

1 **Type III secretion system of *Pseudomonas aeruginosa* affects mucin gene expression via**  
2 **NF- $\kappa$ B and AKT signaling in human carcinoma epithelial cells and a pneumonia mouse**  
3 **model**

4 **Short title:** *Pseudomonas aeruginosa* T3SS affects mucin expression

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6 Ji-Won Park<sup>1</sup>, In-Sik Shin<sup>2</sup>, Sei-Ryang Oh<sup>1</sup>, Un-Hwan Ha<sup>3</sup>, and Kyung-Seop Ahn<sup>1\*</sup>

7  
8 <sup>1</sup> *Natural Medicine Research Center, Korea Research Institute of Bioscience and*  
9 *Biotechnology, 30 Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk*  
10 *28116, Republic of Korea.*

11 <sup>2</sup> *College of Veterinary Medicine, Chonnam National University, Gwangju, 61188, Republic*  
12 *of Korea*

13 <sup>3</sup> *Department of Biotechnology and Bioinformatics, Korea University, Sejong 30016, Republic*  
14 *of Korea.*

15  
16 \*Author to whom correspondence should be addressed

17 Kyung-Seop Ahn<sup>a\*</sup>

18 Natural Medicine Research Center,

19 Korea Research Institute of Bioscience and Biotechnology,

20 30 Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk 28116, Republic of

21 Korea.

22 Tel: +82-43-240-6113; Fax: +82-43-240-6129

23 E-mail: ksahn@kribb.re.kr

24 **Abstract**

25           The type III secretion system (T3SS) in *Pseudomonas aeruginosa* has been linked to  
26 severe disease and poor clinical outcomes in animal and human studies. Of the various T3SS  
27 effector genes, ExoS and ExoT showed mutually exclusive distributions, and these two genes  
28 showed varied virulence. We aimed to investigate whether the ExoS and ExoT effector proteins  
29 of *P. aeruginosa* affect the expression of the proinflammatory mediators Muc7, Muc13, Muc15,  
30 and Muc19 via the NF- $\kappa$ B and AKT signaling pathways. To understand the role of the T3SS,  
31 we used  $\Delta$ ExoS,  $\Delta$ ExoT, and T3SS transcriptional activator ExsA mutants (ExsA:: $\Omega$ ), as well  
32 as A549 cells stimulated with *P. aeruginosa* strain K (PAK). We investigated the effects of  
33  $\Delta$ ExoS,  $\Delta$ ExoT, and ExsA:: $\Omega$  on the development of pneumonia in a mouse model and on  
34 Muc7, Muc13, Muc15, and Muc19 production in A549 cells.  $\Delta$ ExoS and  $\Delta$ ExoT markedly  
35 decreased the neutrophil count in the bronchoalveolar lavage fluid, with a reduction in Muc7,  
36 Muc13, Muc15, and Muc19 expression.  $\Delta$ ExoS and  $\Delta$ ExoT reduced NF- $\kappa$ B and AKT  
37 phosphorylation, together with Muc7, Muc13, Muc15, and Muc19 expression in PAK-infected  
38 mice and A549 cells. In conclusion, *P. aeruginosa* infection induced the expression of Mucus,  
39 and the *P. aeruginosa* T3SS appeared to be a key player in Muc7, Muc13, Muc15, and Muc19  
40 expression, which is further controlled by NF- $\kappa$ B and AKT signaling. These findings might be  
41 useful to devise a novel therapeutic approach for the treatment of chronic pulmonary infections  
42 by targeting ExoS and ExoT.

43

44

45 **Author Summary**

46 *Pseudomonas aeruginosa* is a ubiquitous gram-negative bacterium causing serious infections.  
47 Many clinical isolates of *P. aeruginosa* have a specialized apparatus for injecting toxins into  
48 eukaryotic cells, namely, the type III secretion system (T3SS). The T3SS is a syringe-like  
49 apparatus on the bacterial surface, with 4 effector toxins: ExoS, ExoT, ExoY, and ExoU. We  
50 investigated the effect of ExoS and ExoT of the T3SS of *P. aeruginosa* K strain (PAK). Mucus  
51 plays a vital role in protecting the lungs from environmental factors, but conversely, in muco-  
52 obstructive airway disease, mucus becomes pathologic. We showed that infection with ExoS  
53 and ExoT induced Muc7, Muc13, Muc15, and Muc19 expression in host cells. PAK clinical  
54 strains induce proinflammatory cytokine production through the T3SS, and this involves NF-  
55  $\kappa$ B and SP1/AKT activation in pneumonia mouse models. Mucus induction in response to  
56 ExoS and ExoT infection relied on NF- $\kappa$ B and SP1/AKT activation. Our findings highlight the  
57 roles of Muc7, Muc13, Muc15, and Muc19 in inducing proinflammatory cytokine expression  
58 during ExoS and ExoT exposure in PAK infections, paving the way for a novel therapeutic  
59 approach for the treatment of pulmonary infections.

60

## 61 **Introduction**

62           The gram-negative bacterium *Pseudomonas aeruginosa* uses a complex type III  
63 secretion system (T3SS) to inject effector proteins into host cells [1,2]. The T3SS is a major  
64 virulence determinant that manipulates eukaryotic host cell responses that is present in a broad  
65 range of pathogens. It is a specialized needle-like structure that delivers effector toxins directly  
66 from the bacterium into the host cytosol in a highly regulated manner [3]. This system is  
67 activated on contact with eukaryotic cell membranes, interferes with signal transduction, and  
68 causes cell death or alterations in host immune responses [4]. The T3SS in *P. aeruginosa* has  
69 been linked to severe disease and poor clinical outcomes in animal and human studies [4,5].  
70 The features of this interesting secretion system have important implications for the  
71 pathogenesis of *P. aeruginosa* infections and for other T3SSs. *P. aeruginosa* has four known  
72 effector toxins: ExoS, ExoT, ExoY, and ExoU. These proteins can modify signal transduction  
73 pathways and counteract innate immunity [6,7]

74           Mucins are a major component of the respiratory mucus. Mucins are either membrane-  
75 bound (like MUC1) with a role in sensing external information and transducing it to cells, or  
76 secreted, the type of which is characterized by a high molecular weight and viscosity. They are  
77 glycoproteins secreted by the mucosal and submucosal glands. The mucin molecule consists  
78 of a polypeptide core with branched oligosaccharide side chains, each of which contains 8 to  
79 10 sugars [8,9]. Molecular cross-linking of this structure contributes to the viscoelastic property  
80 of mucus [10]. Despite their recalcitrance, mucins are a main nutrient source for niche-specific  
81 microbiota of the gut and oral cavity. For example, oral streptococci produce a variety of  
82 glycolytic and proteolytic enzymes that liberate bioavailable carbohydrates from salivary  
83 glycoproteins [11,12]. At least 20 human mucin genes have been identified by cDNA cloning:

84 *MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9,*  
85 *MUC12, MUC13, MUC15, MUC16, MUC17, MUC19, MUC20, MUC21, and MUC22*) [13].

