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Effect of *Staphylococcus aureus* in experimental pneumonia mouse model on promotion of mBD-3 expression through activation of the ERK1/2 pathway

Uticaj *Staphylococcus aureus*-a na porast ekspresije mBD-3 posredovan aktivacijom ERK1/2 signalnog puta na eksperimentalnom modelu mišje pneumonije

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Abstract

Background/Aim. *Staphylococcus aureus (S. aureus)* is a gram-positive pathogen that causes various human diseases. *S. aureus* causes pneumonia, which is characterized by localized tissue necrosis. The aim of the study was to explore the expression of mouse β-defensin 3 (mBD-3) induced by *S. aureus* in mouse lungs and the effect of mBD-3 expression on the mitogen-activated protein kinase (MAPK) pathway. **Methods.** An experimental model of *S. aureus* pneumonia in mice was developed, and the expression of mBD-3 and activation of the MAPK pathway were investigated using the methods of immunofluorescence and western blot. **Results.** The experimental model was created successfully. The number of white blood cells was elevated 48 and 72 hrs after the introduction of bacteria through mouse airways, and bronchiolar mucosal hyperemia was observed, along with a large number of white blood cells and mucus in the bronchioles. The mBD-3 expression levels 48 and 72 hrs after the induction of infection were greater than the levels in the control group and 24 hrs after the induction. The amount of phosphorylated extracellular signal-regulated kinase (ERK1/2) was increased 48 and 72 hrs after infection induction, compared with the levels in the control group and 24 hrs after induction. The expression of mBD-3 was lower when ERK1/2 phosphorylation was inhibited by the U0126 inhibitor. **Conclusion.** *S. aureus* in experimental pneumonia mouse model accelerates mBD-3 expression in the mouse lung mainly through an ERK1/2-dependent signaling pathway.

Key words:
defensins; disease models, animal; pneumonia, staphylococcal; signal transduction; staphylococcus aureus.

Apstrakt

Uvod/Cilj. *Staphylococcus aureus (S. aureus)* je gram-pozitivni patogen koji izaziva različite bolesti kod ljudi. *S. aureus* izaziva upalu pluća, koju karakteriše lokalizovana nekroza tkiva. Cilj rada bio je da se ispita ekspresija mišjeg β-defensina 3 (mBD-3) izazvana *S. aureus*-om u plućima miša i efekat ekspresije mBD-3 na signalni put mitogenom-aktivisanih protein kinaza (MAPK). **Metode.** Razvijen je eksperimentalni model pneumonije izazvane *S. aureus*-om kod miševa, a ekspresija mBD-3 i aktivacija MAPK signalnog puta ispitivana je metodama imunofluorescencije i *western blot*-a. **Rezultati.** Eksperimentalni model je uspešno kreiran. Broj belih krvnih zrnaca bio je povećan nakon 48 i 72 sata od unosa bakterija kroz disajne puteve miševa, a uočena je hiperemija bronhiolarne sluzokože uz prisustvo velikog broja belih krvnih zrnaca i sluzi u bronhiolama. Nivoi ekspresije mBD-3 nakon 48 i 72 sata od indukcije infekcije bili su viši od nivoa u kontrolnoj grupi i nakon 24 sata od indukcije. Količina fosforilisane kinaze regulisane ekstracelularnim signalom (*extracellular signal-regulated kinase*, ERK1/2) bila je povećana nakon 48 i 72 sata posle indukcije infekcije u poređenju sa nivoom u kontrolnoj grupi i 24 sata posle indukcije. Ekspresija mBD-3 bila je niža kada je fosforilacija ERK1/2 bila inhibirana primenom inhibitora U0126. **Zaključak.** *S. aureus* na eksperimentalnom modelu mišje pneumonije ubrzava ekspresiju mBD-3 u plućima miša uglavnom posredstvom ERK1/2-zavisnog signalnog puta.

Ključne reči:
defensini; bolest, modeli na životinjama; pneumonija, stafilokokna; signali, transdukcija; staphylococcus aureus.

