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Effects of Ketamine and Cannabinoids on Antimicrobial Peptide Expression and Infection Susceptibility: An *In Vitro* and Animal Study

氯胺酮和大麻素對抗菌肽表達和感染易感性的影響：細胞和動物研究

Submitted to

Beat Drugs Fund Association

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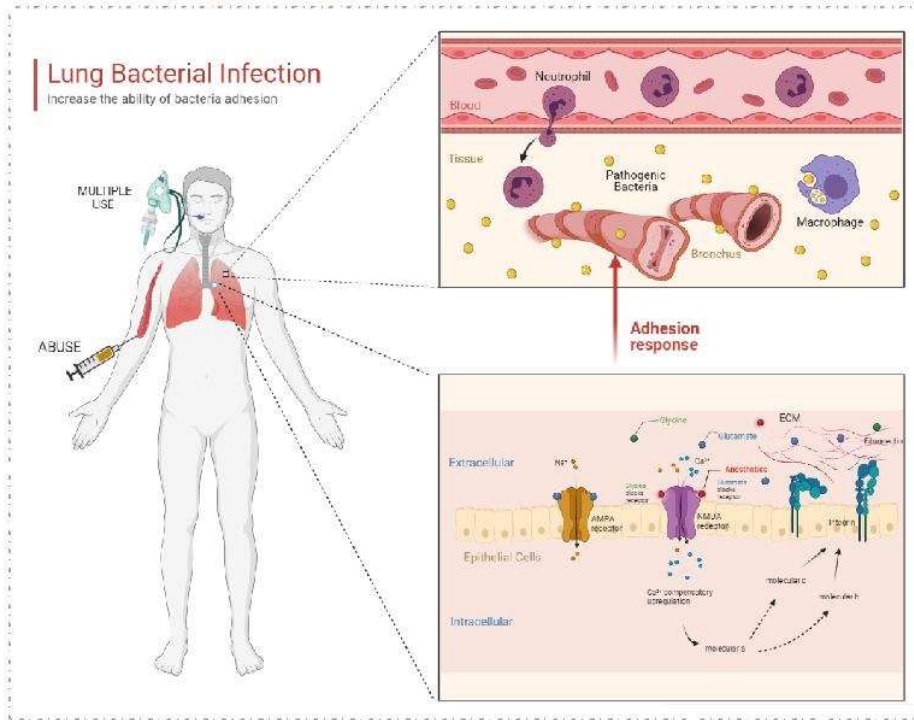
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Graphical Abstract

Effects of Ketamine and Cannabinoids on Antimicrobial Peptide Expression and Infection Susceptibility: An In Vitro and Animal Study

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Clinical Impact:
By clarifying the relationship between ketamine and cannabinoid abuse and pulmonary infection and the possible mechanism, we will promote the general awareness of different sectors of the community about the previously unknown harms of drugs with a view to encouraging and enabling them to play a more active role in drug prevention, early identification and intervention.

Table of Contents

Background

Ketamine, a remarkable molecule, was first synthesized as a replacement for phencyclidine in 1962 by team of Professor Calvin Lee Stevens, an organic chemist. Since it was first evaluated during a clinical trial and initiated as an anaesthetic in 1964[1], ketamine has had an enchanting history in clinic as a dissociative anaesthetic, which was originally used as a military anaesthetic, a tranquilizer for uncooperative children, and in veterinary medicine[2,3]. Until now, in terms of human consumption, it remains the key anaesthetic in veterinary medicine for pain management. Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist. By blocking the NMDA receptor, ketamine can disrupt the normal functioning of glutamatergic neurotransmission, leading to its characteristic dissociative effects[4]. In view of its role in synaptic plasticity, NMDA receptor is the central to brain learning and memory formation[5]. Ketamine also has other pronounced effects at virous receptor sites. It has been shown to increase the expression of brain-derived neurotrophic factor (BDNF) and other neurotrophic factors[6,7], inhibit muscarinic signaling[8] and enhance the synaptic inhibitory effects of gamma-aminobutyric acid (GABA)[9]. Ketamine also produces activation of dopamine release[10] and shows affinity for sigma and opioid receptors[11,12].

Ketamine is now an FDA-approved medication with a range of medical uses, including anaesthesia and pain relief[1,13]. It is also being studied for its potential use in treating depression, posttraumatic stress disorder (PTSD), and other mental health disorders[14-16]. The utility of ketamine in the treatment of mood disorders is of particular interest in repeated randomized clinical trials (RCTs) and meta-analyses reporting replicated rapid and potent replicating antidepressant effects and a reduction in suicidal ideation[17,18]. While ketamine can be a useful medication in certain situations, it also has side effects at high doses, including hallucinations,

dissociation, nystagmus, and increased blood pressure and heart rate[19-21]. As a water and lipid solubility substance, ketamine allows for convenient administration through multiple routes and extensive distribute on throughout the body. It exists as two enantiomers: S-(+)-ketamine and R-(-)-ketamine with differing affinities at the NMDA-receptor. Studies have shown that S-(+)-ketamine is the more potent[22] and effective isomer for producing anaesthesia and analgesia, which is attributed to the higher affinity of the S-(+)-isomer for phencyclidine binding sites on the NMDA receptors, while R-(-)-ketamine has been shown to have more psychotomimetic effects and less analgesic effects compared to S-(+)-ketamine[23]. Ketamine's psychosis-like effects have led to it being used as a pharmacological 'model' of schizophrenia. These same effects have also contributed to it becoming used as an illicit recreational drug, even in early 1970s[24]. With the increase in illicit recreational ketamine use in recent years, new problems have emerged in many parts of the world, particularly for heavy users, including physical harms and addiction[25]. In a reviewing study of 233 emergency room cases of ketamine abusers in Hong Kong, the main symptoms included impaired consciousness in 45%, abdominal pain in 21%, lower urinary tract symptoms in 12% and dizziness in 12%[26].

Cannabis, also known as marijuana, is a plant with an excellent natural source of fiber and various bioactive cannabinoids,that has been used for medicinal and recreational purposes for thousands of years. It contains more than 120 different chemical compounds, the two most well-known cannabinoids are tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is the primary psychoactive compound in cannabis, and it is responsible for the euphoric effect ("high") that people experience when they use cannabis[27,28]. It works by binding to cannabinoid receptors in the brain, which affects various bodily functions, including mood, appetite, and perception[29-31]. On the other hand, CBD has been the subject of many researches in recent years as some believe that it has potential therapeutic benefits. CBD is believed to have anti-

inflammatory, analgesic, anti-anxiety, and antipsychotic properties, among others[32-35]. While the legal status of CBD varies by jurisdiction, according to the law of Hong Kong, CBD has been classified as a dangerous drug since February 1, 2023. Other cannabinoids found in cannabis include cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC), among others[36,37].

Despite the fact that cannabinoids are of great diversity, most of them exist in trace amounts in different forms of substances. Cannabis works by interacting with the body's endocannabinoid system (ECS), which is a complex network of receptors and neurotransmitters that helps regulate various bodily functions, including mood, appetite, pain sensation, and immune function[38,39]. There are two main types of cannabinoid receptors in the body: CB1 and CB2. CB1 receptors are primarily found in the brain and nervous system, while CB2 receptors are primarily found in the immune system and peripheral tissues[40]. Δ9-/Δ8-THC is the main psychoactive ingredient with a dominant composition and high affinity responsible for the activation of cannabinoid receptors in the brain, mainly the CB1 receptor, and in the periphery, CB1 and CB2 receptors. Conversely, other compounds, particularly CBD, may antagonize the activating effect of the CB1 receptor, which is taken up by different agonists[41].

The abuse of ketamine and cannabis has generated serious health concerns. Studies have shown that ketamine abuse associates with the syndrome of cystitis[42]. Cannabis users are prone to oral infections[43]. Ketamine and cannabis abuse may increase the risk of respiratory tract infection[44,45]. The airway injury from marijuana smoking results in a loss of cilia and an increase in mucous secretions that likely lead to impairment in mucociliary clearance, compromising the lung's first line of defense against infection[46]. Additionally, the administration of plant-derived or synthetic cannabinoids impairs pathogen clearance and in

certain cases, increases mortality[47]. The nature and molecular mechanism of immunomodulation change induced by ketamine and cannabis abuse, however, remains obscure. Particularly, in the aspect of aggravating *Staphylococcus aureus* (*S. aureus*, MRSA) associated pneumonia infection. Thus, there is a need to explore the underlying mechanisms by which ketamine and cannabis mediate immunomodulation in relation to susceptibility to respiratory infection in humans.

Aims

1. To determine the effects and molecular mechanisms by which ketamine and cannabinoid inhibit endogenous AMPs expression;

2. To investigate the effects of ketamine and cannabinoid on *S. aureus* colonization, adhesion and survival in human lung epithelial cells and macrophages;

3. To demonstrate the detrimental roles of ketamine and cannabinoid in a mouse model of *S. aureus* infection.

Methods

As cannabidiol could not be obtained by market, thus, in this study, we change to three other active forms of cannabinoids that are used for research purposes, including Arachidonoyl Ethanolamide (cannabinoid receptor (CB1 and CB2) agonist) ("AEA"), 2-Arachidonoyl Glycerol (CB1 receptor agonist) ("2-AG") and (-)-CP 55,940 (non-selective CB agonist) ("CP 55,940"), which had been approved by the Beat Drugs Fund Association.

1.1 Bacterial Culture and Bacterial-Cell Co-Culture Assay

1.1.1 Bacterial strains and growth conditions

A standard *S. aureus* from American Type Culture Collection (ATCC) was kindly provided by the Department of Microbiology, Queen Mary Hospital, The University of Hong Kong. *S. aureus* was grown to stationary phase in trypticase soy broth (TSB) at 37°C for 16 hours with shaking at 220 r.p.m. When necessary, media was supplemented with ampicillin (100 μg/mL), ticarcillin (80 μg/mL), or tetracycline (30 μg/mL). The growth of bacterial cells was monitored by determining the optical density at 600 nm (OD600).

1.1.2. *S. aureus* adherence and invasion of cell

To determine adherent and invading bacteria, human lung epithelial cells A549, 16HBE and macrophages THP-1 were seeded in a 24-well plate. Overnight-grown cultures of *S. aureus* were harvested by centrifugation (3,000 ×g, 10 minutes), washed three times with phosphate-buffered saline (PBS), and finally resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum medium. Cells pre-incubated with either ketamine (20 μM and 40 μM) or non-selective cannabinoid (Anandamide (AEA, 1.0 μM and 5.0 μM), 2-arachidonoylglycerol (2-AG, 10.0 nM and 100.0 nM), CP 55,940 (10.0 nM and 20.0 nM)) for 48 hours were infected with *S. aureus* at a multiplicity of infection (MOI) of 10 for 2 hours. The non-adherent bacterial cells were subsequently removed by washing with PBS for three times, to observe the adhesiveness of the bacteria. The extracellular *S. aureus* adhered to the surface of the cell membrane was killed by incubating with 10mg/L gentamicin for 2 hours, and washed with PBS 3 times to observe the invasiveness of the bacteria. Infected cells were treated with 0.25% trypsin, and lysed by the addition of 1 ml distilled water and serial 10-fold

dilutions were plated on Luria-Bertain (LB) agar to determine the number of colony forming units (CFU) of the adherent and invasion bacteria. Each experiment was performed in triplicate.

1.2 Animal Experiments & Histopathology

1.2.1 Mouse strains

The 6-8 weeks old male C57BL/6 mice were administrated with ketamine (20 mg/kg, i.p.) and cannabinoid (AEA, 40 mg/kg. i.p.; CP, 55940, 5 mg/kg. i.p) [According to the results of *in vitro* experiments, combined with scientific value and valid as ethical requirements for research, and humanistic principles of animal experiments, we selected AEA with best effect from AEA and 2-AG, both of which are endogenous agonists of the canonical cannabinoid receptors CB1 and CB2, for in vivo animal verification.] for 7 days or 3 months, and then were intranasal inoculated with PBS as control or, 1×10^8 CFU *S. aureus* standard strain as infection group. Clinical signs, body weight, and survival of the mice will be monitored before sacrificed after *S. aureus* infection. During the experimental period, the mice were maintained within the Laboratory Animal Services Center, The Chinese University of Hong Kong. All animal experiments performed were covered under Laboratory Animals Ethics Committee's approval.

