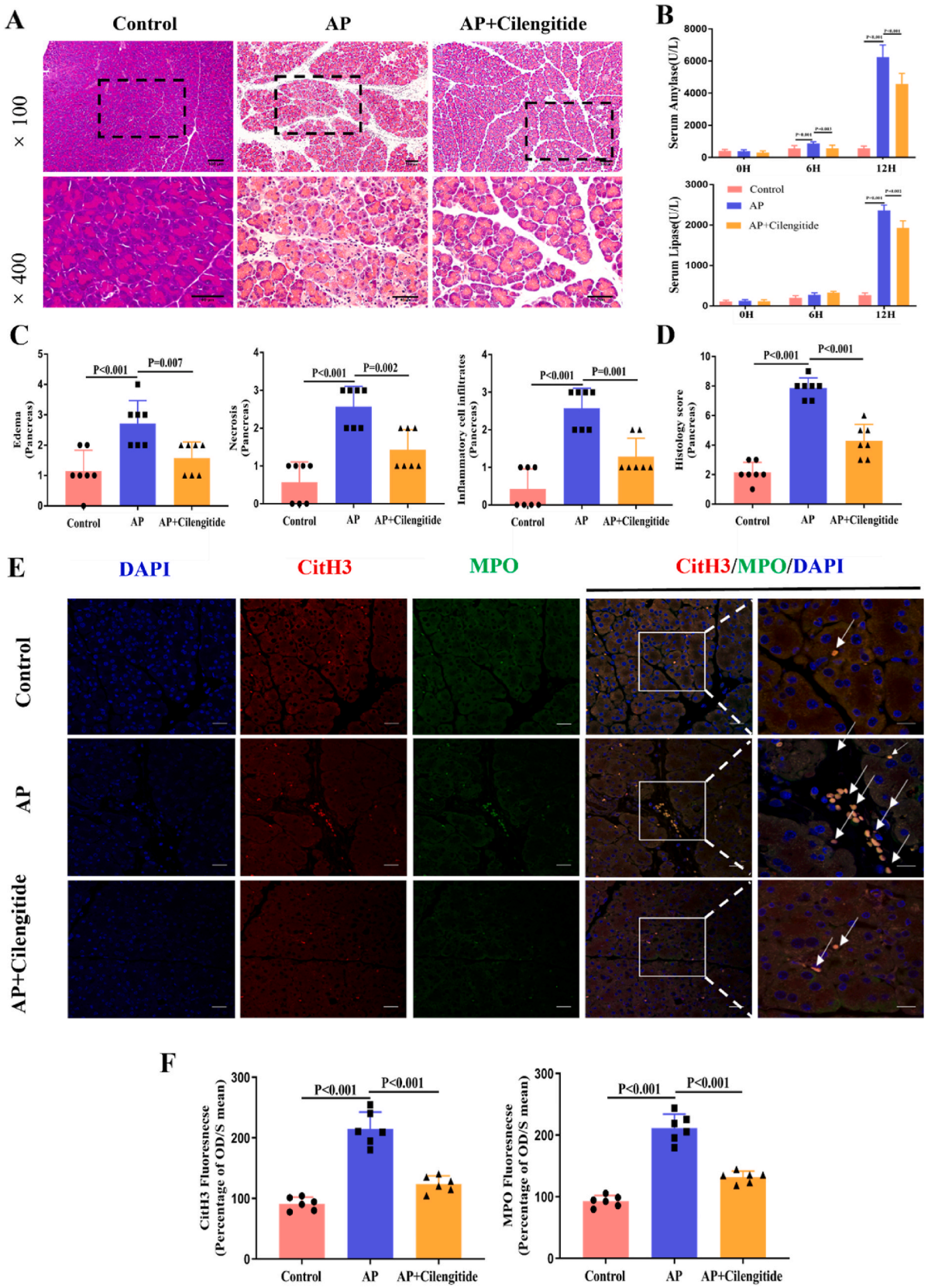

Fig. 6. Integrin αVβ5 inhibitor inhibits NETs formation and mitigates disease severity in MAP mice model. (A) Representative HE staining of pancreatic tissues at magnifications 100× and 400×. Scale Bar = 50 μM. (B) Serum levels of amylase and lipase. (C–D) The pathological scores of pancreatic tissues. (E) Representative immunofluorescence image of CitH3 and MPO at magnifications 400× and 1000×. Scale Bar = 50 μM. (F) Densitometric analysis of CitH3 and MPO fluorescence (N = 7 per group).

inflammatory cell infiltration, acinar cells necrosis and serum levels of amylase and lipase (All $P < 0.05$). In addition, as shown in Fig. 6E–F, cilengitide intervention significantly reduced infiltrating neutrophils and NETs formation in pancreatic tissue, just as the decreased CitH3/MPO colocalization, which were consistent with the results of irisin. All in all, integrin αV/β5 inhibited NETs formation and mitigated disease severity in MAP.