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Introduction

Staphylococcus aureus (*S. aureus*) is an important gram-positive human pathogen responsible for various diseases and represents a leading cause of pneumonia (*S. aureus* pneumonia); its importance as a respiratory pathogen is on the rise [1, 2]. *S. aureus* pneumonia, characterized by localized necrosis and inflammation, is one of the most prevalent *S. aureus*-mediated diseases and occurs in nearly 13.3% of all invasive staphylococcal infections [3]. However, because of the ability of *S. aureus* to develop resistance to a large range of antibiotics, a decrease in the efficiency of current treatments has been reported. Therefore, the development of novel antibiotics or therapeutic strategies against staphylococcal infections is an obvious requirement [4, 5].

Defensins are endogenous cationic peptides and effector molecules for the immune system because of their broad-spectrum antimicrobial activity [6, 7]; they are classified into α and β subfamilies. β-defensins exhibit a broad spectrum of activities against bacteria, viruses, and fungi, particularly at the epithelial interface of mucosal surfaces. β-defensin 3 (BD-3) plays an important role in the inhibition of bacterial infections [8, 9]. In humans, BD-3 has been found in airway surface fluids from patients with psoriasis, suggesting the protein may play a role in fighting local infection [10, 11]. Human BD-3 can also enhance resistance against influenza virus and bacterial infections [12]. Mouse BD-3 (mBD-3) has the same functions as human BD-3 [13].

Mitogen-activated protein kinases (MAPKs) constitute a paradigm of intracellular signaling [14]. They are expressed ubiquitously and control a wide variety of critical cellular functions such as proliferation, differentiation, migration, and apoptosis [15]. In this study, mBD-3 expression in mouse epithelial cells was induced by *S. aureus* in mouse lungs, and the effect of mBD-3 expression on the MAPK pathway was explored.

Methods

Experimental model of S. aureus pneumonia

All procedures for the animal experiment were approved by the Animal Experimental Ethics Committee of Inner Mongolia Medical University (No. YKD2020059), and all protocols complied with relevant guidelines and the Animal Research Guideline. Specific pathogen-free mice were used for *S. aureus* infection experiments at seven weeks of age. The mice were divided into two groups (the control group and the *S. aureus* pneumonia group). The *S. aureus* pneumonia group mice were inoculated with 100 μL of *S. aureus (*ATCC-25923, 10^9 cells/mL) via the nasal route [16–19]. The blood and lung tissue samples were collected 24, 48, and 72 hrs after induction of pneumonia, and the white blood cell counts were analyzed with an Veterinary hematology analyzer (MEK-6450K, NIHON KOHDEN). Mice were humanely sacrificed by euthanasia (chloral hydrate) after treatment.

Hematoxylin and eosin staining

The lung tissue samples were fixed with 10% neutral buffered formalin, embedded in paraffin, and cut into 4 μm thin pieces. The sections were deparaffinized at 65 °C for 4 hrs with gradient ethanol and then stained with hematoxylin or Masson's trichrome staining.

Cell culture

Pulmonary epithelial cells were obtained from the lungs of suckling mice [20–22]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin in a 37 °C incubator with an atmosphere of 5% CO_2 and 95% air. *S. aureus* (10^3 cells/well) was added to the pulmonary epithelial cells (10^6 cells/well), and then the cells were incubated for 4, 8, and 12 hrs, and the inhibitor U0126 (1.5 μM) was added to evaluate the function of phosphorylated ERK1/2.

Immunofluorescence

The lung tissue was frozen in liquid nitrogen and placed in a constant-temperature freezer. A small amount of optimal cutting temperature compound was added for cryosectioning. The sections were dried using a cold air blower and blocked with 5% bovine serum albumin for one hour. Afterward, the frozen sections were incubated with specific antibodies against mBD-3, washed, and labeled with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11008) for one hour. Finally, the samples were imaged and analyzed with fluorescence microscopy (Leica).