1.2.2 *In vivo S. aureus* infection

C57BL/6 mice were infected with *S. aureus* intranasally as previously described[48]. Briefly, bacteria were grown overnight in TSB broth supplemented with 25mg/mL streptomycin and then reinoculated in 1% TSB broth for 24 hours. After 3 hours, the bacteria were harvested by centrifugation (3,000 ×g, 10 minutes), washed three times with PBS and resuspended in medium. The bacteria were resuspended at indicated 1×10^8 CFU/40 μl in 40 ml PBS. Actual inoculum concentrations were determined by plating serial dilutions on *S. aureus* - selective

LB agar plates supplemented with 25 mg/mL streptomycin. Mice were anesthetized by administering ketamine/xylazine/acepromazine intraperitoneally and then subsequently infected intranasally with *S. aureus*.

1.2.3 Tissue and blood sample collection

Mice were sacrificed at indicated time points (7-day for acute infection and 3-month for chronic infection). The right lungs were removed aseptically and homogenized in 0.1% Triton X-100. Serial dilutions of the lysates were plated on LB streptomycin plates for CFU enumeration. Mice blood was collected from the heart in 0.5 M ethylenediaminetetraacetic acid (EDTA)-coated tubes. The left lungs were aseptically resected and divided into three sections for mRNA, protein measurement and histology determination, separately. The tissue used to detect mRNA and protein was collected in sterile tubes and stored in liquid nitrogen, and the part used for tissue sectioning was fixed overnight in 4% paraformaldehyde (4% PFA) for the further hematoxylin-eosin (H&E) and immunofluorescence staining.

1.2.4 Quantitative bacteriology and histopathology

Lung tissues were homogenized in sterile saline using aerosol-proof homogenizers. Aliquots (100 μl) of 10-fold serial dilutions of the homogenates were cultured on *S. aureus*-selective agar plates to quantify the number of viable *S. aureus* organisms in the respective organ[49].

1.2.5 Lung pathological observation and scoring

Mouse lung was fixed immediately in 4% PFA and processed by standard paraffin embedding methods. Sections were cut 4 μm thick, stained with H&E and examined by light microscopy. Lung inflammation and damage were graded with the following pathological scoring system: The histopathological parameters peribronchiolitis, perivasculitis, alveolitis and granuloma

formation will be each semi-quantitatively scored as absent, minimal, slight, moderate, marked or strong, noted as 0, 1, 2, 3, 4, and 5 respectively. Frequency as well as the severity of the lesions were incorporated. Granuloma formation was scored by estimating the occupied area of the lung section[49].

1.2.6 Collection of bronchoalveolar lavage (BAL)

Trachea was exposed through a midline incision. Lungs were lavaged 3 times with 1 ml PBS injected through the trachea, referring to described previously[49]. BAL fluid was spun at 2,000 rpm for 10 minutes. The supernatant was frozen at -80°C and analyzed for various cytokines as described below.

1.3 Exploring the Molecular Mechanisms of Ketamine's Increased *S. aureus* Lung Infection and Adhesion

1.3.1 Epithelial cell and macrophage culture

The human epithelial cell A549 and 16HBE, human monocytic cell line THP-1 and murine macrophage cell line Raw 264.7 were obtained from American Type Culture Collection. A549 and THP-1 cells were cultured in RPMI-1640, 16HBE and Raw 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with 10% fetal bovine serum at 37°C incubator in 5% CO_2.

1.3.2 Western blots

Cultured cells or lung tissues were harvested and washed with the ice-cold 1× PBS and lysed in ice-cold immunoprecipitation assay buffer. The lysed cell debris was pelleted by centrifugation at 13,000 ×g and 4°C for 30 minutes. Protein concentrations in the total lysate

were measured using the standard Bradford assay. 10 μg of protein from the entire cell lysate were separated electrophoretically using SDS-PAGE. The proteins were then transferred to the nitrocellulose membrane in Tris-Glycine transfer buffer and block non-specific binding with freshly prepared 5% nonfat dried milk, which is followed by incubating with a specific primary antibody and then the HRP-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence and imaged using a Western blot digital imaging system. Band intensity was quantified using ImageJ software.

1.3.3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total cell or tissue RNA was extracted with ice-cold TRIzol and then transcribed into cDNA using the PrimeScriptTM RT Reagent Kit. mRNA expression of detected genes was measured by quantitative PCR with the SYBR Premix Ex Taq Kit using the primers listed in **Table S1**. 28S rRNA and 16S rRNA were used as eukaryotic cells and S..aureus internal control separately. Relative mRNA expression levels were calculated using the delta-delta C_T method.

1.3.4 RNA interference

Cells to be transfected were seeded in 6-well plates at 1×10^5 cells per well. Transfections were performed with small interfering RNA specific for NMDAR1, NMDAR2D, fibronectin1 (FN1) and ITGAV using jetPRIME transfection reagent according to the manufacturer's protocol instructions.

1.3.5 Confocal laser scanning microscopy

Treated cells or co-cultured cells were fixed with 4% formaldehyde solution for 30 minutes at room temperature. Dewaxed paraffin tissue sections in xylene and then removed xylene in 100, 95, and 70% ethanol series. Followed with the heat antigen retrieval in a citrate-based antigen

retrieval solution using the microwaves. The cells and tissues were washed 3 times with PBS and incubated with blocking buffer (5%FBS in 1× PBS with 0.25% Triton X-100) for 60 minutes at room temperature and then incubated with the primary antibody overnight at 4 °C, followed by secondary antibody incubation for 60 minutes at room temperature. Cell or tissue images were captured using a confocal microscope and analyzed by the ImageJ software.

1.3.6 Enzyme-linked immunosorbent assay (ELISA)

Plasma was collected by centrifugation at 2,000 ×g and 4°C for 10 minutes. Cell supernatant was harvested by centrifuging cell culture media for 10 minutes at 1,000 rpm and 4°C. BAL was obtained by centrifuging at 10,000 ×g for 15 minutes at 4°C. Antigen expression was analyzed in duplicate by an ELISA kit according to the manufacturer's protocol.

Results

2.1 Ketamine increased *S. aureus* colonization and infection in the lung *in vivo* and *in vitro*

S. aureus USA300 was used to establish intranasal infection pneumonia model C57BL/6 mice as previously described[48,50]. *S. aureus* infection levels in the intranasal acute infection pneumonia model were measured by colony formation assay of quantitative cultures (**Figure 1. A**), real-time RT-PCR (**Figure 1. B**) and immunofluorescence staining (**Figure 1. E, F**). Ketamine impaired and increased *S. aureus* colonization to impair the macroscopic lung pathology were visualized by H&E staining (**Figure 1. C, D**). *S. aureus* infection levels in the intranasal chronic infection pneumonia model were measured by body weight (**Figure S1. A**), Masson's trichrome (**Figure S1. B**), collagen volume fraction courting (**Figure S1. C**), colony

14

formation assay of quantitative cultures (**Figure S1. D**), real-time RT-PCR (**Figure S1. E**), and lung non-heme iron level (**Figure S1. F**).To further assess whether ketamine could enhance the *S. aureus* infection of cell lines, human epithelial cells A549 and 16HBE were treated with ketamine for 48 hours before being infected with *S. aureus* to evaluate the adhesion infection. A549, 16HBE and mouse epithelial cell line LA4 were treated to evaluate the invasion infection. The colony formation assay of quantitative cultures (**Figure 1. G-I** and **Figure S2. A-F**) and immunofluorescence staining (**Figure 1. J-L** and **Figure S1. G-J**) results were consistent with the animal results, the *S. aureus* infection significantly increased in ketamine treated cell lines A549 and 16HBE, indicating that ketamine enhanced *S. aureus* lung infection ability *in vivo* and *in vitro*.
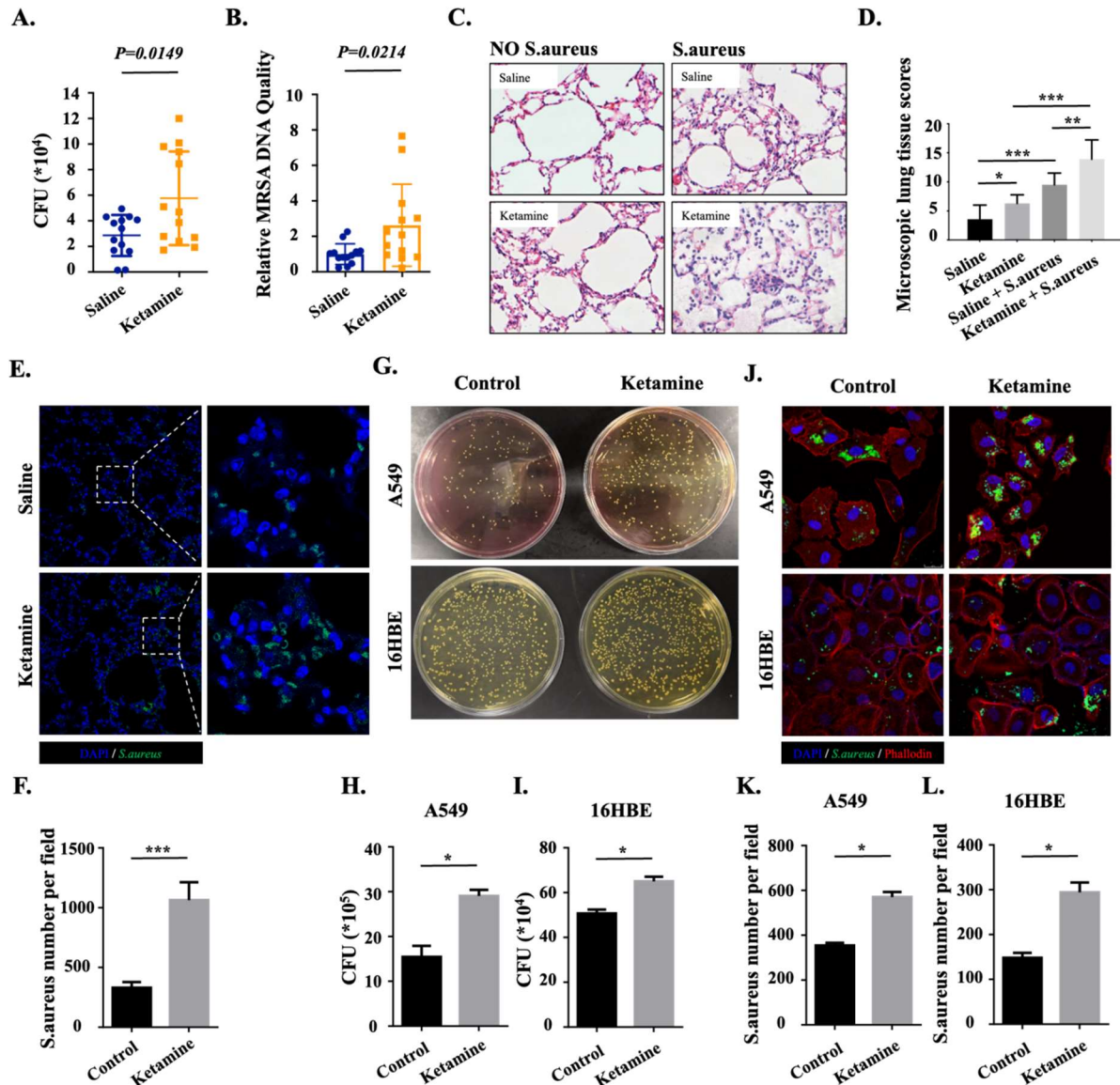
Figure 1. Regular ketamine treatment increased the infection potential and adhesion capacity of *Staphylococcus aureus* in lung in vivo and in vitro. (A-F) C57BL/6 mice were intraperitoneal injected 20 mg/kg ketamine or 0.9% Sodium Chloride as control for 7-day before being infected with 40 μl 1×10^8 CFU *S. aureus* strain intranasally overnight to establish the *S. aureus* pneumonia model. All mice were sacrificed the next day after *S. aureus* infection for 24 hours. (A) Mouse right lung tissues were homogenized for CFUs, n = 13. (B) *S. aureus* levels were quantified by real-time RT-PCR, detecting S. aureus-specific 16S ribosomal DNA. Ketamine impaired the macroscopic lung pathology in the mouse pneumonia model was visualized by H&E staining (C), and the corresponding microscopic lung tissue scores were counted (D), Saline group, n1 = 7; Ketamine group, n2 = 7; Saline + *S. aureus* group, n3 = 10; Ketamine + *S. aureus* group, n4 = 10. Scale bar: 200 μm. (E) Paraffin-embedded sections of mouse lungs from the 7-day groups were stained to visualize *S. aureus* (green) and nuclei (blue). (F)

