

Neuroblast Differentiation-Associated Protein Derived Polypeptides: AHNAK(5758-5775) Induced Inflammatory Reaction in Skin By Mast Cells Activation

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Research Article

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Abstract

Psoriasis is a chronic inflammatory skin disease. Mast cells significantly increase and activate in the lesions and are involved in psoriatic inflammation. Neuroblast differentiation-associated protein (AHNAK) mainly express in skin, esophagus and kidney, which participates in the differentiation of neurons, the formation of cytoskeletal structure muscular regeneration and the calcium homeostasis process. Whether AHNAK is involved in mast cell activation is unclear, and the mechanisms of AHNAK induced skin inflammation also needs investigation. To investigate whether Neuroblast differentiation-associated protein derived polypeptides: AHNAK(5758-5775) activates mast cells and induces skin inflammation contributing to psoriasis, wild-type mice were treated with AHNAK(5758-5775) to observe inflammatory cells infiltrated in skin and cytokines release *in vivo*. Release of inflammatory mediators by mouse primary mast cells, LAD2 cells and human neutrophils were measured *in vitro*. Neutrophils and mast cells were co-cultured to verify AHNAK(5758-5775)' role in inflammation. Molecular docking analysis, molecular dynamics simulation and siRNA transfection were used to prove the receptor of AHNAK(5758-5775). AHNAK(5758-5775) caused skin inflammation in WT mice by recruitment of neutrophils and cytokines release. Moreover, AHNAK(5758-5775) does not directly activate neutrophils PPD, while it is via mast cells. ST2 seems to be a key receptor mediating the activation effect of AHNAK(5758-5775) on mast cells and lead to cytokines release. Altogether, we proposed the novel polypeptide: AHNAK(5758-5775), which might induce inflammation and participated in the occurrence and development of psoriasis by activating mast cells.

Introduction

Psoriasis vulgaris (PV) is a chronic and systemic inflammatory skin disorder. Clinically manifests as well-demarcated erythematous plaques covered with silver thick scales, it affects 2-3% of the world's population nowadays. The onset of PV is antimicrobial peptides (AMP) combine with self-nucleic acids and then activate plasmacytoid DC (pDC), who act as antigen presenting cells and secrete cytokines to promote the differentiation of Th17 and Th22 cells. Most of the current studies focus on the IL-23/Th17 axis and suggest that adaptive immune represented by T cells plays an important role. However, accumulating evidences show that innate immune have an unexpected critical status in PV pathogenesis.

Due to the complex inflammatory molecular and cellular network in psoriasis, we cannot separate the innate and adaptive immunity completely when discussing the pathogenesis. Mast cells (MCs) have been reported to play an irreplaceable role in chronic inflammatory diseases. Studies discovered an increased infiltration of activated MCs and neutrophils in psoriatic lesions, and MCs have been activated in the early stage of the lesions [1–3]. MCs are the main source of IL-22 in psoriasis [3]. Neutrophil infiltration is one of the downstream reactions of MCs activation, secretion of enzymes, cytokines and chemokines like CXCL1/CXCL2, expression of cell-surface molecules by MCs could promote the aggregation of neutrophils [1].

Psoriasis requires the presence of autoantigens to initiate inflammatory responses as an autoimmune skin disease. Endogenous substances such as LL37 and ADAMTSL5 were successively discovered as triggers of psoriasis [4]. Other works demonstrated that peptides such as leptin, catestatin, and some isolated human albumin fragments could activate MCs and then lead to downstream inflammation [5–7]. MCs express several peptide-mediated activated receptors including MrgprX2, ETA receptor, NK1R, NTR and FPRL1 [8].

AHNAK mainly express in skin and other organs. Basically, *AHNAK* is involved in the differentiation of neurons and participates in the formation of cytoskeletal structure muscular regeneration, and calcium homeostasis process [9]. In skin, keratinocytes and fibroblasts in the basal layer are main producers. Present researches mainly focused on its role in tumor invasion, tumor metastasis and tumor inhibition effect [10, 11]. *AHNAK*-derived peptides are effective regulators of the cardiac L-type Ca^{2+} channel [12]. *AHNAK* is highly expressed in MCs and participates in TLR4-mediated MCs activation signaling pathway as well [13]. However, there is no research of *AHNAK* or *AHNAK*-derived peptide in psoriasis or other inflammatory diseases, more related content is waiting for our exploration.

In this study, we found an increase of *AHNAK*-derived peptides. Interestingly, it could activate mast cells via ST2 and participate in neutrophil infiltration. We hypothesized that this might be part of the PV pathogenesis

Materials And Methods

Reagents

AHNAK(5758-5775) was purchased from Nanjing Peptide Biotech Ltd. (Nanjing, China) and was detected by HIGH performance liquid chromatography and mass spectrometry (supplementary Figure 1).

Compound 48/80 (C 48/80) and lipopolysaccharide were purchased from Sigma-Aldrich (St Louis, MO, USA). Tyrode's solution buffer was prepared fresh on the day of use (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l $CaCl_2$, 0.143 g/l $MgSO_4$, 0.162 g/l KH_2PO_4 , 2.383 g/l HEPES, 0.991 g/l glucose, and 1 g/l BSA, pH=7).

Samples of Psoriasis Vulgaris (PV) Patients and Polypeptide Omics Analysis

A single-center and case-control study was adopted. 3 psoriasis vulgaris without no other diseases samples were from Department of Dermatology, Second Affiliated Hospital of Xi'an Jiaotong University. 3 control samples were from Department of orthopedic, Second Affiliated Hospital of Xi'an Jiaotong University, diagnosed as fracture patients with no infection and immune diseases. The samples were frozen with liquid nitrogen to extract peptides and the protein was removed. Polypeptide segments were detected by liquid chromatography-mass spectrometry. And the polypeptide omics analysis was finish by MH BioTech Co.Ltd (Shanghai, China).