The pulmonary epithelial cells grown on poly-L-lysine (Biochrom AG Seromed) coated glass coverslips were fixed with methanol/acetone. The fixed cells were incubated with mBD-3 antibody for one hour at room temperature (RT), followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 for one hour at RT. Finally, the samples were imaged and analyzed with fluorescence microscopy (Leica).

Western blot

The lung tissue or pulmonary epithelial cells were washed twice with cold PBS and lysed with radioimmunoprecipitation lysis buffer (containing a protease inhibitor cocktail). The whole cell lysates were incubated at 4 °C for 30 min, followed by centrifugation (12,000 g for 15 min, at 4 °C). Protein samples were separated with SDS-PAGE electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with specific antibodies against anti-ERK1/2 (Millipore Cat. No. 16-284), anti-phospho-ERK1/2 (Millipore Cat. No. 05-797), anti-c-Jun N-terminal kinase (JNK) (Millipore Cat. No. 04-210), anti-phospho-JNK (Millipore Cat. No. 46-613MAG), anti-p38 kinase (Millipore Cat. No. ABS29), and anti-phospho-p38 (Millipore Cat. No. MABS64).

Statistical analysis

Numerical data were analyzed as the mean ± standard deviation (SD) and compared using the unpaired Student's *t*-test. Differences in values were considered significant at $p < 0.05$.

Results

Effect of S. aureus on white blood cell count and lung tissue

The white blood cell count in the *S. aureus*-infected group was higher than in the control group ($p < 0.05$). Additionally, the white blood cell count was greater after 48 and 72 hrs than after 24 hrs of pneumonia induction ($p < 0.05$). However, there was no significant difference between the numbers 48 and 72 hrs after induction of the infection (Figure 1A).

In the lung tissue stained with hematoxylin and eosin, the presence of more white blood cells corresponded with more bronchiolar mucosal hyperemia 48 and 72 hrs after induction of the infection, and mucus was observed in the bronchioles, unlike the results for the control group and 24 hrs after induction of the infection (Figure 1B).

Expression of mBD-3 and the MAPK pathway in lung tissue

To determine the expression of mBD-3 in the lung tissue, proteins were extracted from the lungs of *S. aureus* pneumonia mice. The results showed that the mBD-3 level of expression was higher 48 and 72 hrs after pneumonia induction than the corresponding expression in the control group and 24 hrs after pneumonia induction (green Figure 2).

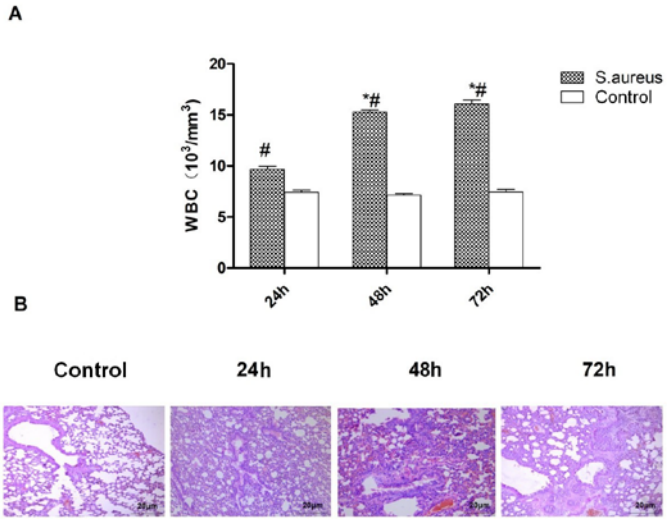

Fig. 1 – A) The white blood cells (WBC) count; B) Hematoxylin and eosin staining of lung tissue from *Staphylococcus aureus* pneumonia mice. * significantly different from the 24-hour data point ($p < 0.05$); # significantly different from the control group at the same time point ($p < 0.05$).