86 However, these mucins cannot maintain homeostasis in intrabronchial respiratory epithelial  
87 cells of patients with weak immunity, and when overexpressed, cause mucous membrane  
88 damage due to excessive secretion of mucus, and need to be blocked. However, the underlying  
89 mechanisms have been clearly clarified for only a few mucins to date. Muc7, Muc13, Muc15,  
90 and Muc19 have been implicated in bronchial inflammation among mucin targets, but the  
91 mechanism of the mechanism has not been studied to date. Especially in immunocompromised  
92 patients and children infected with the opportunistic pathogen *P. aeruginosa*, the risk of  
93 secondary infection is high and leads to not only asthma and airway hypersensitivity as well as  
94 deterioration of existing disease, but also mucus accumulation, which can result in terrible  
95 death due to difficulty of breathing by chronic obstructive pulmonary disease [14,15]. However,  
96 the mechanism underlying this condition is yet to be clarified.

97 NF- $\kappa$ B is also a high expression level transcription factor involved in many  
98 inflammation's formation and development [16]. Moreover, SP1/NF- $\kappa$ B pathway has been  
99 reported connected to cell migration, invasion and EMT (epithelial to mesenchymal transition)  
100 recently [17,18]. Furthermore, SP1 and NF- $\kappa$ B (p65) were found significantly upregulated in  
101 ExoS and ExoT infected cell. The ExsA:: $\Omega$  and  $\Delta$ ST does not increased the expression level  
102 of SP1 and NF- $\kappa$ B (p65) in vitro and in vivo. The expression level of Muc7, Muc13, Muc15,  
103 and Muc19 may be increased through activation of SP1 and NF- $\kappa$ B (p65) due to infection of  
104 ExoS and ExoT.

105 Here, we investigated the effect of ExoS and ExoT of *P. aeruginosa* strain K (PAK) on  
106 the induction of Muc7, Muc13, Muc15, and Muc19 expression, as well as the underlying

107 mechanism, in host cells and a pneumonia mouse model. We expected our study to provide  
108 new insights into the roles of Muc7, Muc13, Muc15, and Muc19 in inducing proinflammatory  
109 cytokine expression in response to ExoS and ExoT exposure in PAK infection.

## 110 **Materials and Methods**

### 111 **Bacterial strains**

112 All strains and plasmids used in this study are listed in Supporting information S1  
113 Table. Chromosomal mutants were all derived from the same parental PAK strain, as indicated  
114 in S1 Table, and were generated by allelic exchange (for details, see the Supporting information  
115 S1 Text). PAK strains vary widely in their expression of virulence genes. The strain used in  
116 this study strongly expresses the T3SS genes. Regions flanking the appropriate mutation were  
117 amplified using chromosomal DNA as a template (unless specified otherwise), joined by  
118 “splicing by overlap extension PCR,” and cloned into the appropriate plasmid using the  
119 indicated restriction enzymes. PAK- $\Delta$ ST is a *P. aeruginosa* clinical isolate that naturally carries  
120 the ExoY gene, but lacks the genes for ExoS and ExoT. We reported that the pUCP18-  
121 PAKexoS (S) and pUCP18-PAKexoT (T) mutant strains are secretion competent and export  
122 the expected effector proteins [19]. Antibiotics were used when necessary at the following  
123 concentrations: for plasmids in *Escherichia coli*, 50  $\mu$ g/mL ampicillin, 15  $\mu$ g/mL gentamicin,  
124 and 25  $\mu$ g/mL kanamycin; for *P. aeruginosa*, 500  $\mu$ g/mL carbenicillin, 100  $\mu$ g/mL gentamicin,  
125 and 100  $\mu$ g/mL tetracycline.

126

### 127 **Human cell culture**

128 A549 adenocarcinomic human alveolar basal epithelial cells, and H292 human airway  
129 epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas,  
130 VA, USA). The cells were maintained in RPMI1640 (Invitrogen, Grand Island, NY, USA)  
131 supplemented with 10% fetal bovine serum (FBS; Invitrogen) in the presence of penicillin (100  
132 U/mL), streptomycin (100  $\mu$ g/mL; Sigma-Aldrich, St. Louis, MO, USA), and HEPES (25 mM),

133 at 37 °C in a 5% CO<sub>2</sub> atmosphere.

134

### 135 ***In-vitro* bacterial infection**

136 For direct bacterial challenge of A549 cells, bacterial strains were grown in tryptic soy  
137 broth (Sigma-Aldrich) at 37 ° C until the OD<sub>600</sub> reached 1. The bacterial cultures were  
138 centrifuged at 7,000 × g for 10 min, washed with PBS, and resuspended at a ratio of 1:20  
139 bacterial cells to A549 cells or H292 cells.

140

### 141 **qRT-PCR analysis**

142 Total RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen) following the  
143 manufacturer's protocol and was used to synthesize cDNA using the AMPIGENE<sup>®</sup> cDNA  
144 Synthesis Kit (Enzo Life Sciences, NY, USA). PCRs were conducted using SYBR Green PCR  
145 Master Mix (KAPA Biosystems, Woburn, MA, USA) and the primers listed in S2 Table.  
146 Reactions were run in a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) using  
147 the following thermal conditions: stage 1, 50°C for 2 min and 95°C for 10 min; stage 2, 95°C  
148 for 15 s and 60°C for 1 min. stage 2 was repeated for 40 cycles. Relative mRNA levels were  
149 calculated using the comparative CT method and normalized to the level of GAPDH.

150

### 151 **Immunoblot analysis**

152 Cells were lysed with 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM sodium  
153 phosphate, 30 mM NaF, 5 μM zinc chloride, 2 mM iodoacetic acid, and 1% Triton X-100 for  
154 10 min at room temperature on ice for 20 min with regular vortexing. The lysate was  
155 centrifuged at 16,000 × g for 10 min at 4 ° C and the supernatant was collected. The protein



156 concentration in the supernatant was measured using bicinchoninic acid (Pierce, Rockford, IL,  
157 USA). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride  
158 membranes.

159 Membranes were blocked in TBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl)  
160 containing 5% nonfat dry milk for 2 h and incubated with the primary antibodies anti-Muc7  
161 (Enzo Life Sciences, Farmingdale, NY, USA), anti-Muc13 (Thermo-Fisher Scientific,  
162 Waltham, MA, USA), anti-Muc15 (Enzo Life Sciences, Farmingdale, NY, USA), anti-Muc19  
163 (Enzo Life Sciences), anti-p65 (Santa Cruz, Dallas, TX, USA), anti-phospho-p65 (Cell  
164 Signaling, Danvers, MA, USA), anti-I $\kappa$ B $\alpha$  (Cell Signaling), anti-phospho-I $\kappa$ B $\alpha$  (Cell  
165 Signaling), anti-AKT (Cell Signaling), anti-phospho AKT (Cell Signaling), anti-SP1 (Santa  
166 Cruz) or  $\beta$ -actin (Thermo-Fisher Scientific) for 18 h at 4 °C. The immunoblots were washed  
167 and incubated with appropriate secondary antibodies and visualized using SuperSignal™ West  
168 Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) or SuperSignal™ West Femto  
169 Maximum Sensitivity Substrate (Pierce).