Animals

Adult male C57BL/6 mice ageing 8 weeks were purchased from the Experimental Animal Center of Xi'an Jiaotong University. Nine mice were used per group.

Histology

10 μ l AHNAK(5758-5775) (20 μ M) prepared in saline were injected subcutaneously into mice ears for 3 days. And saline was set as negative control. Mice were sacrificed through CO₂ inhalation. Ears samples were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E). Avidin-FITC was used for marking mast cells and anti-Ly6G antibody was used for marking neutrophil.

Cytokines Analysis in Mice Skin

10 μ l AHNAK(5758-5775) (20 μ M) prepared in saline were injected subcutaneously into mice ears for 3 days. The samples were frozen with liquid nitrogen and added 0.5 ml saline. Then the ears were cut into pieces. The supernatant was obtained by centrifugation after 30 min ultrasound treated. ELISA Kits were purchased from Sino Biological Inc (Beijing, China).

Mouse Peritoneal Mast Cell (MPMC) Purification and Media Analysis

Adult male C57BL/6 mice were sacrificed through CO₂ inhalation. A total of 12 ml mast cell dissociation medium (MCDM) (15 ml of MCDM: 1.5 ml of 10 \times HBSS, 450 μ l of fetal bovine serum, 150 μ l of 1 M HEPES, 12.9 ml of sterile water, pH = 7.2) was used for two to three sequential peritoneal lavages, then centrifuged at 200 g for 10 min at 4°C. Anti-mouse CD117 and Anti-R-Phycoerythrin (PE) Magnetic (BD Biosciences, New York, USA) was used for isolation and purification mouse peritoneal mast cell (MPMC). MPMC in the pellet were retrieved, and purity was > 95% as assayed by morphology. Then MPMC resuspended in DMEM with 100 ng/ml recombinant mouse stem cell factor (SCF), and used in 4 h.

1 \times 10⁵ MPMC per well, were incubated in a 96-well plate. The culture medium was removed, AHNAK(5758-5775) prepared by Tyrode solution (10, 20, 40 μ M) was added at the indicated concentrations, and the cells were incubated for 1 h for tryptase, histamine, and β -hexosaminidase assay at 37°C with 5% CO₂, while 8 h for cytokines assay by ELISA.

Preparation of Neutrophils From Human Blood and Activation Assay

Blood samples were collected from different authors of this paper. Peripheral neutrophils were separated using a magnetic-activated cell-sorting method. 1 \times 10⁶ neutrophils were incubated in a 96-well plate. The culture medium was removed, AHNAK(5758-5775) prepared by serum-free RPMI 1640 culture medium (10, 20, 40 μ M) was added at the indicated concentrations for 6 h for cytokines assay. LPS (200 ng/mL) was set as a positive control and only serum-free RPMI 1640 culture medium was set as a negative control. The cytokine release analysis by ELISA.

For neutrophils chemotaxis analysis, neutrophils were diluted with serum-free RPMI 1640 medium to a concentration of 1×10^6 cells/ml. 500 μ l of serum-free 1640 medium with AHNAK(5758-5775) (10, 20, 40 μ M) and LPS were added into the lower chamber, and 200 μ l neutrophils were added into a 3 μ m aperture size transwell chamber for 2 h. The cells on the membrane were counted under a microscope, and cells in the lower chamber were counted using a Cellometer Mini (Nexcelom, San Mateo, CA, USA).

Mast Cells Medium Release Assay

1×10^6 LAD2 cells were incubated in a 96-well plate overnight. The culture medium was removed, AHNAK(5758-5775) prepared by Tyrode solution (10, 20, 40 μ M) was added at the indicated concentrations, and the cells were incubated for 1 h for tryptase, histamine, and β -hexosaminidase assay at 37°C with 5% CO₂, while 8 h for cytokines assay. 10 μ g/ml C 48/80 were set as positive control.

Neutrophil-mast Cells Co-culture and Cytokine Release Analysis

Neutrophils were diluted with serum-free RPMI 1640 medium to a concentration of 1×10^6 cells/ml. 1×10^5 cells/ml LAD2 cell (500 μ l) in serum-free 1640 medium with AHNAK(5758-5775) (10, 20, 40 μ M) were added into the lower chamber. 200 μ l neutrophils were added into a 3 μ m aperture size transwell chamber for 2 h to analyze neutrophils chemotaxis, and 6 h for cytokine release analysis by ELISA.

Small interfering (si)RNA Transfection of LAD2 Cells

Specific knockdown was achieved using small interfering (si)RNAs targeting ST2, and non-targeting siRNAs as negative control (NC). The siRNA sequences were as follows: forward, 5'-GGCAUCACAAAUAGCCAAATT-3' and reverse, 5'-UUUGGCUAUUUGUGAUGCCTT-3' for ST2; and forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3' for the control. For transfection, siRNA was delivered at a final concentration of 80 nM using the Lipofectamine® 3000 reagent according to the manufacturer's instructions. Cells were incubated for 48 h to allow knockdown of ST2. ST2 knockout efficiency was detected by RT-qPCR (supplementary Figure 2), and the knockdown-LAD2 cells were used in mast cells medium release assay and neutrophil-mast cells co-culture experiment.

Molecular Docking Analysis and Molecular Dynamics Simulation

Autodock Vina v. 1.1.2 (Scripps Research Institute, San Diego, CA, USA) was used to perform a molecular docking analysis. The Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank was used to obtain target protein crystal structures. Discovery Studio v. 2016 was used to optimize the crystal structures by removing redundant ligands, adding charge, adding hydrogens, and removing water before running docking experiments. Discovery Studio v. 2016 were employed to visualize the docking model.