Thirty visual fields per group were randomly selected to count the bacteria number. Scale bar: 40 µm. (G–L) Human epithelial cells A549 and 16HBE were cultured in medium with ketamine (40 µM, 48 h) and infected with *S. aureus* strain (MOI 10) for 2 hours. The *S. aureus* adhesion infection in cell lines were evaluated by CFUs (G-I) and visualized by immunofluorescence staining (J-L). Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

2.2 Cannabinoids increased *S. aureus* infection in vitro

Two non-selective endocannabinoids AEA and 2-AG, and a synthetic cannabinoid CP 55,940, the ligands for both CB1 and CB2 receptors[51-53], were used to evaluate the effect of cannabinoids on *S. aureus* lung infection. In A549 and 16HBE cell lines, they were cultured with AEA, 2-AG and CP 55,940 for 48 hours severally before being infected with S. aureus, and then the invasion levels effected by cannabinoids in the pneumonia model were measured by colony formation assay of quantitative cultures (**Figure 2. A-D** and **Figure 3. A-D**), real-time RT-PCR (**Figure 2. E** and **Figure 3. E**) and immunofluorescence staining (**Figure 2. F-K** and **Figure 3. F-K**) separately, with the result that S. aureus infection all significantly enhanced in cannabinoid AEA, 2-AG and CP 55,940 severally treated cell lines A549 and 16HBE.
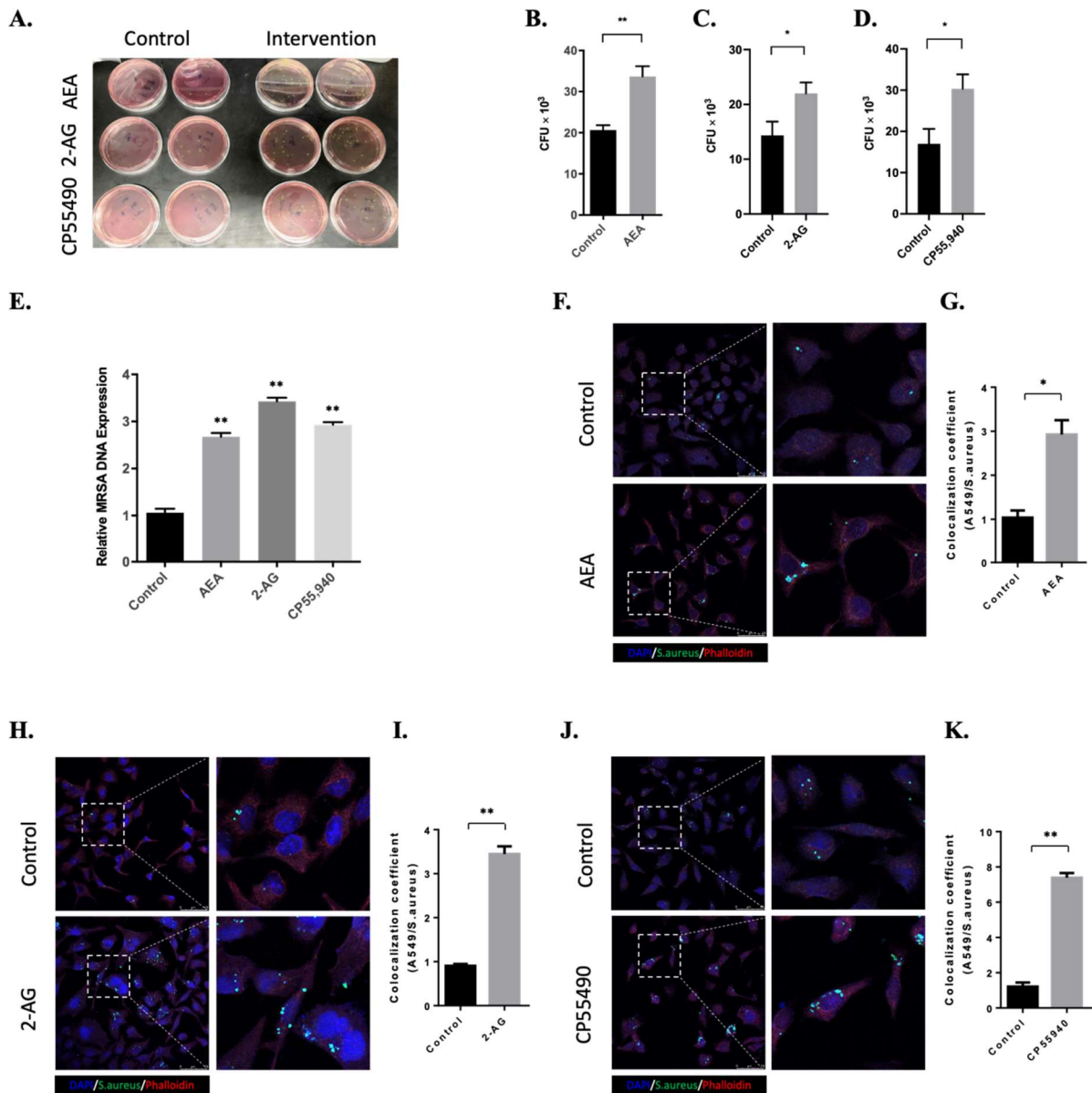
Figure 2. Regular cannabinoids treatment increased the infection potential of *S. aureus* in human epithelial cell line A549. The cells A549 were treated with 5 µM AEA, 100 nM 2-AG, 10 nM CP55,940 for 24 hours, respectively, and then infected with *S. aureus* (MOI 10) for 2 hours, followed by being treated with 10mg/L gentamicin for 2 hours, n = 3. After the infection, A549 cells treated with cannabinoids were collected separately for CFUs (A-D) and DNA expression detection (E). Immunofluorescence staining and the corresponding counting of *S. aureus* number per cell (thirty visual fields per group were selected randomly) were conducted after AEA (F, G), 2-AG (H, I), CP55,940 (J, K) treatment. Scale bar: 20 µm. Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01.
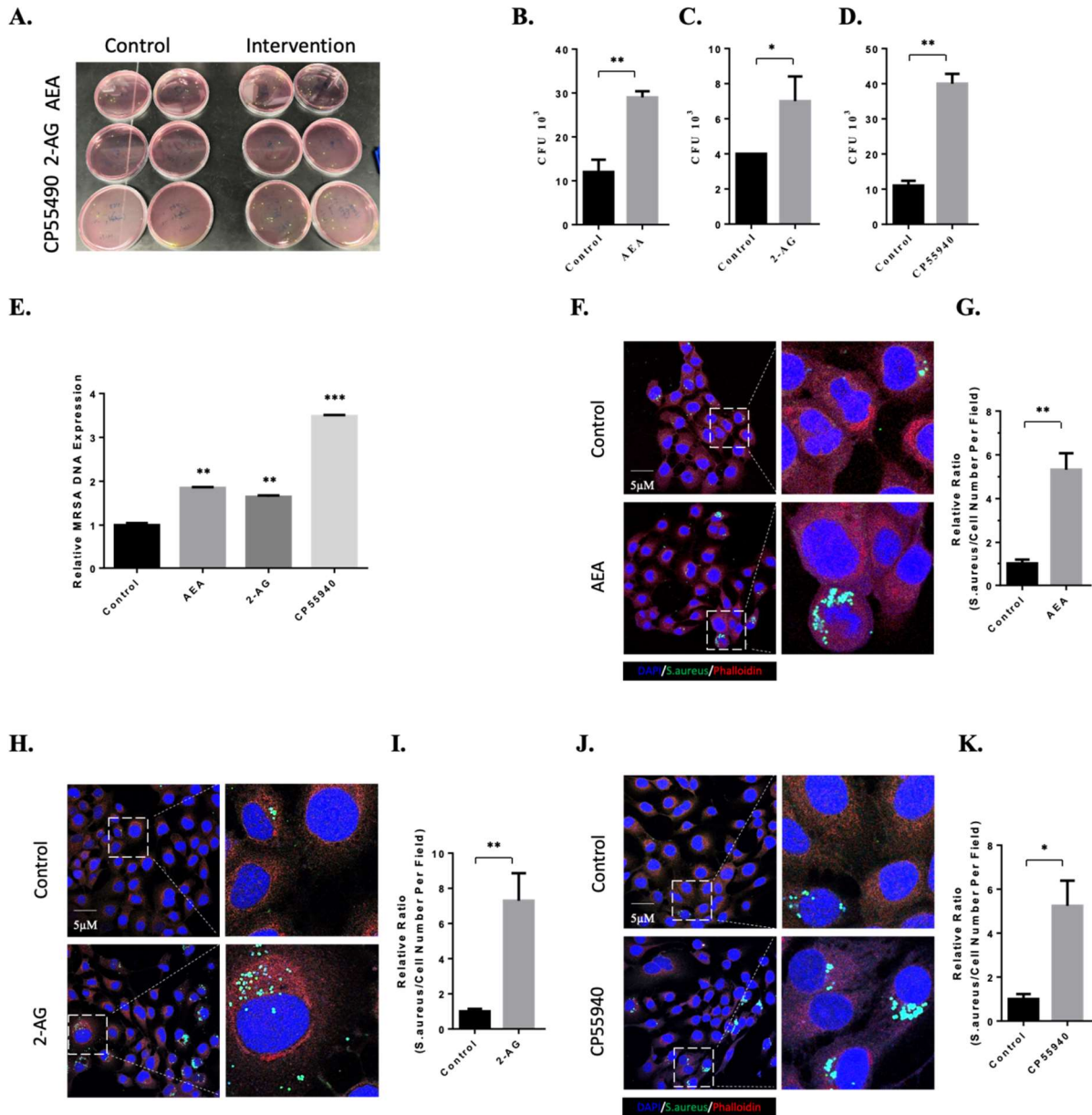
Figure 3. Regular cannabinoids treatment increased the infection potential of *S. aureus* in human epithelial cell line 16HBE. The cell 16HBE were treated with 5 µM AEA, 100 nM 2-AG, 10 nM CP 55,940 for 24 hours, respectively, and then infected with *S. aureus* (MOI 10) for 2 hours, followed by being treated with 10 mg/L gentamicin for 2 hours, n = 3. After the infection, 16HBE cells treated with cannabinoids were collected separately for CFUs (A-D) and DNA expression detection (E). Immunofluorescence staining and the corresponding counting of *S. aureus* number per cell (thirty visual fields per group were selected randomly) were conducted after AEA (F, G), 2-AG (H, I), CP 55,940 (J, K) treatment. Scale bar: 20 µm. Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

2.3 Cannabinoids treatment showed a decrease in the mice lung infection potential of *S. aureus in vivo*

In the pneumonia mouse models separately injected with CP 55,940 and AEA, *S. aureus* infection levels were measured by colony formation assay of quantitative cultures (**Figure 4. A, B** and **Figure 4. D, E**) and murine sepsis score (MSS) (**Figure 4. C** and **Figure 4. F**), the record from observational characteristics before mice were sacrificed[54]. Regular CP 55,940 treatment decreased the *S. aureus* infection in mice lung and regular AEA injection mice showed a decreased tendency in *S. aureus* lung infection compared to the saline injection, which showed the opposite of the results of cells in vitro.