170

### 171 **Promoter analysis**

172 SP1 promoter activity was assessed using a luciferase assay system (Promega,  
173 Madison, WI, USA) according to the manufacturer's instructions, and  $\beta$ -galactosidase  
174 expression (pCH110) was used for normalization. A549 cells were transfected with pGL4.14  
175 and the indicated luciferase SP1 promoter construct.

176 A549 cells were transfected with pGL4.43 (luc2P/NF- $\kappa$ B-RE/Hygro) plasmid using  
177 Lipofectamine 2000 transfection reagent (Invitrogen/Thermo-Fisher Scientific, Carlsbad, CA,  
178 USA) according to the manufacturer's protocol. Twenty hours after transfection, the cells were

179 stimulated with PAK for 2 h, harvested, and assessed for luciferase activity using the ONE-  
180 Glo™ luciferase reporter assay system (Promega) according to the manufacturer's instructions.

181

## 182 **Immunohistochemistry**

183 H292 cells were cultured on Permax plastic chamber slides (Nunc, Rochester, NY,  
184 USA) and fixed in methanol at 4°C for 20 min. The slides were washed three times with PBS,  
185 and blocked with 3% (w/v) BSA in PBS for 30 min. Then, the slides were incubated with anti-  
186 NF-κB p65 subunit (rabbit polyclonal IgG, 1:200 dilution, Santa Cruz) antibody at 4°C for 24  
187 h. The slides were washed to remove excess primary antibody and then incubated with anti-  
188 rabbit Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 2 h at  
189 room temperature, washed with PBS, and then mounted using ProLong Gold Antifade reagent  
190 containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 5 min prior to visualization  
191 by confocal laser scanning microscopy (LSM510; Carl Zeiss, Oberkochen, Germany). All  
192 samples were photographed under the same exposure conditions, and nuclei were quantified  
193 from the images obtained.

194

## 195 **Animal infection**

196 Four-week-old, specific pathogen-free female C57BL/6 mice were purchased from the Orient  
197 Co. (Seoul, Korea) and were used after a week of quarantine and acclimatization. The mice  
198 were allowed access to sterilized tap water and standard rodent chow. Bacteria were  
199 centrifuged and resuspended to the appropriate CFU/mL in PBS as determined by optical  
200 density, and plated out in a serial dilution on nutrient broth agar plates. LPS was injected  
201 intranasally by dissolving 5 µg in 50 µl of PBS. Mice were slightly anaesthetized by

202 intraperitoneal injection of pentobarbital (Virbac, Fort Worth, TX, USA). Bacterial solutions  
203 at appropriate concentration ( $2.5 \times 10^6$  CFU per mouse in 50  $\mu$ L PBS) were then administered  
204 by intranasal instillation (25  $\mu$ L per nostril). Control mice were inoculated intranasally with 50  
205  $\mu$ L of PBS. Survival experiments and analysis of bronchoalveolar lavage fluid (BALF) were  
206 performed as previously described [20,21]

207

## 208 **Histology**

209 After BALF samples were obtained, the mice were sacrificed by intraperitoneal  
210 injection of pentobarbital (50 mg/kg; Hanlim Pharm. Co., Seoul, Korea), and lung tissues were  
211 collected and fixed in 10% (v/v) neutral-buffered formalin. The tissues were embedded in  
212 paraffin, sectioned at 4- $\mu$ m thickness, and stained with hematoxylin and eosin solution  
213 (hematoxylin, Sigma MHS-16; eosin, Sigma HT110-1-32). Quantitative analysis of  
214 inflammation and mucus production was performed in at least 4 squares per slide using an  
215 image analyzer (Molecular Devices Inc., Sunnyvale, CA, USA).

216

## 217 **siRNA-mediated knockdown**

218 siRNAs targeting IKK $\alpha$  and IKK $\beta$  were designed and synthesized by Dharmacon  
219 (Lafayette, CO, USA). A549 cells were resuspended in serum-free DMEM, and  $2.5 \times 10^5$   
220 cells/mL were seeded in a 12-well plate and transfected with siRNA-IKK $\alpha$ /siRNA-IKK $\beta$  or  
221 siRNA-negative control (NC), using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher  
222 Scientific, Inc.). After 6 h, the medium was changed with RPMI1640 containing 10% FBS.  
223 Cells were harvested at 48 h after transfection for RT-qPCR or at 72 h for western blot analysis.  
224 Three different IKK $\alpha$ /IKK $\beta$ -specific siRNAs were screened, and the most efficient one was

225 selected for experiments.

226

### 227 **Ethics Statement**

228 All experimental procedures were carried out in accordance with the NIH Guidelines for the  
229 Care and Use of Laboratory Animals and were approved by the Korea Institute of Bioscience  
230 and Biotechnology Animal Care and Use Committee (IACUC KRIBB-AEC-14094).

231

### 232 **Statistical analysis**

233 Data represent the mean  $\pm$  standard error of the mean (SD). Statistical differences  
234 among groups were determined by one-way ANOVA with repeated measures followed by  
235 Newman–Keuls testing in SPSS 14.0 (IBM Software, Armonk, NY, USA).  $P < 0.05$  was  
236 considered statistically significant.

237 **Results**

238 **Muc7, Muc13, Muc15, and Muc19 expression is increased in PAK-infected A549 and**  
239 **H292 cells**

240 *P. aeruginosa* PAK affected the expression of Muc7, Muc13, Muc15, and Muc19 in a  
241 MOI-and time-dependent manner. As the multiplicity of infection (MOI) of PAK increased,  
242 the expression of Muc7, Muc13, Muc15, and Muc19 in A549 cells increased at the mRNA (Fig  
243 1A–D) and the protein (Fig 1I) level. The mRNA expression of all 4 mucins tended to decrease  
244 after 12 h, while at the protein level, expression decreased after 4 h (Fig 1E–H, J).  
245 Immunohistochemistry (IHC) of H292 cells infected with PAK at MOI 20 confirmed the  
246 increase in Muc7, Muc13, Muc15, and Muc19 (Fig 1K). Thus, Muc7, Muc13, Muc15, and  
247 Muc19 in A549 cells increased in a concentration- and time-dependent manner by PAK  
248 infection.

249

250 **Effects of NF- $\kappa$ B signaling inhibitors BAY11-7082 and LY-294002 on Muc7, Muc13,**  
251 **Muc15, and Muc19 expression and NF- $\kappa$ B p65 and AKT phosphorylation in PAK-**  
252 **infected A549 cells**

253 Bay11-7082 is an I $\kappa$ B $\alpha$  inhibitor and LY-294002 is a well-known inhibitor of PI3K  
254 signaling. We previously reported that PAK increased p65 phosphorylation in an epithelial cell  
255 line [22]. In addition, PAK reportedly increased AKT phosphorylation lung cancer cells [23,24].  
256 Therefore, we tested the effects of the above inhibitors on mucin expression in A549 cells  
257 infected by PAK. Bay11-7082 and LY-294002 suppressed the increases in Muc7, Muc13,  
258 Muc15, and Muc19 mRNA expression induced by PAK (Fig 2A–D). The effect of PAK on  
259 Muc7 expression was hardly affected by the inhibitors while Muc13 expression was less

260 affected. Muc15 and Muc19 were more strongly suppressed in the presence of LY-294002 than  
261 in the presence of Bay11-7082. Similar findings were obtained for protein expression by  
262 western blotting (Fig 2E).