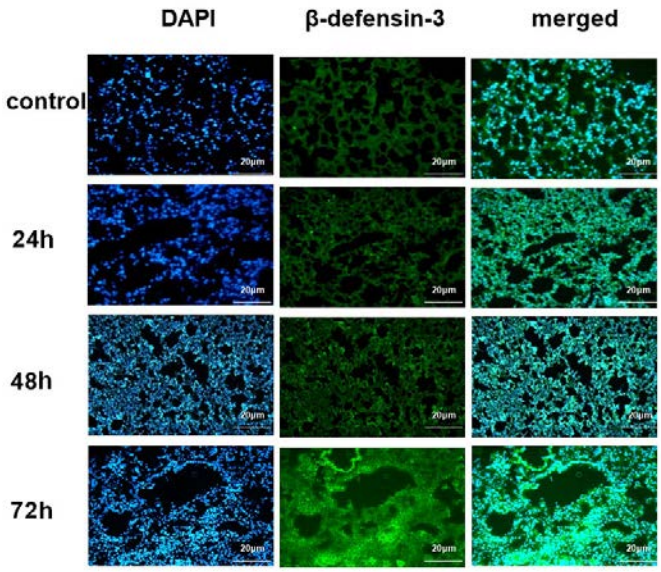

Fig. 2 − Mouse β-defensin 3 expression in lung tissue of *Staphylococcus aureus* pneumonia mice determined by immunofluorescence. DAPI- 4′,6-diamidino-2-phenylindole staining.

To assess MAPK signaling activity, the levels of total and phosphorylated ERK1/2, JNK, and p38 were measured by western blot (Figures 3A and B). The abundance of phosphorylated ERK1/2 was increased 48 and 72 hrs after pneumonia induction compared with the levels in the control group and 24 hrs after the induction (Figure 3B, $p < 0.05$). However, there were no changes in other proteins. Therefore, ERK1/2 may be the key protein that regulates mBD-3 expression.

Expression of mBD-3 and the MAPK pathway in pulmonary epithelial cells

Pulmonary epithelial cells incubated with *S. aureus* were evaluated for the expression of mBD-3 and the changes in the MAPK pathway. The results showed that the expression of mBD-3 in the pulmonary epithelial cells increased as the length of exposure to *S. aureus* increased (green Figure 4A). To assess the MAPK signaling activity in pulmonary

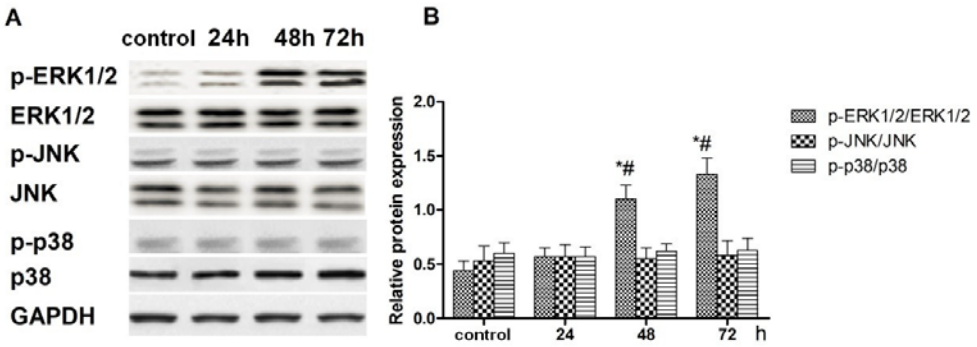

Fig. 3 − Effect of *Staphylococcus aureus* on mitogen-activated protein kinase pathway in lung tissue: A) Representative western blots showing levels of ERK1/2, JNK, p38, p-ERK1/2, p-JNK, p-p38, and GAPDH; B) Histograms summarizing the results shown in A); GAPDH – glyceraldehyde-3-phosphate dehydrogenase; ERK1/2 – extracellular signal-regulated kinase 1/2; JNK – c-Jun N-terminal kinase; p38 – p38 kinase; p-ERK1/2 – phosphorylated ERK 1/2; p-JNK – phosphorylated JNK; p-p38 – phosphorylated p38. Results are expressed as mean ± standard deviation (n = 5); * significantly different from the control group ($p < 0.05$); # significantly different from the 24-hour data point ($p < 0.05$).