The GROMACS 2019 software was used for molecular dynamics simulations. The docking complex was used as the initial conformation for all-atomic molecular dynamics simulation. Amber 99sB-ILDN field parameters were used for both protein and EB molecules with the aid of ACPYPE Server (<https://www.bio2byte.be/acpype/>) Server generates UNK molecular topology file, select dodecahedron solvation box, set the system boundary and compounds in recent distance of 1.0 nm, use TIP3P water model and based on method of VERLET cut random Na⁺ and Cl⁻ added to the complex system to counteract the charge of the protein. Then, the system energy was minimized, the temperature was controlled by NVT and the pressure was controlled by NPT. The system temperature was 300K and the pressure was constant at 101.325kPa. Based on the above equilibrium, the free dynamics of the system was simulated for 100ns.

Statistical Analysis

Data were expressed as mean ± S.E.M. and analyzed one-tail paired Student's t-test. An independent samples analysis of variance was used to determine statistical significance in comparisons of the data using the SPSS software. For the paired comparison samples, the treated groups were compared with the negative control group. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$. For the multiple doses samples, the treated groups compared respectively with the negative control group were calibrated by Bonferroni's test.

Results

AHNAK(5758-5775) Significantly Increased in Psoriasis Patients and Induced Skin Inflammatory Reaction

AHNAK(5758-5775) is derived from neuroblast differentiation-associated protein, which contains amino acids at positions 5758-5775. After removing the proteins, The results of polypeptide omics by LC-MS showed that AHNAK(5758-5775) significantly increased in psoriasis patients than the control samples (Figure 1A). AHNAK(5758-5775) caused inflammatory cells infiltration in the lesions (Figure 1B). It was further found by labeling neutrophils that AHNAK(5758-5775) could recruit neutrophils (Figure 1C). The results of cytokines analysis showed that AHNAK(5758-5775) induced the rise of TNF- α and CXCL2, while showed little influence on IL-23 or IL-17 (Figure 1D). The activation effect of AHNAK(5758-5775) on human neutrophils was further studied *in vitro*. The results showed that AHNAK(5758-5775) had little activation effect on human neutrophils, and did not induce TNF- α , IL-8, IL-1 β or IL-6 release and neutrophils chemotaxis (Figure 2).

AHNAK(5758-5775) Activated Neutrophils via Mast Cells

Mast cells were significantly increased in mouse lesions (Figure 3), so we studied the effect of AHNAK(5758-5775) on mast cells. The results showed that AHNAK(5758-5775) did not induced mast cell degranulation. MPMC did not release tryptase, histamine or β - hexosamine (Figure 3B). However, AHNAK(5758-5775) induced MPMC to synthesize and release MCP-1, TNF- α and CXCL2 (Figure 3C).

Further studies on the effect of AHNAK(5758-5775) on the activation of human mast cells found that AHNAK(5758-5775) activated LAD2 cells and induced release of IL-8, TNF- α and MCP-1, while showed little effect on tryptase, histamine, β -hexosamine, IL-6 or IL-1 β release (Figure 4). Mast cells-neutrophils co-cultured was used to analyze chemotaxis neutrophils mediated by AHNAK(5758-5775). The results showed that AHNAK(5758-5775) induced co-cultured neutrophils release TNF- α , IL-8, IL-1 β and IL-6, and promoted neutrophil migration (Figure 5).

AHNAK(5758-5775) Might Activate Mast Cells via ST2

ST2 can mediated MCs activation but without degranulation reaction. AHNAK(5758-5775) could bind to the pocket of ST2. Ten hydrogen bonds were formed with the residues LYS22, GLN23, ARG35, GLN39, TYR119, THR121, THR135, and ARG198 in the pocket. It was also found that the negative part of peptide AHNAK(5758-5775) binds to the positively charged part of the binding pocket, and the electrostatic force formed can further increase the stability of the complex. (Figure 6A). Root Mean Square Deviation (RMSD) was used to indicate the degree of molecular structure change and to measure the stability of the complex system. RMSD fluctuation of AHNAK(5758-5775)-ST2 systems is less than 0.6 nm. Root Mean Square Fluctuation (RMSF) showed the fluctuation and structural flexibility of amino acid residues of receptor protein. The RMSF value of the amino acid residues binding AHNAK(5758-5775) in ST2 was less than 1.5 nm, reflecting that AHNAK(5758-5775) binding can make the structure of the binding region more stable to a certain extent. Radius of gyration (Rg) measures for compactness of protein. Rg values of AHNAK(5758-5775)-ST2 systems were less than 3.5 nm, which may be related to the fact that ST2 contains more loop structures, or there is conformation transition in the simulation process. The molecular dynamics simulation results indicated that the complexes remained stable with favorable conformations throughout 100 ns. Above all, it suggested that AHNAK(5758-5775) were stable at the binding site of receptors during the interactions (Figure 6B).

Knocking down the expression of ST2 by siRNA showed that the release of IL-8, TNF- α and MCP-1 by NC-LAD2 cells was significantly higher than that of Knockdown-LAD2 cells (Figure 7A). Moreover, activated co-culture NC-LAD2 cells caused more TNF- α and IL-1 β release and neutrophil migration than Knockdown-LAD2 cells (Figure 7B).

Discussion

In addition to T cell-mediated adaptive immune abnormalities, psoriasis also depends on the role of innate immune cells. Neutrophil aggregation in the stratum corneum of psoriasis lesion is called the Munro's microabscesses, which is one of the cardinal histopathological sign of PV. Neutrophils are the most abundant cells of the innate immunity system, new sights think neutrophil extracellular traps (NETs) may be the early source of self-DNA, RNA and LL37 that activate dendritic cells, and then produce inflammatory mediators such as IFN- γ [14] [15]. Though it is not generally believed that neutrophils are specific in psoriasis vulgaris, some studies detected that they were recruited early before the appearance of skin lesions [16].