263 Phosphorylation of NF- $\kappa$ B and AKT was assessed in PAK-infected A549 cells in the  
264 presence or absence of each inhibitor. In PAK-infected A549 cells, luciferase assay indicated  
265 that transcription factor activities of NF- $\kappa$ B and SP1 increased. In the presence of Bay11-7082  
266 and LY-294002 these PAK-induced transcription factor activities of NF- $\kappa$ B and SP1 were  
267 significantly suppressed as indicated by luciferase analysis and western blot analysis (S1A–D  
268 Fig). In PAK-infected A549 cells, the proinflammatory cytokines IL-8 and IL-6 increased,  
269 while Bay11-7082 and/or LY294002 significantly suppressed this response as indicated by  
270 qRT-PCR and ELISA of IL-6 and IL-8 (S2A–D Fig). Thus, PAK-induced NF- $\kappa$ B and SP1  
271 signal transduction as well as IL-8 and IL-6 of proinflammatory cytokine expression is  
272 suppressed by the use of the inhibitors BAY11-7082 and LY-294002 in A549 cells.

273

274 **ExsA:: $\Omega$  mutant does not induce expression of Muc7, Muc13, Muc15, and Muc19 via NF-**  
275  **$\kappa$ B p65 and AKT phosphorylation in PAK-infected A549 cells**

276 ExsA:: $\Omega$  is a mutant that has no functional T3SS. We compared the induction of  
277 expression of the 4 mucins in A549 cells between PAK and exsA:: $\Omega$ . As expected, mRNA  
278 expression of all 4 mucins was significantly induced by wild-type PAK, while exsA:: $\Omega$  did not  
279 induce their expression as compared to non-infected control cells (Fig 3A–D). Similar findings  
280 were obtained for mucin protein expression (Fig 3A–D). AKT and SP1 phosphorylation was  
281 less strongly induced by exsA:: $\Omega$  than by wild-type PAC as indicated by western blotting (Fig  
282 3E). SP1 and NF- $\kappa$ B transcription factor activities were significantly induced by wild-type

283 PAC but not by *exsA::Ω* as indicated by luciferase assays (Fig 3F, H). Similar to AKT and SP1  
284 phosphorylation, p65 and IκBa phosphorylation was less strongly induced by *exsA::Ω* than by  
285 wild-type PAC as indicated by western blotting (Fig 3I). IHC confirmed the findings for SP1  
286 and p65 (Fig 3G, J). There are multiple reports that PAK affects NF-κB signaling, but there  
287 was no report that PAK phosphorylated p65, IκBa and SP1 to affect Muc7, Muc13, Muc15,  
288 and Muc19 in experiments using A549 cells.. Taken together, these results indicated that a  
289 functional T3SS is required for PAK to exert its effects on SP1/AKT signaling, NF-κB  
290 signaling, and mucin gene expression.

291

292

293 **ExoS and ExoT of T3SS are required for the increase in Muc7, Muc13, Muc15, and**  
294 **Muc19 and inflammatory cytokine expression in A549 cells**

295 Next, we assessed the roles of ExoS and ExoT in the induction of mucin expression  
296 by using a mutant strain defective in these two proteins ( $\Delta$ ST) and complementation strains of  
297  $\Delta$ ST with either of the proteins restored (S, expressing ExoS only, and T, expressing ExoT  
298 only). We observed that ExoS and ExoT are critical for the expression of the 4 mucins gene in  
299 A549 cells as  $\Delta$ ST could not induce Muc7, Muc13, Muc15, and Muc19 expression, while the  
300 induction of mucin expression was partially restored in the complementation strains restored  
301 their expression induction. Real-time PCR and ELISA indicated that IL-6 expression was  
302 restored to approximately 90% and 80% of the PAK-induced level in case of infection with  
303 ExoS and ExoT, respectively, and TNF- $\alpha$  was restored to about 60% and 55% of the PAK-  
304 induced level in case of infection with ExoS and ExoT, respectively (S3A–D Fig). Thus, in  
305 accordance with previous reports, the expression of Muc7, Muc13, Muc15, and Muc19 and

306 proinflammatory cytokines induced by PAK in A549 cells, requires ExoS and ExoT. In cells  
307 treated with ExsA:: $\Omega$ , the increase in IL-6 and TNF- $\alpha$  was suppressed as compared to cells  
308 treated with wild-type PAK.  $\Delta$ ST totally lost the ability to induce IL-6 and TNF- $\alpha$ , while this  
309 ability was restored in the complementation strains expressing either ExoS (S) or ExoT (T)  
310 (S3A–D Fig).

311

### 312 **ExoS and ExoT of T3SS are required for the increase in Muc7, Muc13, Muc15, and** 313 **Muc19 expression via NF- $\kappa$ B p65 and AKT phosphorylation in A549 cells**

314 Next, we investigated whether ExoS and ExoT are related to known SP1/AKT and  
315 NF- $\kappa$ B signals, which are involved in pneumonia and lung diseases, using the mutant strains.  
316 The results showed that S and T induced the activation of the SP1/AKT pathway and AKT and  
317 p65 phosphorylation in A549 cells nearly to the level the wild-type PAK strain did, while  $\Delta$ ST  
318 completely the ability to do so (Fig 5A–D). In particular, it was confirmed that increased SP1,  
319 known as a transcription factor for AKT and increases the phosphorylation of I $\kappa$ B $\alpha$  (Fig. 5A–  
320 D). These results indicated that ExoS and ExoT are important virulence factors of *P.*  
321 *aeruginosa* and are very important for the induction of the inflammatory response in host cells.

322

### 323 **Effect of PAK infection in a pneumonia mouse model and the roles of ExoS and ExoT** 324 **therein**

325 We determined the PAK infection level in a C57BL/6 mouse model. It is known that  
326 PAK infection at specific MOI induces an inflammatory in mice. Accordingly, we tested PAK  
327 infection at different MOIs to select the most appropriate one. Pure LPS was used as a control,  
328 and mice were subjected to infection with PAK alone (PAK) or together with LPS treatment



329 (PAK+LPS). PAK or LPS was diluted with PBS to inject into mice. Experiments were  
330 conducted using 7-8 mice per group. The protein levels of Muc13, Muc15, and Muc19 were  
331 increased in lung tissues of PAK+LPS-infected mice (S4A–C Fig). Hematoxylin and eosin  
332 (H&E) staining of lung tissues revealed that inflammatory cells were increased in PAK-  
333 infected and in PAK+LPS-treated mice. In particular, treatment of PAK+LPS together with  
334 LPS and PAK alone resulted in a much greater increase in inflammatory cells. (S5 Fig).