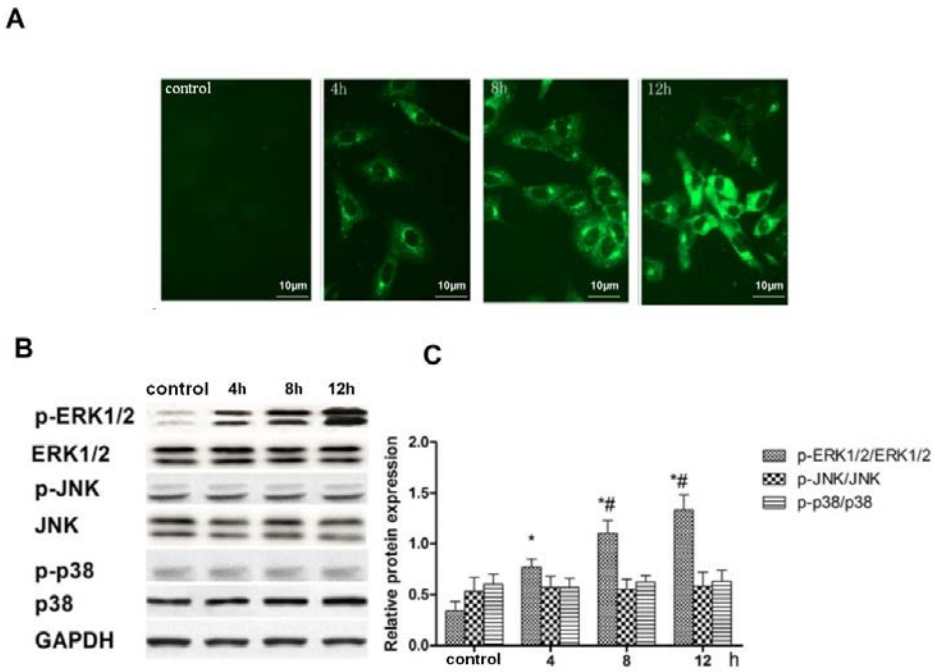

Fig. 4 − Effect of *Staphylococcus aureus* on mouse β-defensin 3 expression and mitogen-activated protein kinase pathway in pulmonary epithelial cells: A) mBD-3 expression determined by immunofluorescence; B) Representative western blots showing levels of ERK1/2, p38, p-ERK1/2, p-JNK, p-p38, and GAPDH; C) Histograms summarizing the results shown in B). For abbreviations see under Figure 3.
Results are expressed as mean ± standard deviation (n = 5); * significantly different from the control group ($p < 0.05$); # significantly different from the 4-hour data point ($p < 0.05$).