MCs are considered of linkers of innate and adaptive immunity, they could release cytokines, enzymes, chemokines and histamines when activated. Expressing cell surface molecules to interact with other cells such as endothelial cells, neutrophils, keratinocytes and T-cell subsets to participate in inflammation. The number of MCs, especially those that produce IL-8, TNF- α and IFN- γ , was found increased in psoriasis lesions [17]. Generally, it is believed that IL-22 mainly originated from TH17, TH22, and TC22 cells in psoriasis, but researchers proposed that MCs are major producers of IL-22 and can also secrete IL-17 in 2015[3]. They can also release mast cell extracellular traps (MCETs) in psoriasis, playing a role similar to that of NETs [18, 19]. Apparently, increasing effort has made to identify MCs' function as pro-inflammatory cells in psoriasis.

Suppression of tumorigenicity 2(ST2), is a member of the interleukin-1 receptor-like protein-1 (IL1RL1) family. It plays roles in both tumors and inflammatory diseases. As an immune response molecular, it exists mainly in: T-lymphocytic cell line (LyT), macrophages, primary mast cells, dendritic cells[20]. IL-33 is an important ligand of ST2, it is defined as the IL-33/ST2 pathway and participates in many autoimmune diseases, and the current studies on ST2 and psoriasis are mainly limited to this pathway. Notably, elevated serum IL-33 levels in PV patients are effective activators of MCs, and then the releasement of IL-1, IL-6, IL-13, TNF- α , CCL2, and CCL3 could cause neutrophil infiltration. In the phorbol ester-induced psoriasis model, the ST2^{-/-} mouse model showed less inflammation than the wild type [21–23]. These results suggest that MCs activation via ST2 is of great significance in PV.

We detected the psoriatic lesions and found that the AHNAK-derived peptides significantly increased in the lesions compared with the normal skin. It has low tissue specificity and can be produced by keratinocytes in the skin[24]. As we discovered, neutrophil infiltration could be find in the mice ears after injection the peptide solution. However, our experiment proves this effect is not direct, and there may be other involved procedures. The immunofluorescence confirms that mast cells indeed activated in mice skin lesions. The activation of mast cells induced by antimicrobial peptides, complements, neuropeptides, cytokines and chemokines belongs to IgE-independent pathway[25]. AHNAK(5758-5775) could activate mast cells through an IgE-independent way. Co-cultured of MCs and neutrophils showed several inflammatory mediators and chemokines released by mast cells could lead to the recruitment of neutrophils. The known ST2 is the target receptor of the peptide on mast cells. In spite that few studies mentioned or confirmed whether mast cells are directly involved in neutrophil infiltration in psoriasis, it is not difficult to speculate the molecular network and interactions between them.

In summary, we propose a novel peptide derived from neuroblast differentiation-associated protein (AHNAK) from the psoriatic lesions, it may act as an autoantigen in the first step of psoriatic inflammatory activation. AHNAK(5758-5775) activate MCs via ST2 receptor and participate in neutrophil infiltration. The discovery of those kinds of endogenous peptides will make better understanding of the pathogenesis of psoriasis and focus attentions on the role of innate immune cells in psoriasis.

Declarations

ACKNOWLEDGEMENTS

Not Applicable

AUTHOR CONTRIBUTION

Study design, Delu Che and Songmei Geng; Sample collection, Tong Zhou and Tao Jia. Mice experiments and cytokines analysis, Xiangjin Song and Lei Zhang. Mice cell purification and Media Analysis, Delu Che and Lei Zhang. Human cell experiments, Delu Che, Xiangjin Song and Xueshan Du. Data analysis, Yi Zheng and Xueshan Du. All authors contributed to drafting of the manuscript and adopted the final version.

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AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are included within the article and the supplementary information file.

Ethics Approval This study was registered at Chinese Clinical Trail Registry, and the registration number is ChiCTR2100052365. The ethical approval (2021-1517) was approved by Ethics Committee at Xi'an Jiaotong University and was conformed to the ethical standard. Animal experiments in this study were approved by Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2021-1025), and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

Consent to Participate All specimen in this study had signed informed consent.

Consent for Publication All the authors have read the manuscript and agreed to submit the paper to *Inflammation* journal.

Conflict of Interest The authors declare no competing interests.