335 When we tested the  $\Delta$ ST, S, and T strains, Muc7, Muc13, Muc15, and Muc19 mRNA  
336 and protein expression were significantly increased by S and T, but not  $\Delta$ ST, as indicated by  
337 qPCR and western blotting (Fig 6A–E). Similar findings were achieved for proinflammatory  
338 cytokine expression (data not shown). Thus, mucin gene expression in the mouse lungs in  
339 response to *P. aeruginosa* infection is controlled by ExoS and ExoT.

340

#### 341 **Roles of ExoS and ExoT in AKT activation in mice**

342 Proinflammatory cytokine production was increased through the T3SS of PAK, and  
343 this involves AKT activation in pneumonia mouse models. To confirm the role of SP1 in S-  
344 and T-infected pneumonia model mice, we evaluated the level of AKT phosphorylation by  
345 western blot analysis using antibodies to SP1, AKT, and pAKT. Non-treated as well as  $\Delta$ ST-  
346 treated cells displayed weak AKT phosphorylation, whereas S- and T-treated monolayers  
347 showed a significant increase in SP1 translocation, which was detectable as of 30 min and was  
348 sustained for 1 h (Fig 7A).

349

#### 350 **IKK- $\alpha$ and IKK- $\beta$ are necessary for NF- $\kappa$ B phosphorylation in response to S and T** 351 **infection**

352           The I $\kappa$ B kinase enzyme complex is part of the upstream NF- $\kappa$ B signal transduction  
353 cascade. The I $\kappa$ B kinase (IKK) is an enzyme complex that is involved in propagating the  
354 cellular response to inflammation [25]. Small interfering (si)RNA is one of the experimental  
355 tools for functional analyses. We used siRNAs to knock out IKK $\alpha$  and IKK $\beta$  expression. The  
356 presence of control siRNA (siNC) did not affect the increase in muc7, Muc13, Muc15, and  
357 Muc19 expression induced in A549 cells by PAK infection. However, knockout of IKK $\alpha$  and  
358 IKK $\beta$  suppressed the increases in muc7, Muc13, Muc15, and Muc19 by 50% (S6A–D Fig ).  
359 ELISA of proinflammatory cytokines IL-6 and IL-8 revealed similar effects as those observed  
360 for the mucins (S7A, B Fig).

361           Next, we conducted experiments using the strains S and T. A549 cells infected with  
362 PAK, ExoS, or ExoT after siNC transfection induced increased muc7, Muc13, Muc15, and  
363 Muc19 expression as compared to the negative control. However, in A549 cells infected with  
364 PAK, ExoS, or ExoT after knockout of IKK $\alpha$  and IKK $\beta$ , the increases in Muc7, Muc13, Muc15,  
365 and Muc19 were suppressed by 40–60% (Fig 8A–D).

366 **Discussion**

367 T3SS gene expression is induced by contact with eukaryotic cells or under specific  
368 environmental conditions [26]. ExoS and ExoT are toxins with adenosine diphosphate  
369 ribosyltransferase and Rho guanosine triphosphatase activities [27]. They are similar in  
370 structure, but the toxicity of ExoT is less potent than that of ExoS [28,29]. When activated,  
371 these proteins destroy the actin filaments that make up the cytoskeleton of the host, thus  
372 inhibiting phagocytosis and finally killing the host cell [30]. We found that S and T  
373 complementation strains were extremely efficient in lysing eukaryotic cells, and this  
374 observation corresponded with their highly toxic phenotypes in a pneumonia mouse model. S  
375 and T were found to be as damaging as the T3SS-proficient PAK strain in mice. Histological  
376 analysis *in vivo* showed that S- and T-infected lungs had damage lesions.

377 *P. aeruginosa* ExoS and ExoT were suggested to be associated with lung injury and  
378 mucus accumulation in mice [31,32], but these types of virulence mechanisms have never been  
379 investigated at the molecular level. The way in which these major virulence determinants of *P.*  
380 *aeruginosa* function to cause severe disease during infection was not clear until date. Therefore,  
381 the effects of NF- $\kappa$ B and SP1/AKT activities on the expression of Muc7, Muc13, Muc15, and  
382 Muc19 in S- and T-infected pneumonia model mice were investigated. Expression and delivery  
383 of Muc7, Muc13, Muc15, and Muc19 were found to be improved by S and T infection.  
384 Examination of infected A549 cells revealed that the expression of Muc7, Muc13, Muc15, and  
385 Muc19 is dependent on NF- $\kappa$ Bp65 and SP1/AKT phosphorylation. In particular,  
386 overexpression of the inflammatory cytokines (IL-6, IL-1 $\beta$ , and TNF- $\alpha$ ) in response to  
387 activation of ExoS- and ExoT-induced NF- $\kappa$ B and AKT was associated with the pathogenesis  
388 mechanism in the pneumonia mouse model. In addition, proinflammatory cytokine expression

389 as well as expression of Muc7, Muc13, Muc15, and Muc19 was increased upon infection with  
390 strains S and T. Our results suggest that ExoS and ExoT play important roles in the genetic  
391 etiology associated with acute and chronic infections.

392 *P. aeruginosa* is known to have an important influence on the activation of NF- $\kappa$ B and  
393 SP1 in pulmonary diseases and to regulate the production of cytokines, matrix  
394 metalloproteinases (MMPs), and mucins [4,33]. Muc7, Muc13, Muc15, and Muc19 gene and  
395 protein expression is facilitated by several cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , secreted by  
396 macrophages [8,34]. It has been reported that IL-6, IL-8, and TNF- $\alpha$  secreted by lung  
397 lymphocytes specifically induce the secretion of Muc7, Muc13, Muc15, and Muc19 to promote  
398 destruction of lung tissue [35,36]. Muc7, Muc13, Muc15, and Muc19, IL-1 $\beta$ , and TNF- $\alpha$   
399 expression levels in response to infection were significantly reduced in A549 cells transformed  
400 with siRNA targeting IKK- $\alpha$  and IKK- $\beta$ . This shows that Muc7, Muc13, Muc15, and Muc19  
401 are required for the expression of proinflammatory cytokines. Since mucin expression is known  
402 to increase respiratory irritation in pulmonary infections, it further implies cytokine-induced  
403 pathogenicity, as observed in the lung biopsy specimens.

404 The results generated in the current study increase our understanding of the  
405 pathogenesis of pneumonia by demonstrating that the T3SS, and specifically, ExoS and ExoT,  
406 disrupt the host response in the lungs. Most importantly, ExoS and ExoT of T3SS induce the  
407 activation of NF- $\kappa$ B and AKT, and are able to induce the expression of Muc7, Muc13, Muc15,  
408 and Muc19 in the lungs. Although these 4 mucins have not been studied thoroughly yet, they  
409 are considered important inflammatory mediators in inflammation of the respiratory tract.  
410 Treatment of chronic respiratory inflammation is limited to vaccination against commonly  
411 occurring respiratory pathogens, pharmacological bronchodilation, or respiratory infection

412 antibiotics to alleviate dyspnea. Our research results provide a basis for future therapies to  
413 prevent and confound lung immunopathology through increasing our understanding of the  
414 molecular mechanism of pneumonia.