In this study, we just used three cannabinoids (AEA, 2-AG and CP 55,940) to conduct the relevant experiment in vitro and in vivo. Even the main cannabinoids are THC and cannabidiol (CBD), besides them, more than 100 other cannabinoids have been defined. Unlike cannabinoids, such as THC or CBD that come from the outside of the body, AEA and 2-AG both are endocannabinoids that are produced by our body naturally, and CP 55,940 is a synthetic cannabinoid. So these three cannabinoids results could not represent the whole cannabinoid or CBD. We admit that there is limitation from animal study and further mechanism is needed to explore the other cannabinoids' effects.
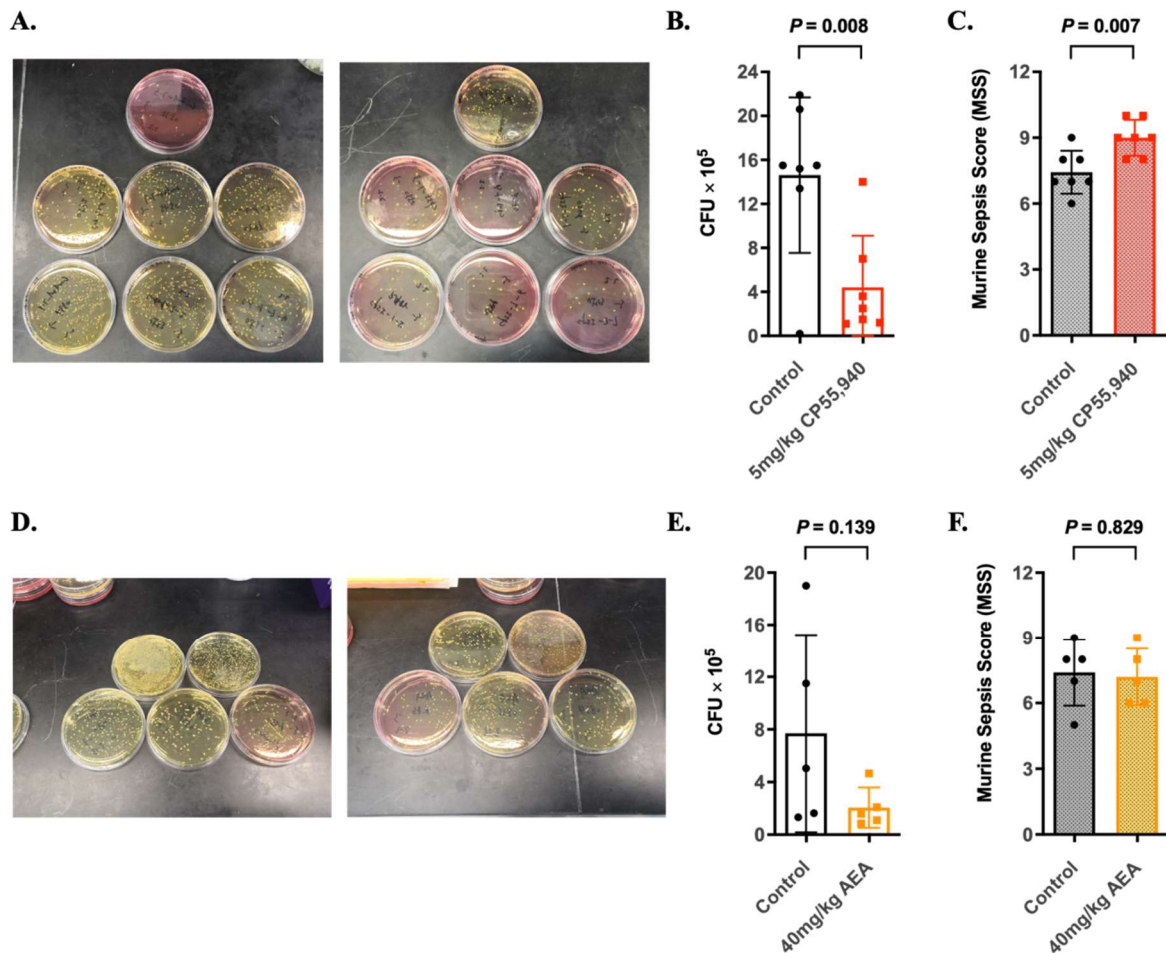
Figure 4. Regular cannabinoid treatment decreased the mice lung infection potential of *S. aureus* in vivo. C57BL/6 mice were intraperitoneal injected 5mg/kg CP55,940 or 0.9% Sodium Chloride as control for 7-day (A-C), and 40mg/kg AEA or 0.9% Sodium Chloride as control for 7-day (D-F), then infected with 20 μl 1× 108 CFU *S. aureus* strain intranasally overnight to establish the *S. aureus* pneumonia model. All mice were sacrificed within(?) 24 hours after *S. aureus* infection. (A, B) Mouse right lung tissues were homogenized for CFUs. (C) Murine Sepsis Score (MSS) was conducted before mice sacrifice. CP55,940 group: n = 7; AEA group: n = 5. Results are presented as means ± S.E.M. from three independent experiments.

2.4 NMDAR knockdown increased the infection capacity of *S. aureus* in vitro

Ketamine, as a non-competitive NMDA receptor (NMDAR) antagonist[55] that acts by an open channel block mechanism[56], and that accounts for most of its actions. To verify the influence of ketamine exerting the NMDA receptor blocker effect on *S. aureus* lung infection, we

constructed the NMDAR critical subunit Grin1 and Grin2D knockdown gene. The knockdown effects were verified in human epithelial cell line 16HBE. Grin1 and Grin2D knockdown efficiency were evaluated by real-time RT-PCR (**Figure 5. A** and **Figure 5. E**) and western blot (**Figure 5. C, D** and **Figure 3. G, H**). Combined with the colony formation assay of quantitative cultures (**Figure 5. B** and **Figure 5. F**), results showed NMDAR knockdown effectively increased the *S. aureus* infection in cell 16HBE.
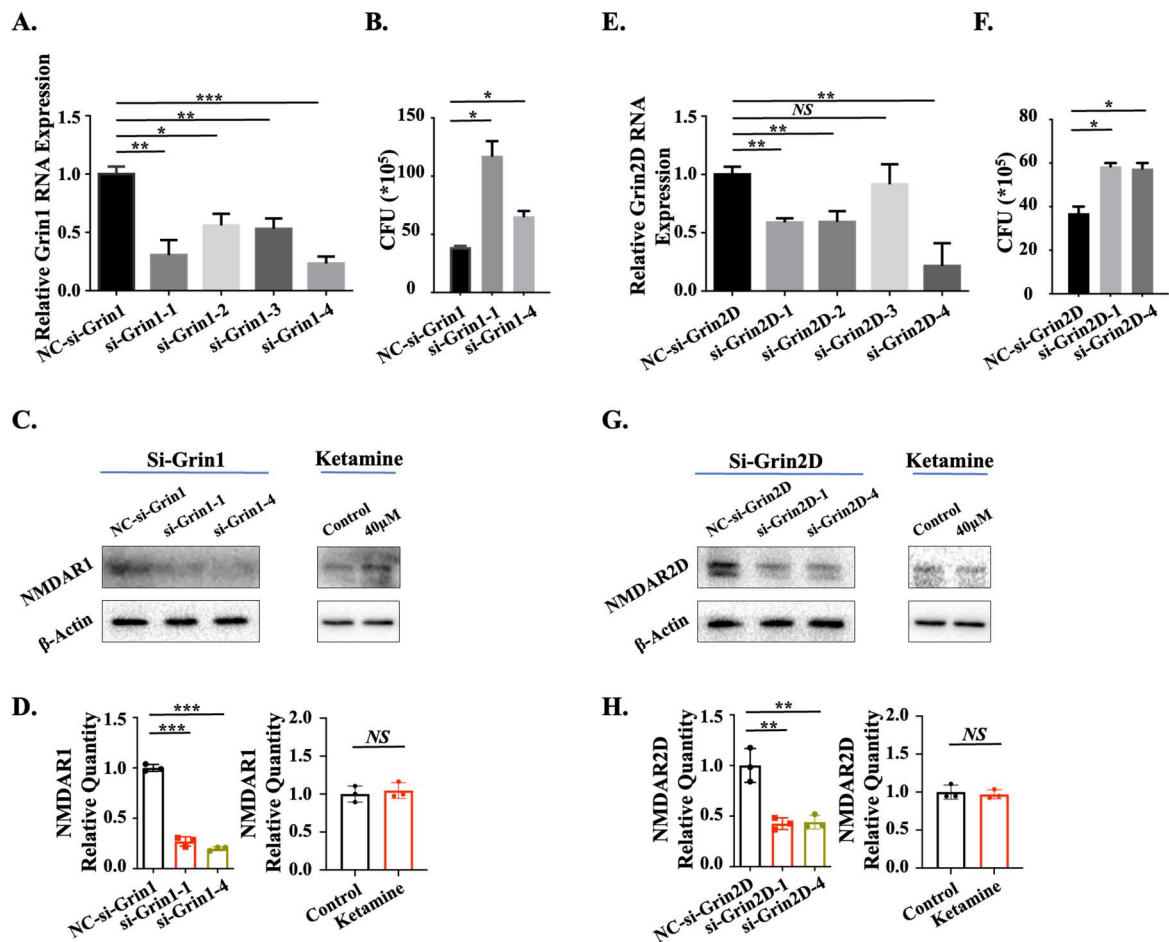
Figure 5. Knockdown NMDAR critical subunit Grin1 and Grin2D increased the adhesion capacity of *Staphylococcus aureus* in vitro. The Grin1 (A-D) and Grin2D (E-H) knockdown effects and functions were detected in cell line A549. Cells were transfected with Grin1-specific siRNA and Grin2D-specific siRNA for 24 hours. Cells were harvested to examine the Grin1 mRNA level (A) and Grin2D mRNA level (E) to assess the knockdown efficiency. Cells transfected with Grin1-specific siRNA and Grin2D-specific siRNA were infected with *S. aureus* (MOI 10) for 2 hours, and then extracellular *S. aureus* infection levels were measured by CFUs (B, F). The knockdown efficiencies of Grin1 and Grin2D at protein level were also confirmed by western blot (C, G)

and the corresponding grayscale value (D, H). Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

2.5 Molecular IL 1B, IL 6, TNF-alpha, AMPs (Cathelicidin (LL-37), HAMP), NF-kB, iron level, collagens and EMTs expression change treated with ketamine in vitro and in vivo.