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Figures

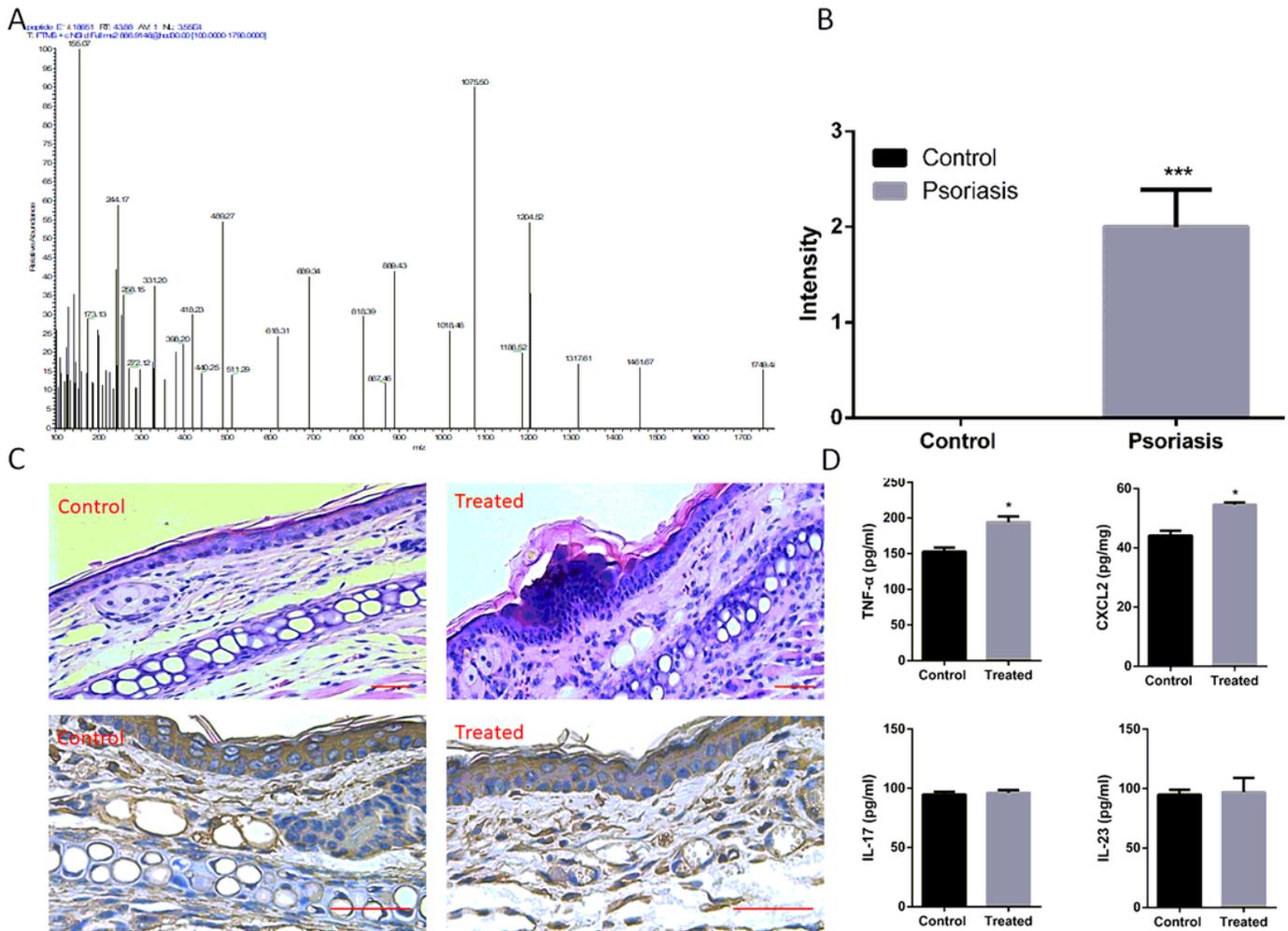


Figure 1

AHNAK(5758-5775) significantly induced skin inflammatory reaction. A: AHNAK(5758-5775) significantly increased in psoriasis patients than the control samples analysis by LC-MS. B: AHNAK(5758-5775) caused inflammatory cells infiltration in the lesions after hypodermic injection in mice. C: AHNAK(5758-5775) caused recruitment of neutrophils in mice skin. D: AHNAK(5758-5775) induced up-regulated the level of TNF- α and CXCL2 in mice skin, while showed little influence on IL-23 or IL-17. (n=9, data are expressed as mean \pm S.E.M. and analyzed one-tail paired Student's t-test. Differences were considered significant at * $p < 0.05$ and *** $p < 0.001$).

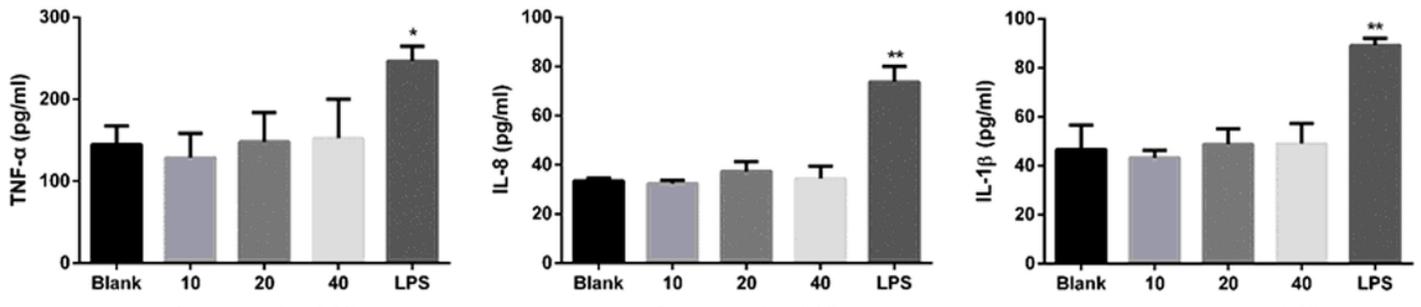


Figure 2

AHNAK(5758-5775) showed little activation effect on human neutrophils. AHNAK(5758-5775) did not induce TNF- α , IL-8, IL-1 β or IL-6 release and neutrophils chemotaxis *in vitro*.