415

#### 416 **Acknowledgments**

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420 (KGM 1221814) of the Republic of Korea.

421

422 **Figure Captions**

423 **Fig 1. Muc7, Muc13, Muc15, and Muc19 expression in PAK-infected A549 and H292 cells.**

424 (A–D) PAK MOI- and (E–H) time-dependent increases in Muc7, Muc13, Muc15, and Muc19  
425 mRNA expression as measured by qRT-PCR. Data are presented as the mean  $\pm$  SD. “–,” A549  
426 cells treated with PBS; PAK, A549 cells infected with *P. aeruginosa*. #Significantly different  
427 from the normal control group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. negative control (PBS)  
428 Western blots showing PAK MOI- (I) and time-dependent (J) regulation of Muc7, Muc13,  
429 Muc15, and Muc19 protein expression. (K) Intracellular staining of Muc7, Muc13, Muc15, and  
430 Muc19 in PAK-infected H292 cells (magnification, 400 $\times$ ). Nuclei were stained with DAPI.

431

432

433 **Fig 2. Inhibitory effects of BAY-11-7082 and LY-294002 on the expression of Muc7,**

434 **Muc13, Muc15, and Muc19 in PAK-infected A549 cells.** A549 cells were pretreated with  
435 Bay11-7082 or LY-294002 and then infected with PAK (MOI 20) for 4 h. (A–D) Muc7, Muc13,  
436 Muc15, and Muc19 mRNA expression as assessed by qRT-PCR. (E) Western blot of Muc7,  
437 Muc13, Muc15, and Muc19 protein expression and quantitative data. Western blot bands were  
438 quantified using ImageJ software. The data are presented as the mean  $\pm$  SD. “–,” A549 cells  
439 treated with PBS; PAK, A549 cells infected with *P. aeruginosa*; Bay, Bay11-7082; LY, LY-  
440 294002; #Significantly different from the normal control group, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P$   
441  $< 0.001$ , significantly different from the respective controls.

442

443 **Fig 3. ExsA:: $\Omega$  affects Muc7, Muc13, Muc15, and Muc19 expression in A549 and H292**

444 **cells.** The expression levels of Muc7 (A), Muc13 (B), Muc15 (C), and Muc19 (D) mRNA and

445 protein were determined by qRT-PCR and western blotting, respectively. A549 cells were  
446 treated with PAK and ExsA:: $\Omega$  for 1 h, followed by 2 washes for 15 min and further treatment  
447 for 4 h. PAK exposure increases the phosphorylation of AKT (E, F) and p65 (H, I) compared  
448 with the vehicle control group in A549 cells. Transcription of SP1 (G) and NF- $\kappa$ B (p65) (J)  
449 into the nucleus were confirmed by immunocytochemistry (ICC) in H292 cells. The data are  
450 presented as the mean  $\pm$  SD. “–,” A549 cells treated with PBS; PAK, A549 cells infected with  
451 *P. aeruginosa*; ExsA:: $\Omega$ , A549 cells infected with ExsA:: $\Omega$  (no T3SS). #Significantly different  
452 from the non-infected control cells, \* $P < 0.05$ , significantly different from PAK-only-infected  
453 cells.

454

455 **Fig 4. ExoS and ExoT are required and each sufficient to induce Muc7, Muc13, Muc15,**  
456 **and Muc19 expression in A549 and H292 cells.** (A–D) Muc7, Muc13, Muc15, and Muc19  
457 mRNA and protein expression as determined by qRT-PCR and western blotting, respectively.  
458 (E–F) The cellular localization of Muc7, Muc13, Muc15, and Muc19 as determined by  
459 immunocytochemistry in H292 cells. “–,” control A549 and H292 cells treated with PBS only;  
460 PAK, A549 or H292 cells infected with *P. aeruginosa*; ExsA:: $\Omega$ , A549 or H292 cells infected  
461 ExsA:: $\Omega$  (no T3SS);  $\Delta$ ST, PAK- $\Delta$ ST mutant; Mock, PAK $\Delta$ STmt-pUCP18; S, PAK $\Delta$ STmt-  
462 pUCP18-PAKexoS; T, PAK $\Delta$ STmt-pUCP18-PAKexoT (MOI = 200) #Significantly different  
463 from the non-infected control cells, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly different  
464 from PAK-only-infected cells.

465

466 **Fig 5. ExoS and ExoT are required and each sufficient to increase NF- $\kappa$ B and AKT**  
467 **signaling in A549 cells.** A549 cells were infected with the indicated strains for 1 h. (A) AKT

468 and p-AKT expression as detected by western blotting. Quantification of p-AKT and SP1  
469 expression using RAS-4000. (B) A549 cells were transfected with SP1 luciferase (Luc) reporter  
470 plasmid (0.1  $\mu$ g). (C) NF- $\kappa$ B expression as detected by western blotting. Quantification of p-  
471 p65 and p-I $\kappa$ B- $\alpha$  expression using RAS-4000.  $\beta$ -actin was used as the internal control. (D)  
472 A549 cells were transfected with expression NF- $\kappa$ B luciferase (Luc) reporter plasmid (0.1  $\mu$ g).  
473 At 24 h after transfection, A549 cells were treated with the strains at MOI = 20 for 1 h, and  
474 then, luciferase activity was measured. The data were normalized to  $\beta$ -galactosidase activity.  
475 All data are representative of 3 independent experiments. Luciferase activities were measured  
476 24 h after the transfection. “-,” control A549 cells treated with PBS only; PAK, A549 cells  
477 infected with *P. aeruginosa*; ExsA:: $\Omega$ , A549 cells infected with ExsA:: $\Omega$  (no T3SS);  $\Delta$ ST,  
478 PAK- $\Delta$ ST mutant; Mock, PAK $\Delta$ STmt-pUCP18; S, PAK $\Delta$ STmt-pUCP18PAKexoS; T,  
479 PAK $\Delta$ STmt-pUCP18PAKexoT (MOI = 20).

480

481 **Fig 6. ExoS and ExoT are required to induce Muc7, Muc13, Muc15, and Muc19**  
482 **expression in lung tissue.** (A–D) Muc7, Muc13, Muc15, and Muc19 mRNA levels as  
483 determined by qRT-PCR. (E) Western blot showing Muc7, Muc13, Muc15, and Muc19 protein  
484 expression and quantitative data. NC, control mice treated with PBS only; PAK, mice infected  
485 with *P. aeruginosa*; ExsA:: $\Omega$ , mice cells infected ExsA:: $\Omega$  (no T3SS);  $\Delta$ ST, PAK- $\Delta$ ST mutant;  
486 Mock, PAK $\Delta$ STmt-pUCP18; S, PAK $\Delta$ STmt-pUCP18PAKexoS; T, PAK $\Delta$ STmt-  
487 pUCP18PAKexoT (MOI  $2.5 \times 10^6$ ) #Significantly different from the non-infected control cells,  
488 \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly different from PAK-only-infected cells.