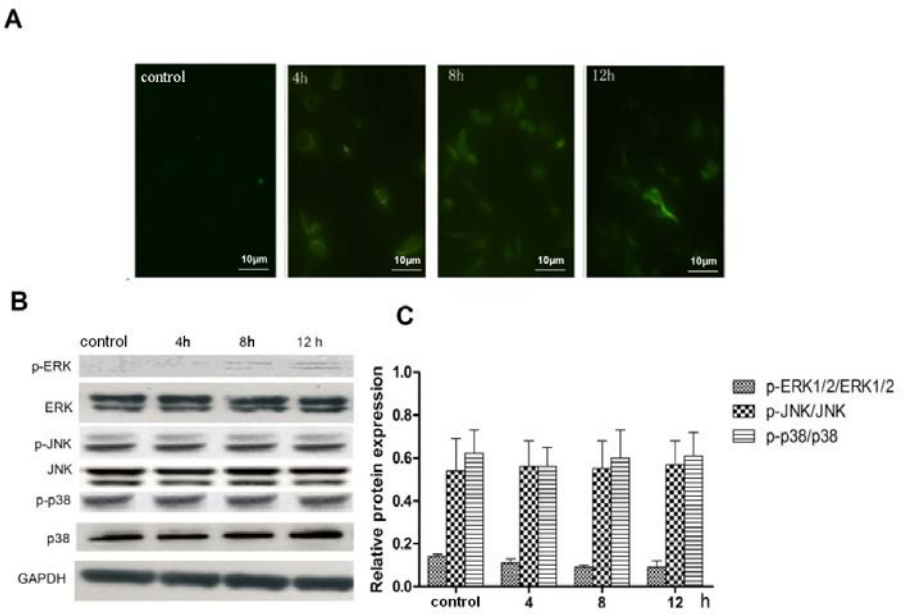

Fig. 5 − Effect of *Staphylococcus aureus* on mouse β-defensin 3 expression and mitogen-activated protein kinase pathway after pERK1/2 inhibition in pulmonary epithelial cells: A) mBD-3 expression determined by immunofluorescence; B) Representative western blots showing levels of ERK1/2, JNK, p38, p-ERK1/2, p-JNK, p-p38, and GAPDH; C) Histograms summarizing the results shown in B). For abbreviations see under Figure 3. Results are expressed as mean ± standard deviation (n = 5).

epithelial cells, the levels of total and phosphorylated ERK1/2, JNK, and p38 kinase were measured by western blot analysis 4, 8, and 12 hrs after the beginning of incubation. The phosphorylated ERK1/2 was highest at 8- and 12-hour points (Figures 4B and C, $p < 0.05$), suggesting that ERK1/2 plays a key role in mBD-3 expression. To test this theory, U0126 (a specific inhibitor of ERK1/2 phosphorylation) was used to silence ERK1/2 expression. The result showed a decrease in the expression of mBD-3 (Figure 5A) when ERK1/2 phosphorylation was inhibited, while the expression of other proteins remained unchanged (Figures 5B and C).

Discussion

S. aureus is a globally distributed pathogen that can induce serious diseases in many species and cause infections in lung tissue, soft tissue, the bloodstream, etc. [13, 23]. Since mBD-3 is a natural antimicrobial peptide, it can inhibit the growth of bacteria and viruses. An experimental model of *S. aureus* pneumonia in mice was successfully developed [24, 25], and in this study the effect of mBD-3 expression on the MAPK pathway was explored.

The white blood cell count was higher 48 and 72 hrs after induction of infection compared to the control group and the 24-hour point. Bronchiolar mucosal hyperemia was observed, along with the presence of a large number of white blood cells and mucus in the bronchioles. *S. aureus* infection was found in the lungs.

In the lungs of mice with *S. aureus* pneumonia, the mBD-3 expression was higher, and the level of phosphorylated ERK1/2 was increased 48 and 72 hrs after the onset of infection in comparison to the 24 hrs after the onset of infection. That indicated that the levels of mBD-3 expression and phosphorylated ERK1/2 increased over time. We also evaluated the activity of MAPK signaling pathways. MAPKs are a superfamily of serine/threonine kinases that includes ERK1/2, JNK, and p38 [26–28]. These kinases are involved primarily in the activation of nuclear transcription factors that control cell proliferation, differentiation, and apoptosis. Our results suggest that *S. aureus* accelerates mBD-3 expression via the ERK1/2 signaling pathway, not through the activation of JNK or p38. The level of phosphorylated ERK1/2 in pulmonary epithelial cells exposed to *S. aureus* was highest 8 and 12 hrs after the beginning of incubation with the bacteria. Furthermore, the blocking of ERK1/2 by chemical inhibition suppressed mBD-3 expression. When *S. aureus* invaded the lung epithelial cells, the mBD-3 secreted from the cells enhanced the resistance against infection, and the phosphorylated ERK1/2 promoted mBD-3 secretion.