4. Discussion

Our study verified for the first time that irisin had the clear protective effect on the formation of NETs in AP. In addition, we also clarified the function of integrin αVβ5 in the formation of NETs and in AP mice model. These results explained the anti-inflammatory effect of irisin on the other hand, and also suggested that integrin αVβ5 was the key target for targeting the formation of NETs in AP.

Neutrophil recruitment was the rate-limiting step in pancreatic tissue and distant organs injury [31,32]. This was evidenced in a study by Oiva et al. in which it was found that neutrophils were involved in early local pancreatitis and distant organ injury [33]. With the release of inflammatory cytokines, neutrophils, and macrophages, there was increased leukocyte activation and infiltration in the tissues, elevated neutrophil granular enzyme MPO activity, and exacerbated tissue damage, leading to severe acute pancreatitis [34]. Therefore, AP, a non-infectious inflammatory disease, was selected for our study to verify the inhibitory effect of irisin on the NETs formation. In our study, we demonstrated that irisin had a protective effect on AP through two different AP models. *In vivo* experiments, we found that irisin could inhibit the infiltration of neutrophils in pancreatic tissue and reduce the activity of MPO, thereby alleviating pancreatitis. These results fully demonstrated the potential therapeutic effect of irisin on inflammatory diseases.

Recently, several studies showed that irisin had immune effects on various types of cells, including immunotherapy, immune regulation, etc [16,35–37]. Wang et al. [36] found that irisin could improve the immune regulatory response of viral myocarditis by regulating Treg/Th17 cells. In addition, Myint [37] et al. demonstrated that irisin acted as the novel ligand for integrins αLβ2 and α4β7, thereby promoting lymphocyte adhesion. In our study, we focused on the effect of irisin on neutrophils, mainly to observe its effect on the formation of NETs. Through abundant *in vivo* and *in vitro* experiments, we fully verified that irisin could significantly inhibit NETs formation in AP. Above results once again confirmed that the immune regulatory effect of irisin from the perspective of neutrophils.

As we all know, integrins are composed of transmembrane cell adhesion molecules composed of noncovalently associated α/β heterodimers [38]. Oguri et al. [39] showed that the complex consisting of CD81 with integrins αVβ1 and αVβ5 could mediate the activation of integrin/FAK signaling pathway in response to irisin. At the same time, another study found that irisin interacted with integrin αVβ5 on intestinal epithelial cells *in vitro* and *in vivo*, and it was verified that integrin αVβ5 was the receptor of irisin [30]. Our study found that the expression level of integrin αVβ5 was elevated in neutrophils during acute inflammation. *In vivo* experiments, we also found that inhibition of integrin αVβ5 could significantly reduce the infiltration of neutrophils in mouse pancreatic tissue, inhibit the formation of NETs, and alleviate pancreatic tissue damage. These results confirmed that inhibiting the activation of integrin αVβ5 could inhibit the formation of NETs *in vitro* and *in vivo*, indicating that integrin αVβ5 was one of the necessary factors for NETs formation, which was consistent with that of irisin. Therefore, we found that irisin and its receptor integrin αVβ5 were important intervention targets for the formation of NETs.

Hakkim et al. [40] demonstrated firstly that the Raf-MEK-ERK signaling pathway played a key role in PMA-induced formation of NETs. Many subsequent studies found that PMA induction could lead to the NADPH oxidase activation and generation of ROS, which in turn stimulated the phosphorylation of ERK and P38/MAPK, ultimately inducing the formation of NETs [41]. In our study, through RNA-Seq and subsequent functional verification, we analyzed that irisin inhibited the formation of NETs via P38/MAPK pathway. Our study also showed that irisin reduced the levels of cytokines produced during NETs formation. Generally known, P38/MAPK is a key inflammatory signaling pathway. Ko et al. [42] pointed out that rapamycin could down-regulate the levels of IL-6, IL-8 and MCP-1 by inhibiting the activation of the IL1-p38 MAPK-NF-κB pathway. At the same time, another study found that the combination therapy of Pien Tze Huang (PTH) and TAK242 could inhibit the release of IL-1β, IL-6, TNF-α and MCP-1 by regulating the TLR4/NF-κB/MAPK signaling pathway, and reduce the volume of cerebral infarction, to improve the neurological deficit caused by middle cerebral artery occlusion [43]. Our study showed that irisin significantly reduced the level of inflammatory factors produced during the formation of NETs, which may be related to the inhibition of P38/MAPK signaling pathway.

As the cleavage product of FNDC5, irisin is an endogenous small natural peptide with good safety. Compared to the membrane binding protein FNDC5, irisin has the better and more extensive potential clinical application space, which needs to be verified in subsequent clinical trials.

5. Conclusion

In conclusion, our findings confirmed for the first time that irisin could inhibit the formation of NETs via the integrin αVβ5-P38/MAPK pathway. In the future, irisin may provide better theoretical possibilities for the treatment of AP and other inflammatory diseases related to NETs. Integrin αVβ5 may also be a key target for targeting the formation of NETs.

Author contributions

Fei Han, Zi-fan Ding and Xiao-lei Shi contributed equally to this work.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102787.

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