489

490 **Fig 7. ExoS and ExoT are required and each sufficient to increase AKT/SP1 signaling in**



491 **lung tissue.** Western blot showing pAKT, AKT, and SP1 protein expression and quantitative  
492 data. NC, control mice treated with PBS only; PAK; ExsA:: $\Omega$  (no T3SS),  $\Delta$ ST, S, and T (MOI  
493  $2.5 \times 10^6$ ). #Significantly different from the non-infected control cells, \* $P < 0.05$ , \*\* $P < 0.01$ ,  
494 significantly different from PAK-only-infected cells.

495

496

497 **Fig 8. Muc7, Muc13, Muc15, Muc19, and proinflammatory cytokine expression require**  
498 **the NF- $\kappa$ B pathway activated via IKK- $\alpha$  and IKK- $\beta$  in A549 cells.** (A–D) qRT-PCR  
499 analyses showing Muc7, Muc13, Muc15, and Muc19 mRNA expression upon the addition of  
500 siRNAs targeting IKK- $\alpha$  and IKK- $\beta$ . (E) Western blot showing Muc7, Muc13, Muc15, and  
501 Muc19 protein expression upon the addition of siRNAs targeting IKK- $\alpha$  and IKK- $\beta$  as  
502 determined by western blotting and quantitative data. (F–H) Cytokine levels as determined by  
503 ELISA. Data are the mean  $\pm$  SEM of 3 different experiments performed in duplicate.

504

505

506

507 **Supporting information**

508 **S1 Fig. Effects of PAK exposure on the phosphorylation of NF- $\kappa$ B and SP1/AKT in A549 cells.**

509 **S2 Fig. Effects of inhibitors on the expression of proinflammatory cytokines in PAK infected A549**  
510 **cells.**

511 **S3 Fig. Effects of ExoS and ExoT exposure of proinflammatory cytokines in PAK infected A549**  
512 **cells.**

513 **S4 Fig. Effects of PAK and LPS exposure of the Muc13, Muc15, and Muc19 in mice.**

514 **S5 Fig. Histopathology of mouse lungs 20 h after inoculation with *P. aeruginosa* and LPS.**

515 **S6 Fig. Muc7, Muc13, Muc15, and Muc19 in A549 after siRNA-mediated downregulation of IKK-**  
516  **$\alpha$  and IKK- $\beta$ .**

517 **S7 Fig. Proinflammatory cytokine in A549 after siRNA-mediated downregulation of IKK- $\alpha$  and**  
518 **IKK- $\beta$ .**