Conclusion

Overall, our results provide evidence that the *S. aureus* signaling pathway accelerates mBD-3 expression mainly through an ERK1/2-dependent pathway in mouse lungs. Thus, our findings suggest that mBD-3 expression is regulated by the ERK1/2 pathway.

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R E F E R E N C E S

1. *Fode P, Stegger M, Andersen PS*. Human β-defensin 3 (DEFB103) and its influence on Staphylococcus aureus nasal carriage. Int J Infect Dis 2011; 15(6): e388–94.
2. *Kisich KO, Howell MD, Boguniewicz M, Heizer HR, Watson NU, Leung DY*. The constitutive capacity of human keratinocytes to kill Staphylococcus aureus is dependent on beta-defensin 3. J Invest Dermatol 2007; 127(10): 2368–80.
3. *Wu Q, Gui P, Yao S, Zhu H, Li J, Li Y*. Expression of β-defensin-3 in lungs of immunocompetent rats with methicillin-resistant Staphylococcus aureus ventilator-associated pneumonia. J Surg Res 2011; 169(2): 277–83.
4. *Pascoal MA, Marson FAL, Paschoal IA, Levy CE*. Influence of pancreatic status, CFTR mutations, Staphylococcus aureus and/or Pseudomonas aeruginosa infection/colonization on lung function in cystic fibrosis during a 2-year follow-up period. Wien Klin Wochenschr 2020; 132(19–20): 572–80.
5. *Wu Y, Nie Y, Huang J, Qiu Y, Wan B, Liu G*, et al. Protostemonine alleviates heat-killed methicillin-resistant Staphylococcus aureus-induced acute lung injury through MAPK and NF-κB signaling pathways. Int Immunopharmacol 2019; 77: 105964.
6. *Mücke PA, Ostrzinski A, Hammerschmidt S, Maaß S, Becher D*. Proteomic Adaptation of Streptococcus pneumoniae to the Antimicrobial Peptide Human Beta Defensin 3 (hBD3) in Comparison to Other Cell Surface Stresses. Microorganisms 2020; 8(11): 1697.
7. *Sechet E, Telford E, Bonamy C, Sansonetti PJ, Sperandio B*. Natural molecules induce and synergize to boost expression of the human antimicrobial peptide β-defensin-3. Proc Natl Acad Sci U S A 2018; 115(42): E9869–78.
8. *Chen L, Lv Z, Gao Z, Ge G, Wang X, Zhou J,* et al. Human β-defensin-3 reduces excessive autophagy in intestinal epithelial cells and in experimental necrotizing enterocolitis. Sci Rep 2019; 9(1): 19890.
9. *Priyadharshini VS, Ramírez-Jiménez F, Molina-Macip M, Renteria-Rosales C, Santiago-Cruz J, Zarate-Segura P,* et al. Human Neutrophil Defensin-1, -3, and -4 Are Elevated in Nasal Aspirates from Children with Naturally Occurring Adenovirus Infection. Can Respir J 2018; 2018: 1038593.
10. *Dhingra H, Kaur K, Singh B*. Engineering and characterization of human β-defensin-3 and its analogues and microcin J25 peptides against Mannheimia haemolytica and bovine neutrophils. Vet Res 2021; 52(1): 83.
11. *He W, Wei D, Zhang J, Huang X, He D, Liu B,* et al. Novel bone repairing scaffold consisting of bone morphogenetic Protein-2 and human Beta Defensin-3. J Biol Eng 2021; 15(1): 5.
12. *Cooper CA, Tizard ML, Stanborough T, Moore SC, Chandry PS, Jenkins KA*, et al. Overexpressing ovotransferrin and avian β-defensin-3 improves antimicrobial capacity of chickens and poultry products. Transgenic Res 2019; 28(1): 51–76.