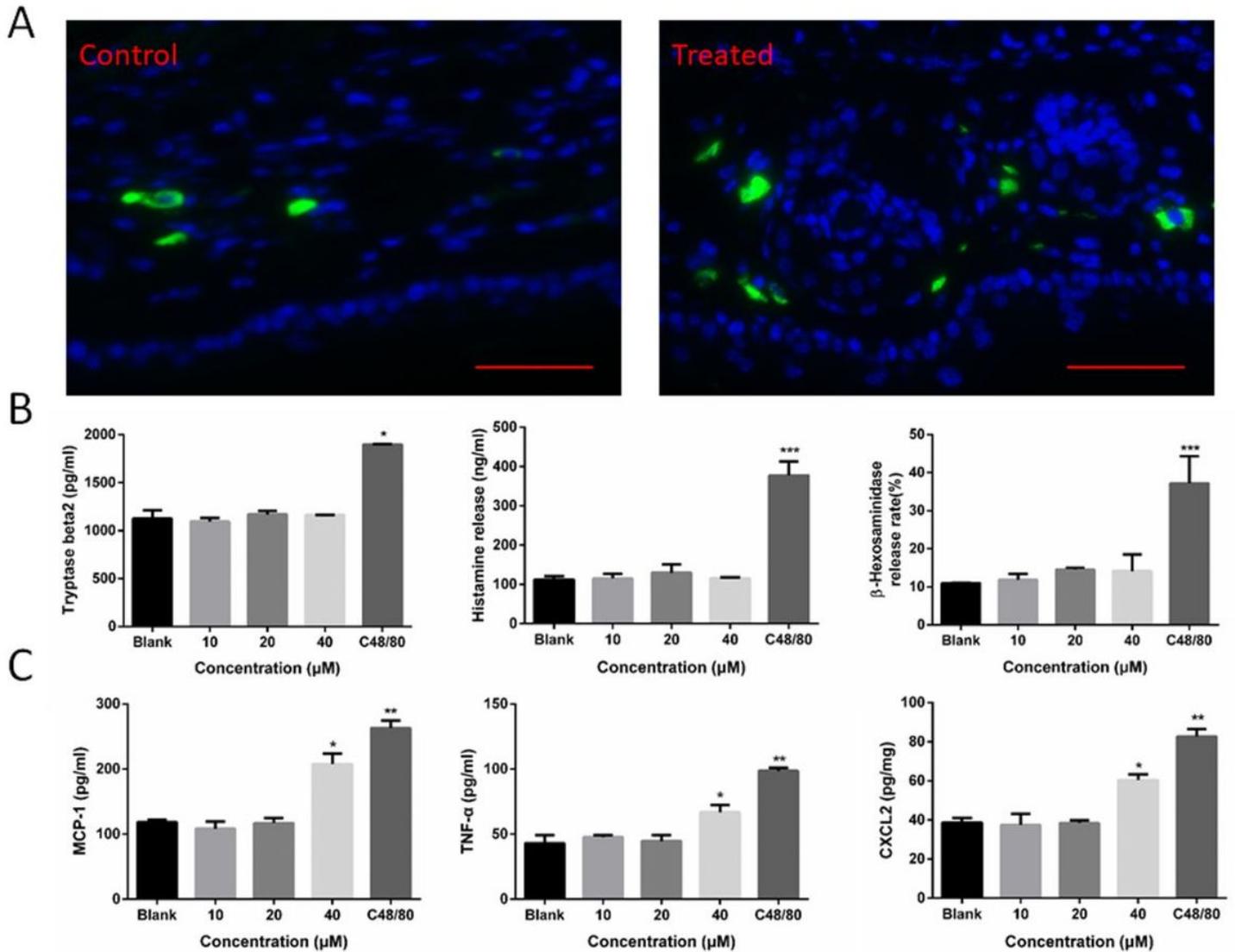


Figure 3

AHNAK(5758-5775) showed activation effect on mouse mast cells. A: Mast cells increased significantly after AHNAK treatment in mouse lesions (5758-5775). B: AHNAK(5758-5775) did not induced MPMC degranulation and release little tryptase, histamine or β -hexosamine. C: AHNAK(5758-5775) induced MPMC to synthesize and release MCP-1, TNF- α and CXCL2. (data are expressed as mean \pm S.E.M. and were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.0125$, ** $p < 0.0025$, and *** $p < 0.00125$.)

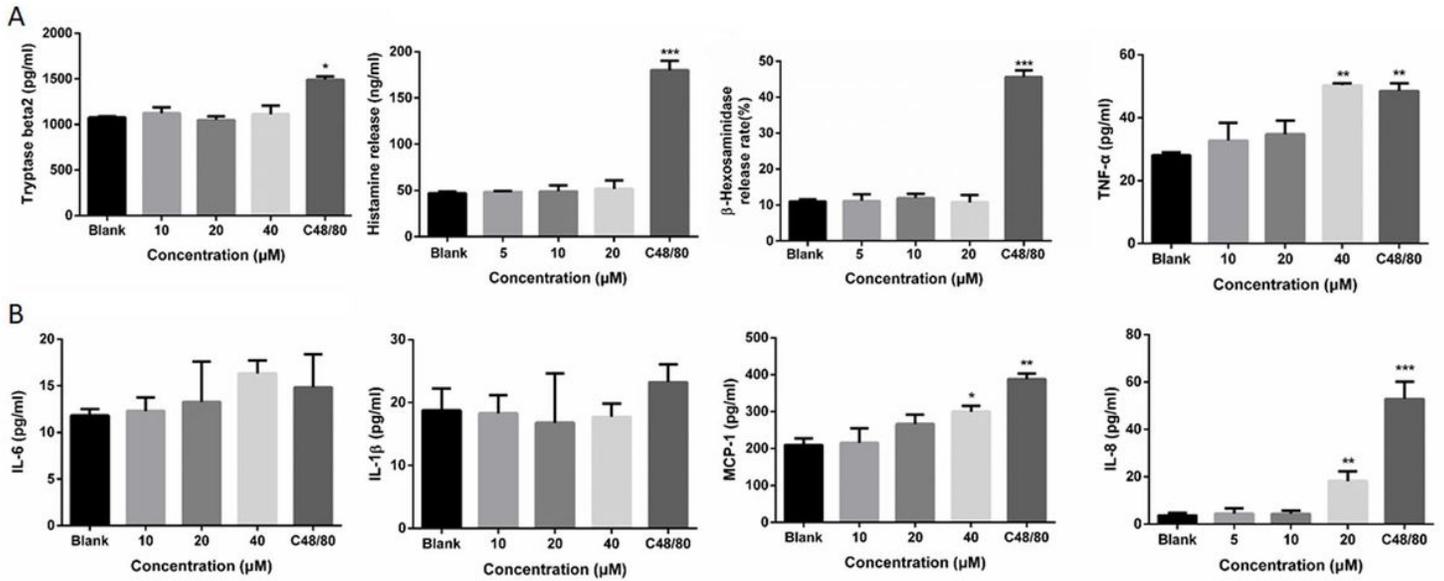


Figure 4

AHNAK(5758-5775) induced mast cells release cytokines. A: AHNAK(5758-5775) showed little effect on tryptase, histamine, β -hexosamine, IL-6 or IL-1 β release. B: AHNAK(5758-5775) activated LAD2 cells and induced IL-8, TNF- α and MCP-1 release. (data are expressed as mean \pm S.E.M. and were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.0125$, ** $p < 0.0025$, and *** $p < 0.00125$.)