Inflammatory cytokine IL 1B, IL 6 and TNF-α, antimicrobial peptides (AMPs) LL-37 and HAMP, and the relative molecules NF-kB and iron level were detected to evaluate the ketamine effect in the lung in vitro and in vivo. Human epithelial cell lines A549, 16HBE and human leukemia monocytic cell line THP-1 were used in the detection. Results showed the ketamine treatment decreased IL 1B, IL 6 and TNF-α mRNA expression in cell lines A549, 16HBE and THP-1 (**Figure 6. A-C**), and the IL 1B, IL 6 and TNF-α protein expression levels in BAL were statistically non-significant **(Figure 6. D-F)**. After the ketamine culture, LL-37 and HAMP showed decreased tendency in A549 and THP-1 cell lines, but not statistically significant **(Figure 6. G, H)**. NF-kB mRNA expression in cell line A549 increased significantly and get non-statistically significant in THP-1 (**Figure 6. I, J**). The total iron level in mouse lung tissues and cell lines A549 and 16HBE showed an increased trend with ketamine treatment but statistically non-significant (**Figure 6. K-M**). We next examined the main structural protein collagen and epithelial-mesenchymal transitions (EMTs). After ketamine treatment, collagen I and collagen IV in cell lines A549 increased significantly (**Figure S3. A**), showed no statistically significant difference in cell line 16HBE (**Figure S3. B**). Collagen I expression decreased, and collagen IV increased in mouse lung tissue (**Figure S3. C**). Additionally, we further verified the expression by detecting the change of collagen after knocking down FN1 and ITGAV, results showed that FN1 knockdown increased collagen I and collagen IV (**Figure S3. D**), ITGAV knockdown decreased collagen I and collagen IV (**Figure S3. E**), and

knockdown FN1 and ITGAV at the same time increased collagen I but decreased collagen IV (**Figure S3. F**). EMTs detection results showed that epithelial marker TJP1 decreased both in cell lines A549 and 16HBE (**Figure S3. G, H**), and among 28 detected mesenchymal markers, 3 molecules VIM, FGF1 and SOX10 increased both in A549 and 16HBE cell lines (**Figure S3. I, J**).
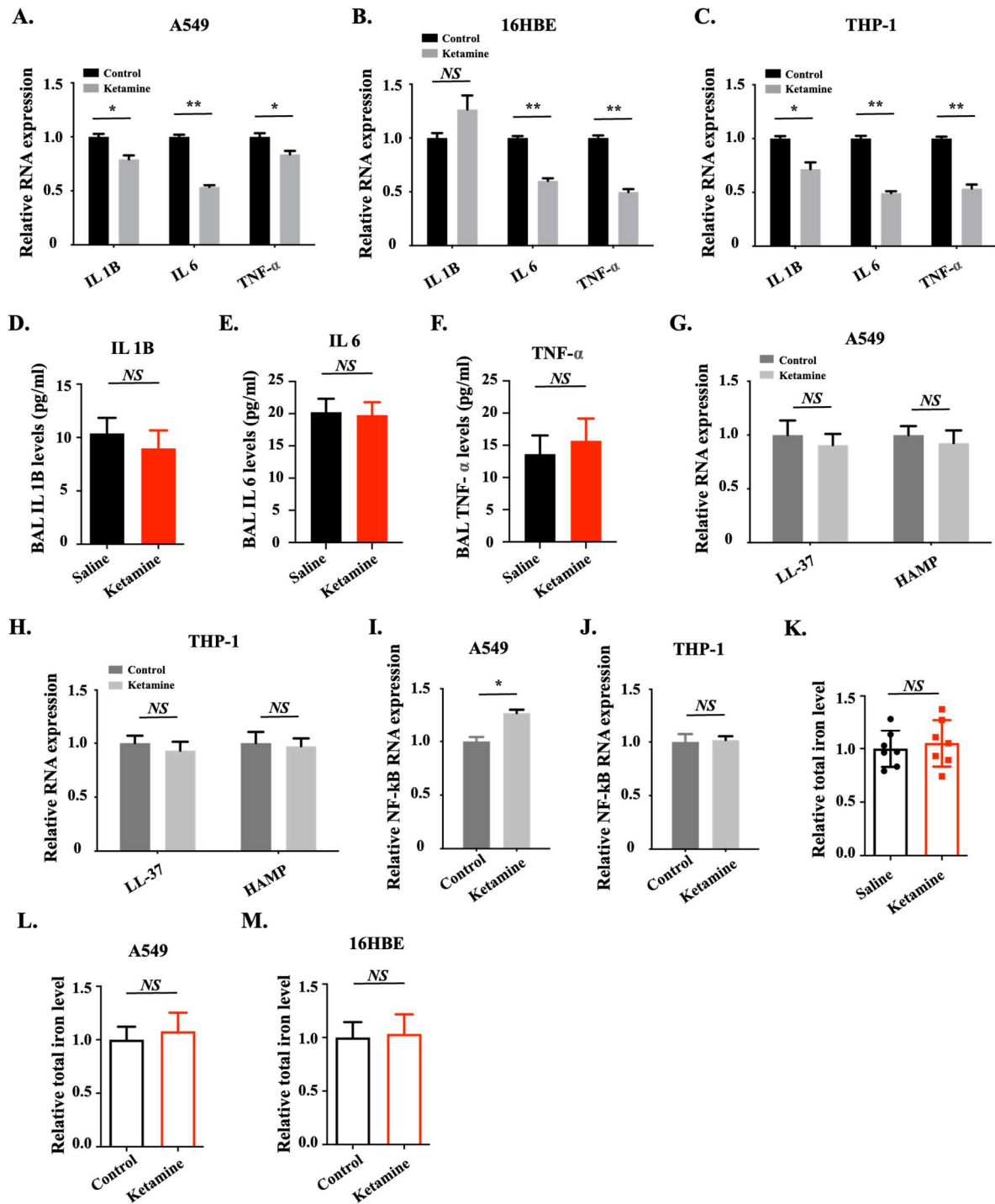
Figure 6. The inflammatory and AMPs molecular mechanism investigation of ketamine treatment in lung cell lines and tissues. C57BL/6 mice were intraperitoneal injected 20mg/kg ketamine or 0.9% Sodium Chloride as control for 7-day before their lung tissue was collected. Human cells A549, 16HBE and THP-1 were cultured in medium with ketamine (40 μM, 48 hours), and washed cells 3 time with PBS before collection. Inflammatory cytokines IL 1B, IL 6 and TNF-α mRNA in cell lines A549, 16HBE and THP-1 were detected by RNA expression detection (A-C), and IL 1B, IL 6 and TNF-α levels in mice BAL were detected by ELISA assay (D-F), n=7. The

mRNA expression of cathelicidin LL-37, hepcidin encoding gene HAMP and AMP related NF-kB in cell lines A549 and THP-1 were tested by RT-qPCR (G-J). Iron levels in mice lung tissues and cell lines A549 and 16HBE were detected by iron assay kit (K-M). Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01.

2.6 Ketamine increased *S. aureus* pulmonary infection by upregulating FN1 and ITGAV expression in vivo and in vitro

The key adhesion protein-fibronectin1 (FN1) and the vitronectin receptor-integrin alpha V (ITGAV) are the major components of the extracellular matrix (ECM). In animal lung tissue treated with ketamine, FN1 and ITGAV showed significantly increased as revealed by immunofluorescence staining and quantitative analysis, respectively (**Figure 7. A, B** and **Figure 7. C, D** for acute model; **Figure S1. H, I** and **Figure S1. G, K** for chronic model). We further determined if FN1 and ITGAV increased *S. aureus* pulmonary infection and adhesion. By visualizing colocalization of FN1/ *S. aureus* and ITGAV/*S. aureus* respectively (**Figure 7. E, F** and **Figure 7. G, H** for acute model; **Figure S1. L, M** and **Figure S1. N, O** for chronic model), we found that their colocalization coefficients did not change significantly. Simultaneously, we confirmed the protein expression of FN1 and ITGAV in vivo and in vitro after ketamine treatment. Consistently, western blots of FN1 and ITGAV both showed significant increase in tissue and whole cell lysates (**Figure 7. I-K** for acute model, **Figure S1. F, G** for chronic model and **Figure 7. L-N** for cell lines).
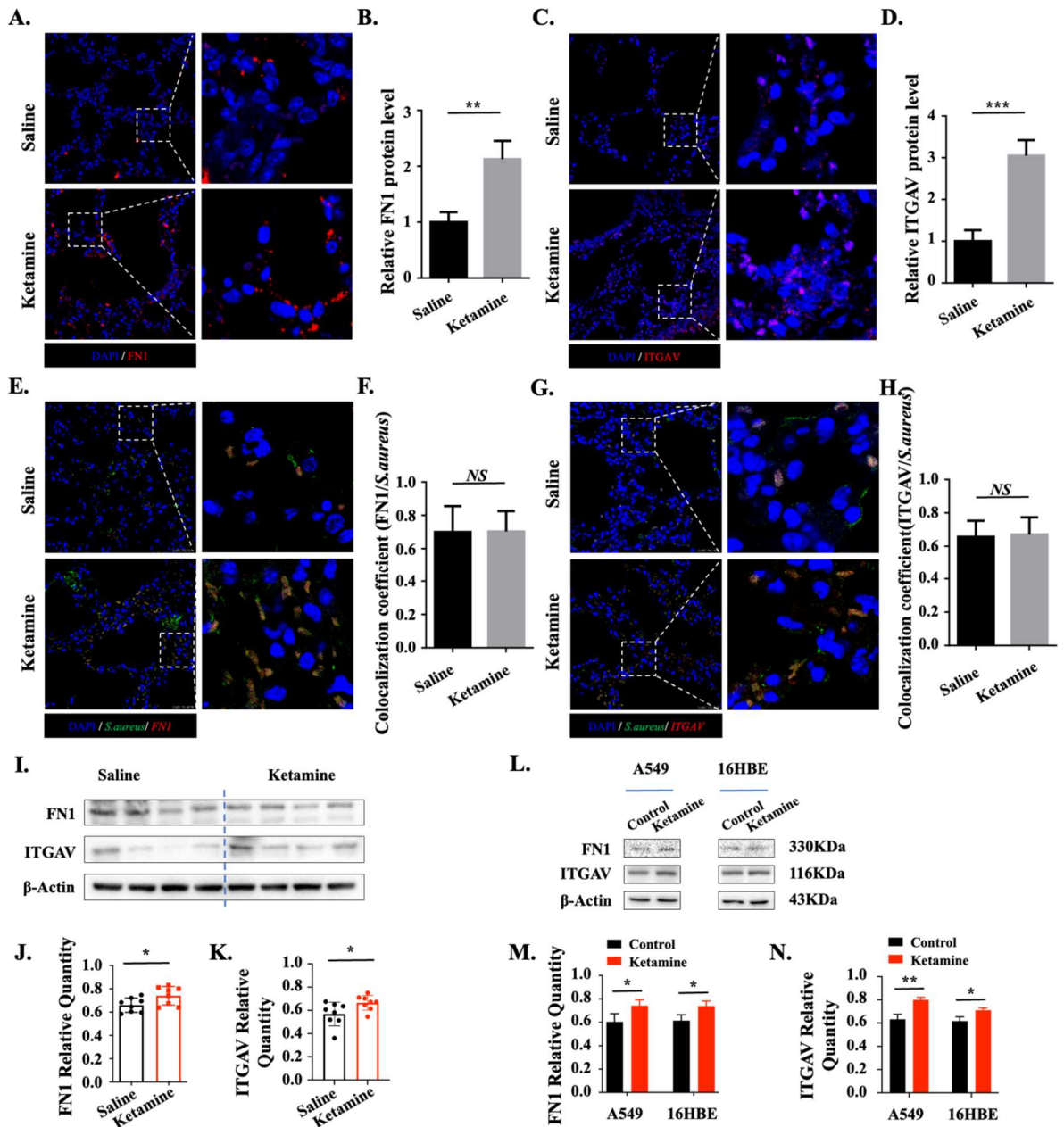
Figure 7. Ketamine increased *Staphylococcus aureus* pulmonary infection by upregulating FN1 and ITGAV expression in vivo and in vitro. C57BL/6 mice (n = 8 per group) were intraperitoneal injected 20mg/kg ketamine or 0.9% Sodium Chloride as control for 7-day before their lung tissue was collected. For mouse lung tissue, the immunofluorescence staining was conducted separately to visualize FN1 (red), ITGAV (red) and nuclei (blue) (A, C). And colocalization coefficient of FN1/*S. aureus* (Red/green), ITGAV/*S. aureus* (Red/green) and nuclei (blue) (E, G). Scale bar: 40 μm. Relative counting followed the staining and thirty visual fields per group were randomly selected to count the protein level (B, D), and colocalization coefficient (F, H). For the human epithelial cells A549 and 16HBE treated with ketamine, western blot was conducted to detect FN1 and ITGAV expression at the

same time (L), FN1 and ITGAV protein levels were qualified (M, N). Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

2.7 FN1 and ITGAV knockdown reduced the adhesion of *Staphylococcus aureus* to A549 and 16HBE cell lines in vitro

Instead, we constructed the FN1 and ITGAV knockdown gene to verify their effects on *S. aureus* pulmonary infection by transfecting siRNA in cell lines A549 and 16HBE. After confirming the FN1 and ITGAV knockdown success in both A549 and 16HBE cell lines (**Figure 8. A, B** and **Figure 9. A, B**), two knockdown lines of each gene were selected to evaluate the function. In cell lines A549 and 16HBE after FN1 and ITGAV knockdown, *S. aureus* infection levels were measured by colony formation assay of quantitative cultures (**Figure 8. C-E** and **Figure 9. C-E**) and quantitative real-time RT-PCR (**Figure 8. F, G** and **Figure 9. F, G**), results showed that FN1 and ITGAV knockdown significantly reduced the adhesion of *S. aureus* to A549 and 16HBE cell lines.