13. *Zhang Y, Zhao Y, Liu H, Yu W, Yang F, Li W*, et al. Mouse β-Defensin 3, A Defensin Inhibitor of Both Its Endogenous and Exogenous Potassium Channels. Molecules 2018; 23(6): 1489.
14. *Wang Z, Zhu M, Wang M, Gao Y, Zhang C, Liu S*, et al. Integrated Multiomic Analysis Reveals the High-Fat Diet Induced Activation of the MAPK Signaling and Inflammation Associated Metabolic Cascades via Histone Modification in Adipose Tissues. Front Genet 2021; 12: 650863.
15. *Okkenhaug K*. How to resist PI3Kδ inhibition: activate MAPK! Blood 2021; 138(1): 3–4.
16. *Wang L, Li Q, Li J, Jing S, Jin Y, Yang L,* et al. Eriodictyol as a Potential Candidate Inhibitor of Sortase A Protects Mice From Methicillin-Resistant Staphylococcus aureus-Induced Pneumonia. Front Microbiol 2021; 12: 635710.
17. *Meijer MT, de Vos AF, Peters Sengers H, Scicluna BP, Roelofs JJ, Abou Fayçal C,* et al. Tenascin C Has a Modest Protective Effect on Acute Lung Pathology during Methicillin-Resistant Staphylococcus aureus-Induced Pneumonia in Mice. Microbiol Spectr 2021; 9(1): e0020721.
18. *Liu ZZ, Yang YJ, Zhou CK, Yan SQ, Ma K, Gao Y,* et al. STING Contributes to Host Defense Against Staphylococcus aureus Pneumonia Through Suppressing Necroptosis. Front Immunol 2021; 12: 636861.
19. *Murphy AG, O'Keeffe KM, Lalor SJ, Maher BM, Mills KH, McLoughlin RM*. Staphylococcus aureus infection of mice expands a population of memory γδ T cells that are protective against subsequent infection. J Immunol 2014; 192(8): 3697–708.
20. *Nayak PS, Wang Y, Najrana T, Priolo LM, Rios M, Shaw SK*, et al. Mechanotransduction via TRPV4 regulates inflammation and differentiation in fetal mouse distal lung epithelial cells. Respir Res 2015; 16(1): 60.
21. *Sinha M, Lowell CA*. Immune Defense Protein Expression in Highly Purified Mouse Lung Epithelial Cells. Am J Respir Cell Mol Biol 2016; 54(6): 802–13.
22. *Lam HC, Choi AM, Ryter SW*. Isolation of mouse respiratory epithelial cells and exposure to experimental cigarette smoke at air liquid interface. J Vis Exp 2011; (48): 2513.
23. *Wu S, Huang J*. Resveratrol alleviates Staphylococcus aureus pneumonia by inhibition of the NLRP3 inflammasome. Exp Ther Med 2017; 14(6): 6099–104.
24. *Jiang Y, Wang Y, Wang B, Yang D, Yu K, Yang X,* et al. Antifungal activity of recombinant mouse beta-defensin 3. Lett Appl Microbiol 2010; 50(5): 468–73.
25. *Lee SE, Lee JS, Kim MR, Kim MY, Kim SC*. Topical retinoids induce β-defensin 3 expression in mouse skin. Int J Dermatol 2010; 49(9): 1082–4.
26. *Li J, Liu X, Wang Q, Huangfu J, Schuman MC, Lou Y*. A Group D MAPK Protects Plants from Autotoxicity by Suppressing Herbivore-Induced Defense Signaling. Plant Physiol 2019; 179(4): 1386–401.
27. *Griukova A, Deryabin P, Sirotkina M, Shatrova A, Nikolsky N, Borodkina A*. P38 MAPK inhibition prevents polybrene-induced senescence of human mesenchymal stem cells during viral transduction. PLoS One 2018; 13(12): e0209606.
28. *An Z, Huang X, Zheng C, Ding W*. Acinetobacter baumannii outer membrane protein A induces HeLa cell autophagy via MAPK/JNK signaling pathway. Int J Med Microbiol 2019; 309(2): 97–107.

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