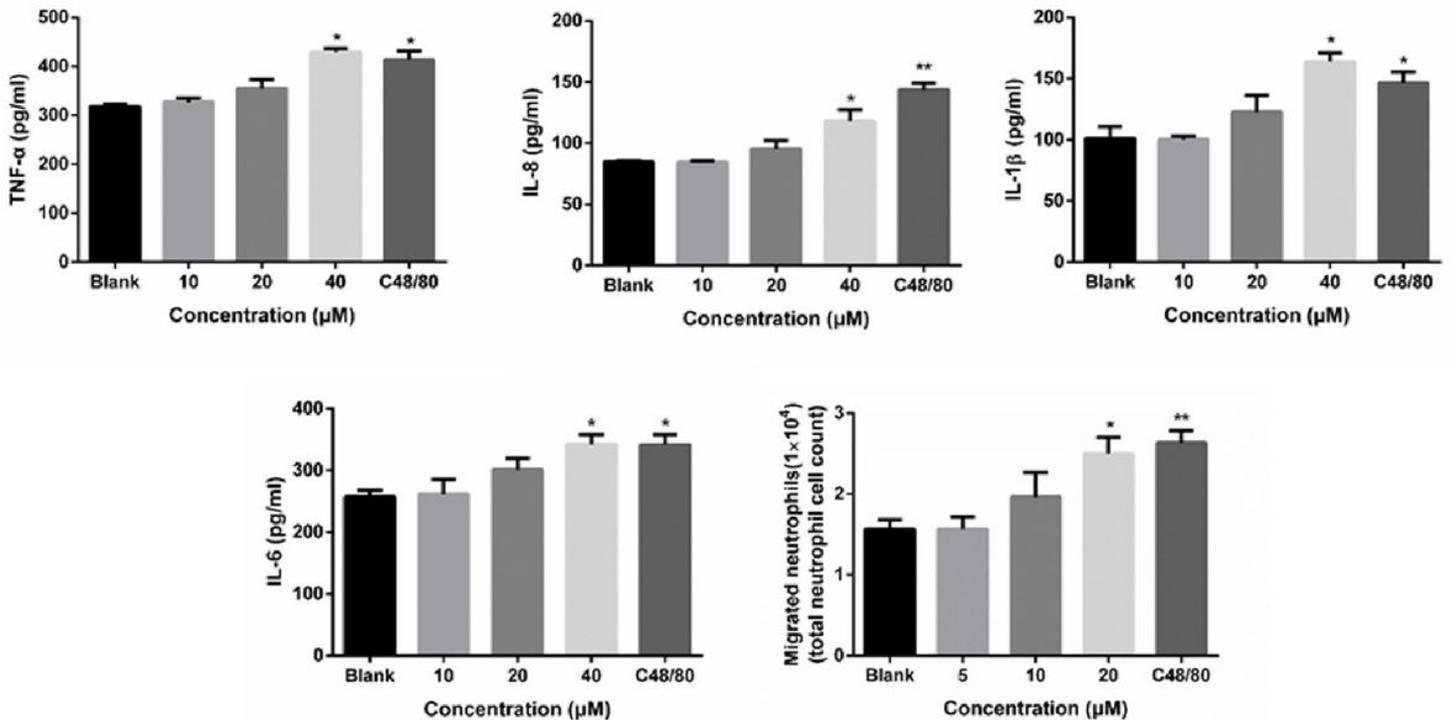


Figure 5

AHNAK(5758-5775) activated neutrophils via mast cells. AHNAK(5758-5775) induced neutrophils which co-cultured with mast cells release TNF- α , IL-8, IL-1 β and IL-6 and promoted neutrophil migration. (data are expressed as mean \pm S.E.M. and were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.0125$, and ** $p < 0.0025$).