Figure 8. FN1 knockdown reduced the *Staphylococcus aureus* adhesion to A549 and 16HBE cell lines. The FN1 knockdown efficiency was detected in cell lines A549 (A) and 16HBE (B) by RNA expression detection. Cells transfected with Si-FN1-2 and Si-FN1-3 for 24 h were infected *S. aureus* (MOI 10), and after 2 h, cells were harvested. The A549 and 16HBE cell lysis plated on *S. aureus*-selective LB agar plates were incubated for 24 hours at 37 °C to count colony number (C-E). And *S. aureus* DNA levels were measured by real-time RT-PCR to

detect *S. aureus*-specific 16S ribosomal DNA in A549 (F) and 16HBE (G) cell lines. Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.
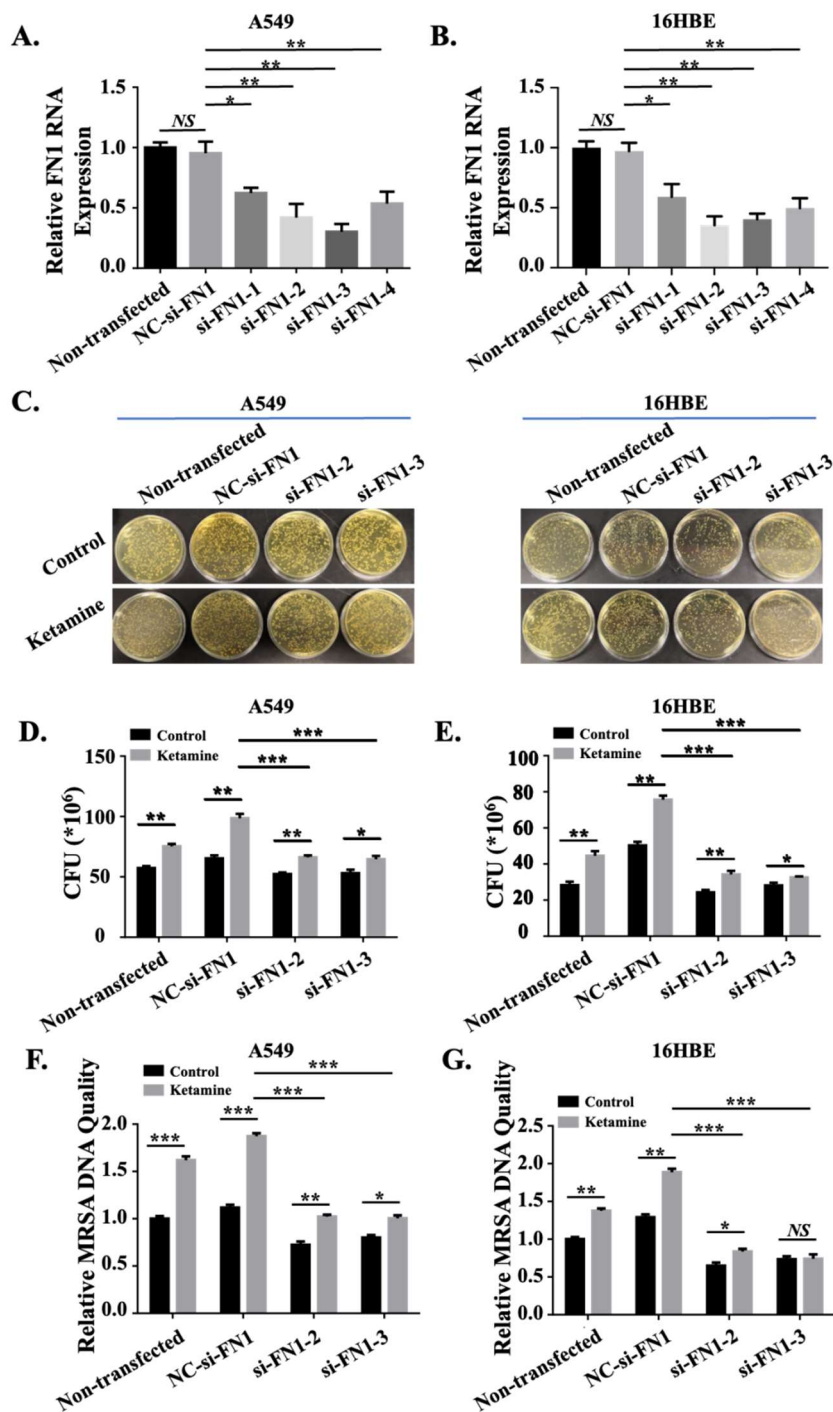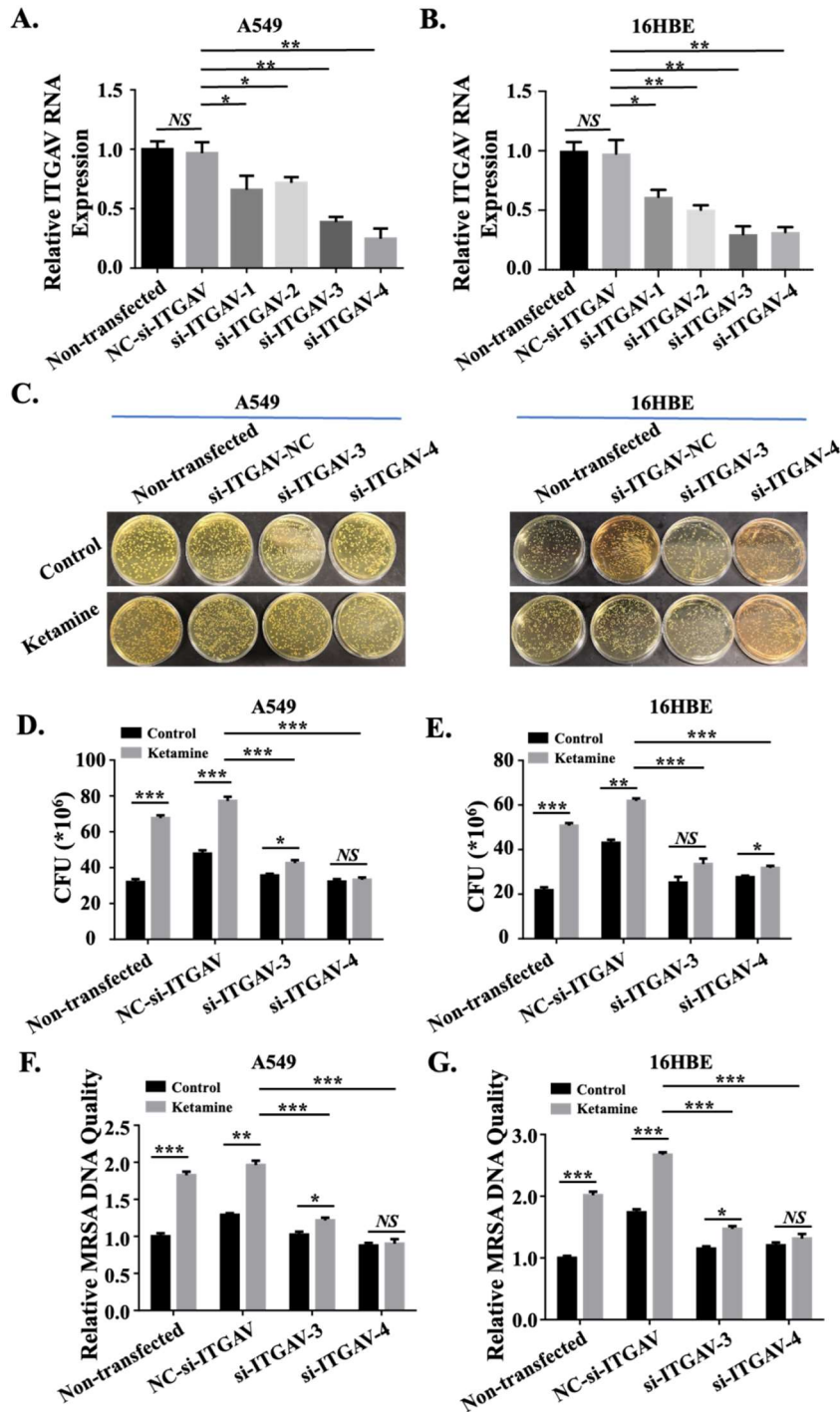

Figure 9. ITGAV knockdown reduced the *Staphylococcus aureus* adhesion to ketamine treated A549 and 16HBE cell lines. The ITGAV knockdown efficiency was detected in cell lines A549 (A) and 16HBE (B) by RNA expression detection. Cells transfected with Si- ITGAV-3 and Si- ITGAV-4 for 24 hours were infected *S. aureus*

(MOI 10), and after 2 hours, cells were harvested. The A549 and 16HBE cell lysis plated on *S. aureus*-selective LB agar plates were incubated for 24 hours at 37 °C to count colony number (C-E). And *S. aureus* DNA levels were measured by real-time RT-PCR to detect *S. aureus*-specific 16S ribosomal DNA in A549 (F) and 16HBE (G) cell lines. Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

Data processing and analysis

All data were expressed as the mean ± standard error of the mean (S.E.M.). To determine statistical significance between groups, comparisons were made using two-tailed t-tests. Analyses of multiple groups were done by one-way analysis of variance (ANOVA) followed by the Tukey's t-test. For all statistical tests, a P value less than 0.05 was considered statistically significant.

Discussions

In this study, we validated that ketamine high doses usage increased the *S. aureus* pulmonary infection *in vivo* and *in vitro*. Ketamine abuse in recent years has shown apparent increases globally, and the adverse event reports for illicit use have been published in the United Stated, United Kingdom, Australia and Eastern Europe[57-63]. A retrospective survey on ketamine abusers showed 18.8% of symptoms were diagnosed with upper respiratory tract infection, ranked second one amongst their illnesses following the epigastric pain (25%)[44]. *S. aureus* is a ubiquitous pathogen that is implicated in a variety of respiratory tract infections and produces a potent pro-inflammatory response that is often associated with high morbidity and

mortality[64,65]. Our study results showed high doses ketamine use increased *S. aureus* infection and adhesion in lung.

Our study found that these three cannabinoids increased the *S. aureus* infection in human epithelial cells. The cannabinoid is being paid attention to by more and more areas due to the increasing recreational abuse and health hazards. From 1 February 2023, cannabidiol had been listed as a dangerous drug in Hong Kong[66]. Studies show that cannabidiols abuse induces acute lung inflammation and associates with respiratory failure[67,68]. Given the cannabidiol could not be obtained by market, thus, in this study, we change to three other active forms of cannabinoids that are used for research purposes, however, the discrepancy of the function action of cannabinoids was shown from our *in vitro* and *in vivo* experiments. Whether it is due to the chemical structure of different cannabinoids warrants further exploration.

As the NMDA receptor antagonist, ketamine upregulated FN1 and ITGAV expression. Integrins are evolutionary old cell adhesion receptors[69], many of their ligands are large multi-adhesive ECM molecules. As an important ECM number, fibronectin mediates many different cellular interactions and binds to integrins[70-72]. The ability to targeted binding to the human fibronectin is a well-known property that has been reported for many bacteria[73,74]. Concordantly, in our study, we showed that FN1 and ITGAV expression both increased after ketamine treatment, and the colocalization coefficients of FN1/ *S. aureus* and ITGAV/*S. aureus* did not change significantly. These findings suggest that ketamine increased *S. aureus* lung infection and adhesion by upregulating FN1 and ITGAV. For further investigation into the detailed mechanisms of their upstream regulatory molecules and the interaction way between molecules, more other research is needed to discover and explore in the future.

This work has uncovered the previously unappreciated role of the ketamine in Methicillin-Resistant *Staphylococcus Aureus* (MRSA) -associated pneumonia. In this regard, ketamine abuse increased the susceptibility of MRSA infection both *in vitro* and *in vivo*, especially aggravated lung tissue damage experimental mouse model. Give the MRSA is not a common causative agent for pulmonary infection, we suggest this topic would warrant a detailed investigation on other pathogenetic bacteria or virus infection. Nevertheless, by understanding and harnessing this novel mechanism of ketamine facilitating MRSA infection, we would be able to promote the general awareness of a broader cross-section of the community of the hidden drug problem, as well as to encourage and enable them to play a more active role in drug prevention, early identification in effective therapeutic targets to pulmonary infection intervention.

Supplemental material

3.1 DNA sequence of primers

Table S1. DNA sequence of primers.

Gene name	Primers	Sequence (5′ -> 3′)
Human		

Gene		Sequence
Grin1	Forward	CCAGTCAAGAAGGTGATCTGCAC
	Reverse	TTCATGGTCCGTGCCAGCTTGA
Grin2D	Forward	GAACATCCGCAGCAACTATCCC
	Reverse	GCAGCATCGTAGATGAAGGCGT
FN1	Forward	ACAACACCGAGGTGACTGAGAC
	Reverse	GGACACAACGATGCTTCCTGAG
ITGAV	Forward	AGGAGAAGGTGCCTACGAAGCT
	Reverse	GCACAGGAAAGTCTTGCTAAGGC
ACTB	Forward	CACCATTGGCAATGAGCGGTTC
	Reverse	AGGTCTTTGCGGATGTCCACGT
IL 1B	Forward	CCACAGACCTTCCAGGAGAATG
	Reverse	GTGCAGTTCAGTGATCGTACAGG
IL 6	Forward	AGACAGCCACTCACCTCTTCAG
	Reverse	TTCTGCCAGTGCCTCTTTGCTG
TNF α	Forward	CTCTTCTGCCTGCTGCACTTTG
	Reverse	ATGGGCTACAGGCTTGTCACTC
NF-kB	Forward	TGAACCGAAACTCTGGCAGCTG
	Reverse	CATCAGCTTGCGAAAAGGAGCC
Mouse		
FN1	Forward	CCCTATCTCTGATACCGTTGTCC
	Reverse	TGCCGCAACTACTGTGATTCGG
IITGAV	Forward	GTGTGAGGAACTGGTCGCCTAT
	Reverse	CCGTTCTCTGGTCCAACCGATA
ACTB	Forward	CATTGCTGACAGGATGCAGAAGG
	Reverse	TGCTGGAAGGTGGACAGTGAGG
IL 1B	Forward	TGGACCTTCCAGGATGAGGACA
	Reverse	GTTCATCTCGGAGCCTGTAGTG

IL 6	Forward	TACCACTTCACAAGTCGGAGGC
	Reverse	CTGCAAGTGCATCATCGTTGTTC
TNF α	Forward	GGTGCCTATGTCTCAGCCTCTT
	Reverse	GCCATAGAACTGATGAGAGGGAG
NF-kB	Forward	TCCTGTTCGAGTCTCCATGCAG
	Reverse	GGTCTCATAGGTCCTTTTGCGC
Bacteria		
MRSA (sa442)	Forward	GTCGGGTACACGATATTCTTCACG
	Reverse	CTCGTATGACCAGCTTCGGT
16S rRNA	Forward	AGAGTTTGATCCTGGCTCAG
	Reverse	GGTTACCTTGTTACGACTT

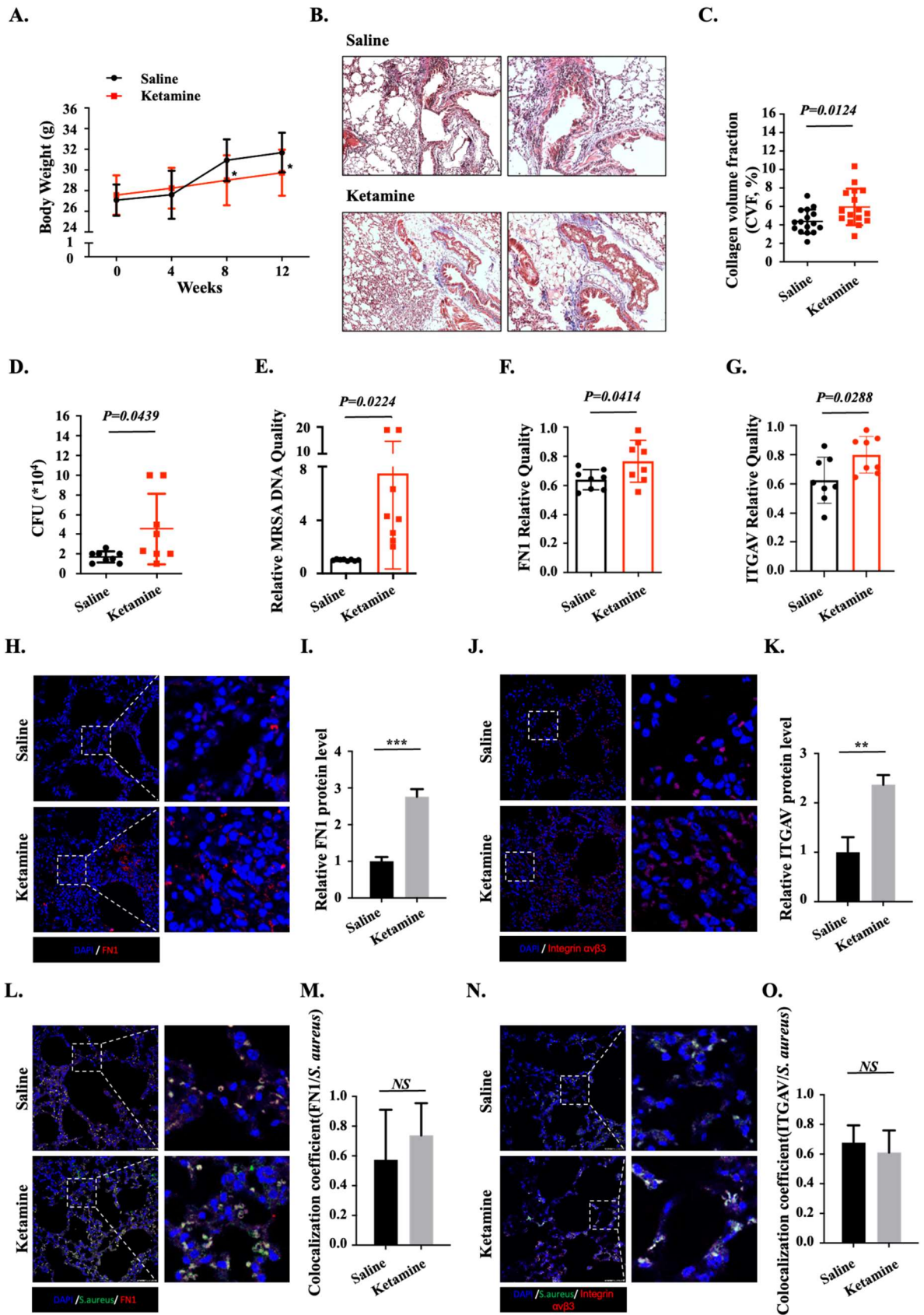## 3.2 Supplemental figures

Figure S1. Uninterrupted ketamine injection increased the S. aureus chronic infection in mice lung. C57BL/6

mice were intraperitoneal injected 20mg/kg ketamine or 0.9% Sodium Chloride as control for 3 months before

their lung tissue was collected. Mice body weight were measured before being sacrificed (A), n = 7. Masson staining was conducted to visualize the collagen tissue change (B), sixteen field were selected randomly for collagen volume fraction counting (C). Mouse right lung tissues were collected and homogenized for CFUs (D), and S. aureus levels were quantified by real-time PCR (E), n = 8. FN1 and ITGAV protein levels were qualified (F, G), n = 8. At the same time, immunofluorescence staining was conducted separately to visualize FN1 (red), ITGAV (red) and nuclei (blue) (H, J). And colocalization coefficient of FN1/S. aureus (Red/green), ITGAV/S. aureus (Red/green) and nuclei (blue) (L, N). Scale bar: 40 μm. Relative counting followed the staining and thirty visual fields per group were randomly selected to count the protein level (I, K), and colocalization coefficient (M, O). Results are presented as means ± S.E.M. from three independent experiments. **P < 0.01; ***P < 0.001.
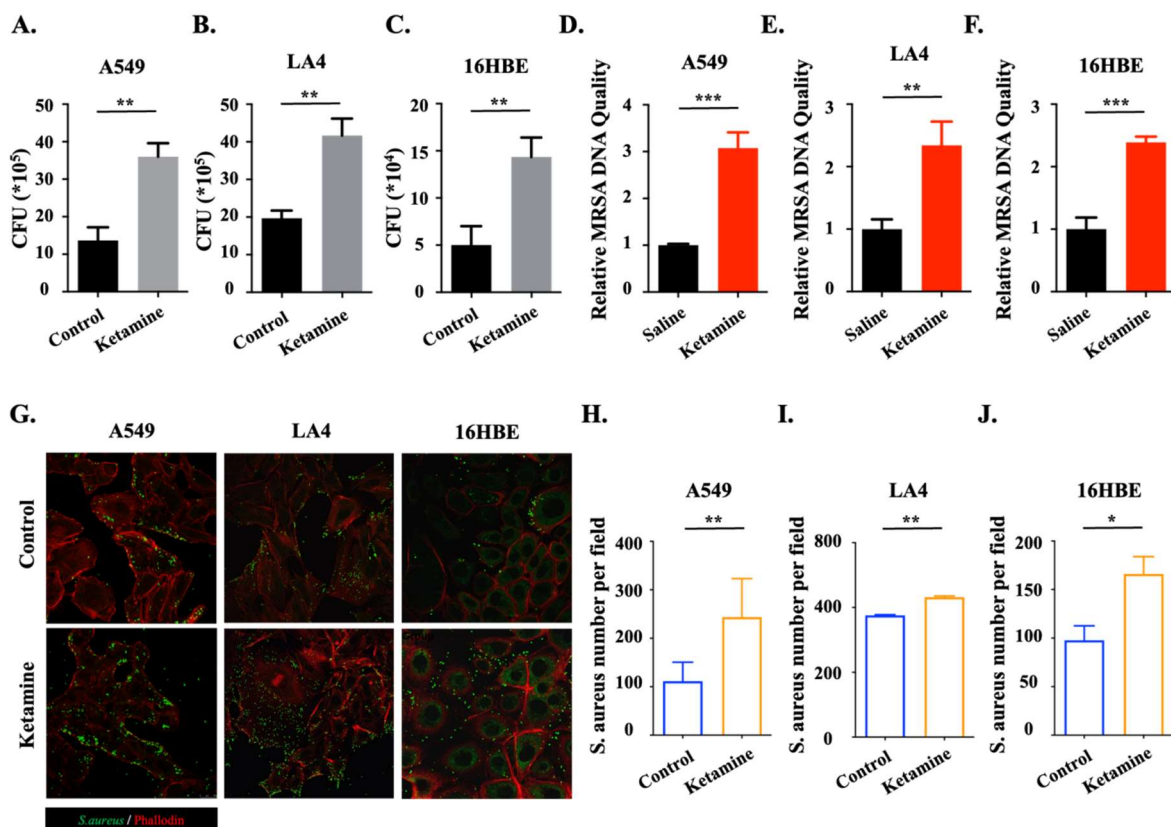
Figure S2. Regular ketamine treatment increased the invasion capacity of *Staphylococcus aureus* in lung cells in vitro. Human and mouse epithelial cells A549, LA4 and 16HBE were cultured in medium with ketamine (40 μM, 48 hours) and infected with S. aureus strain (MOI 10) for 2 hours, washed cells 3 time with PBS, followed by being treated with 10mg/L gentamicin for 2 hours. The S. aureus invasion infection in cell lines were evaluated