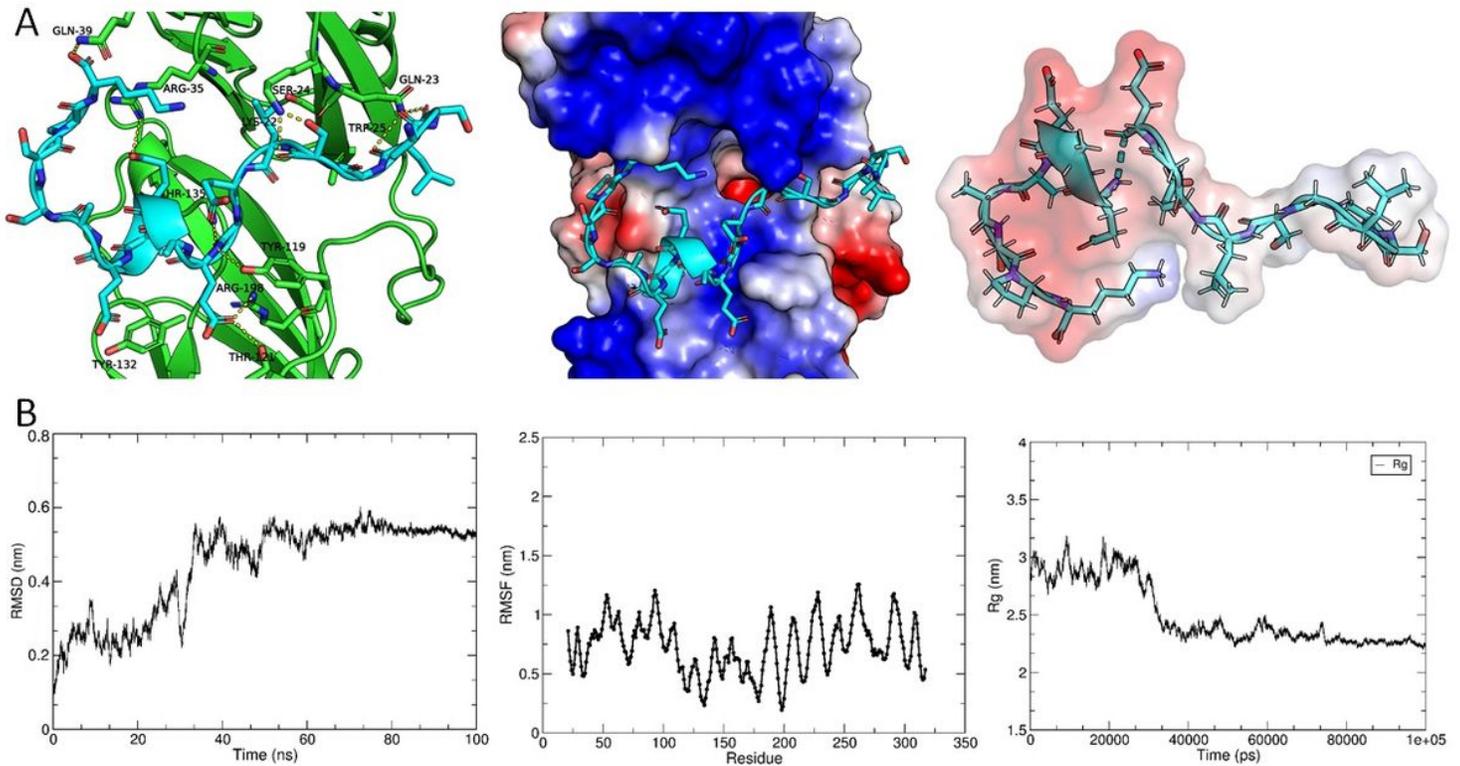


Figure 6

AHNAK(5758-5775) might activate mast cells via ST2. A: AHNAK(5758-5775) could bind to the pocket of ST2 analyzed by molecular docking. B: AHNAK(5758-5775) were stable at the binding site of receptors during the interactions analyzed by molecular dynamics simulation.

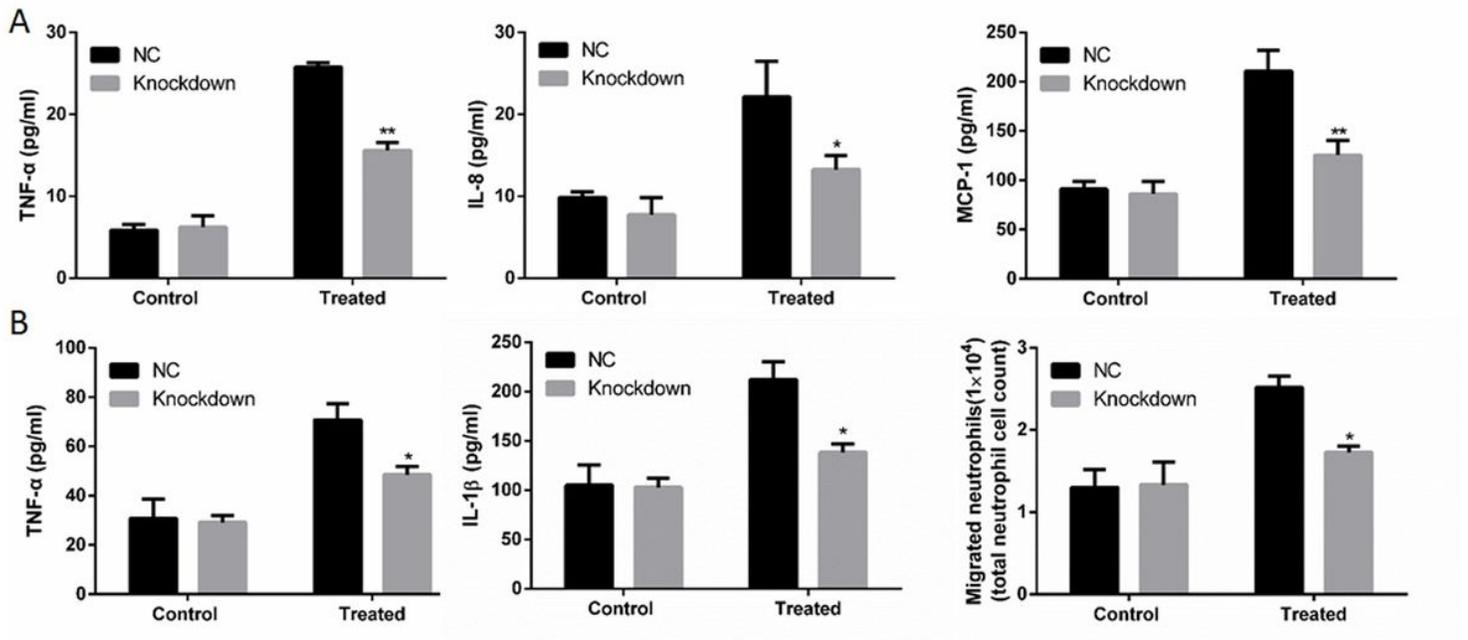


Figure 7

The activation effect of AHNAK(5758-5775) on mast cell reduced after knockdown of the ST2 expression. A: AHNAK(5758-5775) induced-release of IL-8, TNF-α and MCP-1 by NC-LAD2 cells was significantly higher than that of Knockdown-LAD2 cells. B: AHNAK(5758-5775) activated neutrophil co-culture with NC-LAD2 cells induced more TNF-α and IL-1β release and neutrophil migration than Knockdown-LAD2 cells. (data are expressed as mean ± S.E.M. and were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.0125$, and ** $p < 0.0025$).

Supplementary Files

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- [supplementaryFigure1.tif](#)
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