by CFUs (A-C), qualified by real-time PCR (D-F), and visualized by immunofluorescence staining (G-J). Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.
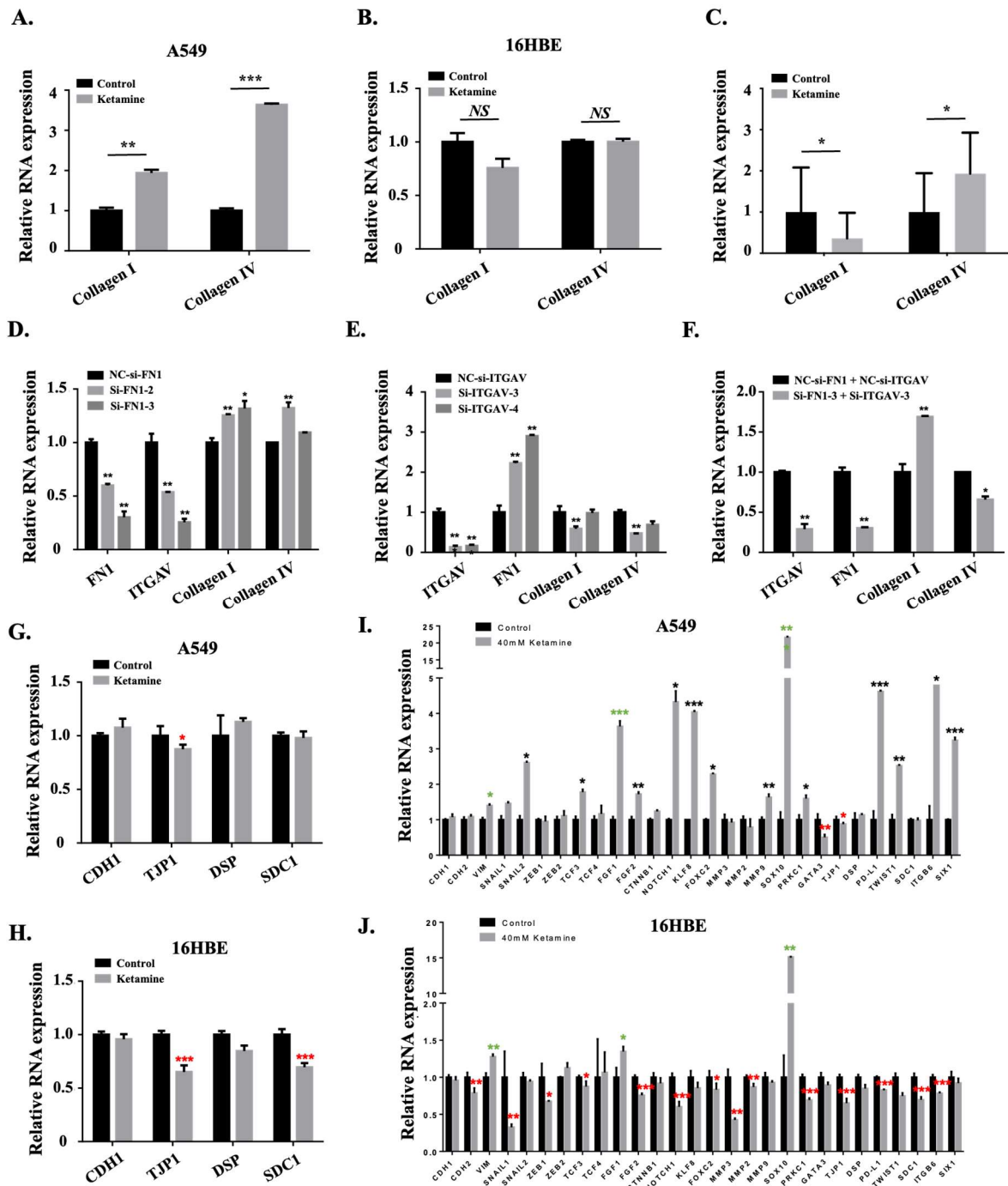
Figure S3. The collagens and EMTs mechanism investigation of ketamine treatment in lung cell lines and tissues. C57BL/6 mice were intraperitoneal injected 20 mg/kg ketamine or 0.9% Sodium Chloride as control for 3 months

before their lung tissue was collected. Human epithelial cells A549 and 16HBE were cultured in medium with ketamine (40 μM, 48 hours), and washed cells 3 time with PBS before collection. Collagen I and collagen IV in cell lines A549, 16HBE and mice lung tissues (n=7) were detected by RNA expression detection (A-C), further the collagen I and collagen IV expression after FN1 and ITGAV knockdown in A549 were detected by RT-qPCR (D-F). Epithelial markers (G, H) and mesenchymal markers (I, J) mRNA expression in cell lines A549 and 16HBE were tested by RNA expression detection. Results are presented as means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

3.3 Deliverable table

|	Name of activity	Aim and Content	Results
1	Evaluating the effect of ketamine and cannabinoid receptor agonists (AEA, 2-AG, CP 55,940) on AMPs expression in *S. aureus* infection *in vitro*.	THP-1 and A549 cells pre-incubated with ketamine and cannabinoid receptor agonists (AEA, 2-AG, CP 55,940) will be subsequently infected with *S. aureus*. AMP mRNA expression and protein secretion from THP-1 and A549 cells will be determined.	1. After 40 μM ketamine pre-treatment for 48 hours, AMPs (cathelicidin and hepcidin) showed declined trend but no statistically significant difference in mRNA expression in A549 and THP-1 cell line. 2. There were no AMP (cathelicidin) mRNA expression difference in cell line A549 after AEA and CP 55,940 treatment separately.
2	Evaluating the effect of ketamine in clearance *S. aureus* colonization and survival *in vitro*.	Ketamine and cannabinoid receptor agonists (AEA, 2-AG and CP 55,940) will reduce the production of first line of AMPs (cathelicidin and hepcidin) and thereby increase the *S. aureus* infection susceptibility in *S. aureus*-infected THP-1/A549 cells. Ketamine and cannabinoid receptor agonists (AEA, 2-AG and CP 55,940) will also increase the survival of both adherent and invasive *S. aureus*.	Ketamine increased the *S. aureus* infection susceptibility (both adhesion and invasion) in cell lines A549 and 16HBE.

3	Evaluating the effect of cannabinoid receptor agonists (AEA, 2-AG and CP 55,940) on *S. aureus* colonization and survival in vitro.	Refer to the above content.	Cannabinoid receptor agonists AEA, 2-AG and CP 55,940 all increased the *S. aureus* invasion susceptibility in cell lines A549 and 16HBE. After the invasion measurement in vitro, animal experiments were conducted.
4	Identifying the transcription factors related to ketamine/ cannabinoid receptor agonists (AEA, 2-AG and CP 55,940) suppression of AMPs.	Several novel transcription factors bound to CAMP and HAMP genes whose activates are regulated by ketamine and cannabinoid receptor agonists (AEA, 2-AG and CP 55,940) will be verified *in vitro*.	1. Ketamine increased S. aureus pulmonary infection by upregulating FN1 and molecule integrin (ITGAV) expression in vivo and in vitro. 2. Molecular IL-1B, IL 6, TNF-alpha, AMPs (LL-37, HAMP), NF-kB, iron level, collagens and EMTs expression change treated with ketamine in vitro and in vivo.
5	Evaluating the effect of ketamine and cannabinoid receptor agonists (AEA and CP 55,940): Quantitative bacteriology and histopathology; Lung pathological scoring, Collection of bronchoalveolar lavage	As compared with control group, Ketamine plus *S. aureus* infection or cannabinoid receptor agonists (AEA and CP 55,940) plus *S. aureus* mice will exhibit more severe lung damage with increased epithelial cell apoptosis,	1. Ketamine increased *S. aureus* infection, microscopic lung tissue score and impaired the macroscopic lung pathology in necrotizing pneumonia in mice;

	(BAL); L-1β, IL-6, TNF-a ELISA Assay.	extensive infiltration of mixed neutrophils and mononuclear cells in the airway and alveolar lumen upon the development of *S. aureus* pneumonia. In addition, there will be more severe dilation and more mixed inflammatory cell infiltration in the perivascular and peri-bronchial space in the lung. These changes will be accompanied by an increase in *S. aureus* colonization and decrease in mouse survival time.	2. Chronic ketamine injection decreased the mice body weight and increased the *S. aureus* lungs infection; 3. BAL after ketamine injection was collected successfully for the next molecules measurement. 4. The IL-1β, IL 6 and TNF-alpha showed no significant change in mouse bronchoalveolar lavage fluid (BALF). 5. CP 55,940 decreased *S. aureus* infection and murine sepsis score in mice, and AEA showed the same decreased tendency (AEA and 2-AG both are endogenous agonists of the canonical cannabinoid receptors CB1 and CB2). 6. The BAL after AEA and CP 55,940 injection was collected successfully.
6	Evaluating the effect of ketamine and the molecular mechanism of ketamine suppresses endogenous	As compared with control group, ketamine plus *S. aureus* infection.	1. Ketamine treatment decreased IL-1β, IL-6 and TNF-a mRNA expression in A549, 16HBE and THP-1

	AMPs expression; Collection of bronchoalveolar lavage (BAL); Cathelicidin ELISA Assay.		cell lines, no significant change in mouse BAL. 2. NF-kB increased in A549, but had non-statistical significant in THP-1 cell line. No significant different of Cathelicidin Assay.
7	Further evaluating the effect of ketamine: Collection of lung tissue; Heart blood; Masson's trichrome; Iron level assay; The further molecular mechanism and possible regulatory pathways.		1. Masson's trichrome based on mice lung tissue showed ketamine injection increased the collagen tissue. 2. The Iron level after ketamine treatment showed increased trend in A549, 16HBE and lung tissues, but had no statistically significant difference. 3. After ketamine treatment, molecules collagen I and collagen IV increased in cell A549, but no significant change in 16HBE cell line. In mice tissue, collagen I decreased and collagen IV increased. 4. EMT-related molecules expression measurement showed that epithelial

		44	marker TJP1 decreased both in cell lines A549 and 16HBE after ketamine treatment, only 3 among 28 tested mesenchymal markers both increased in A549 and 16HBE. 5. Ketamine increased FN1 and ITGAV expression in mice lung tissue, and we verified that ketamine increased *S. aureus* pulmonary infection by upregulating FN1 and ITGAV in cell lines A549 and 16HBE (by knocking down FN1 and ITGAV expression respectively).

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