519

520 **Reference**

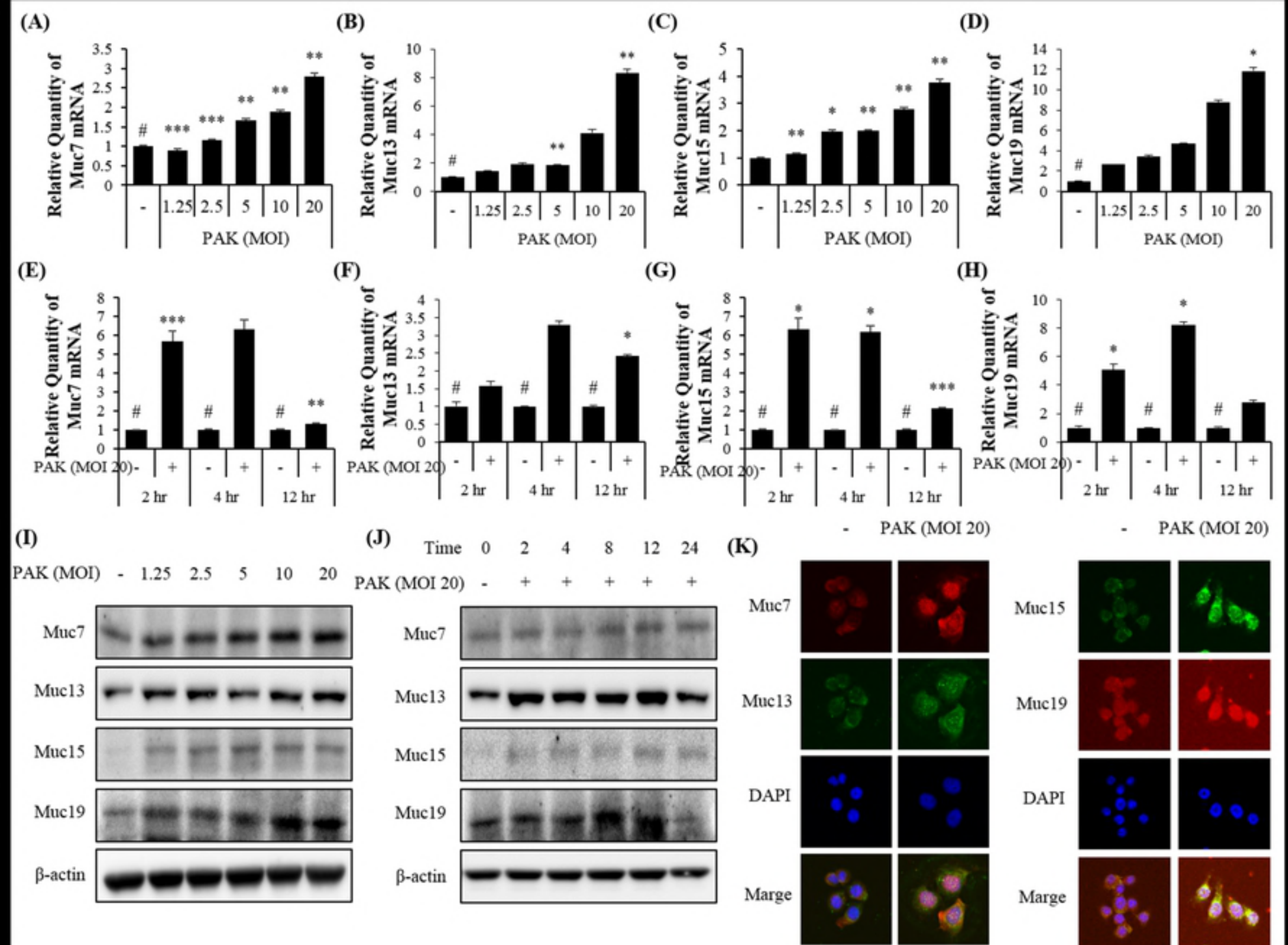
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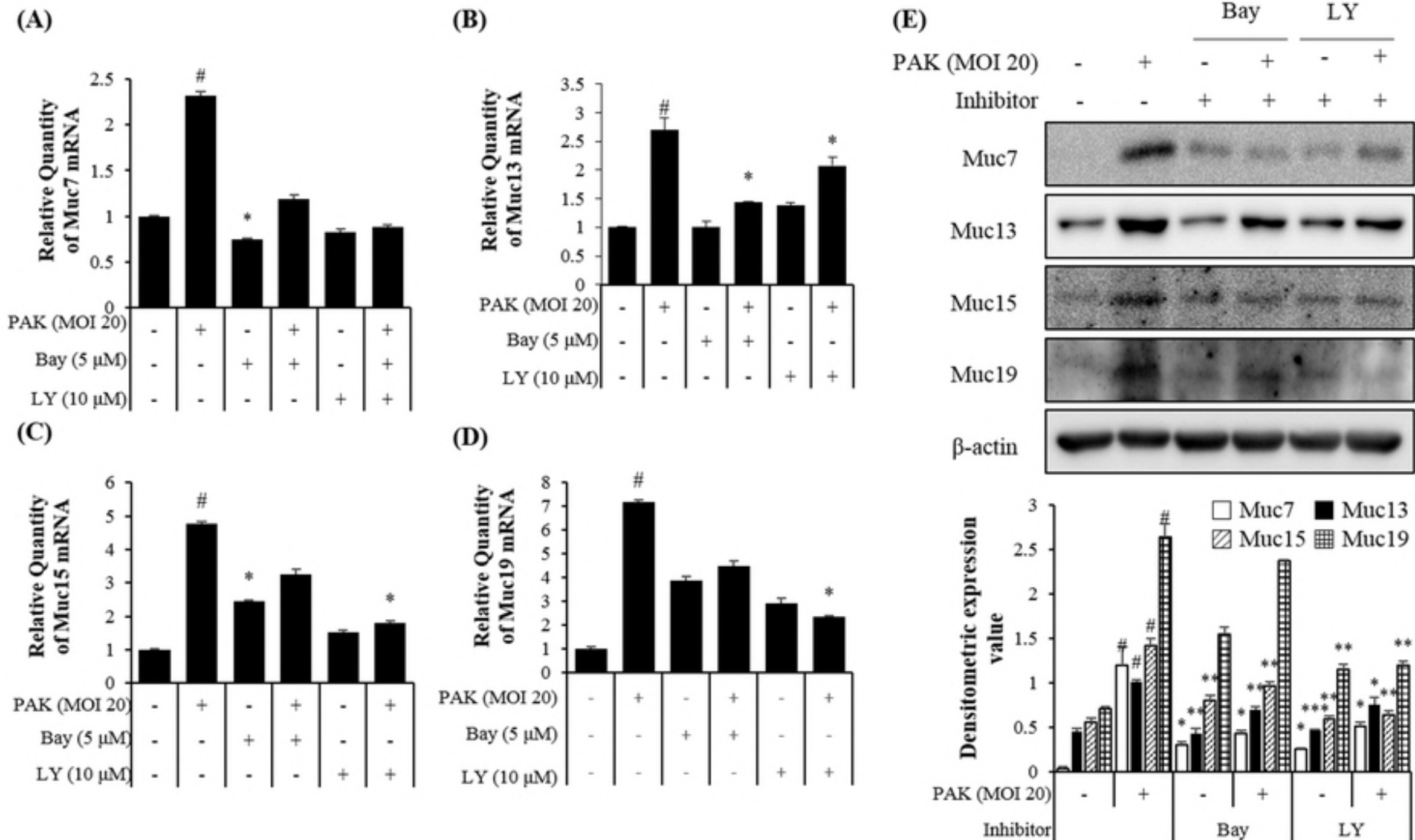
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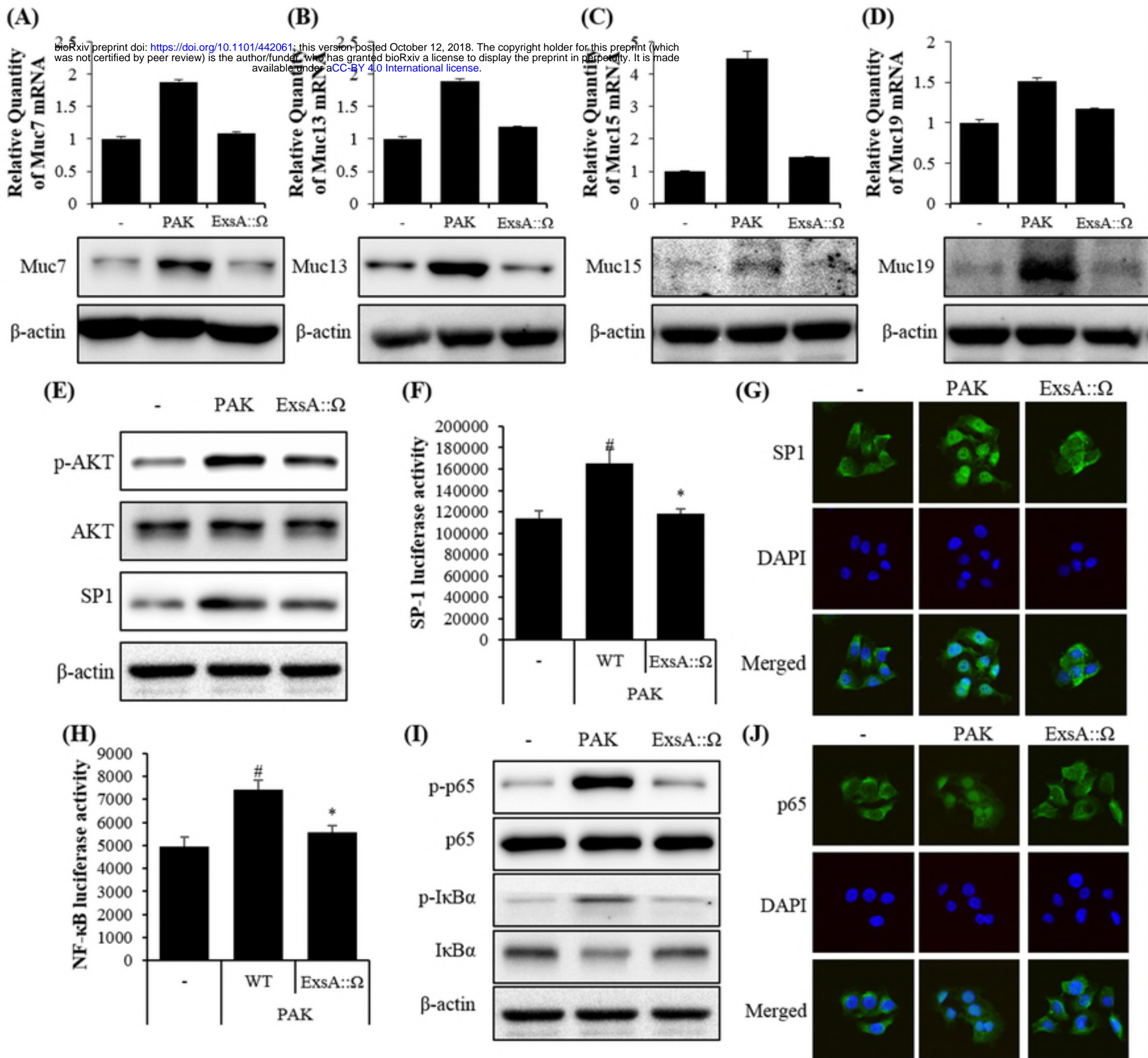


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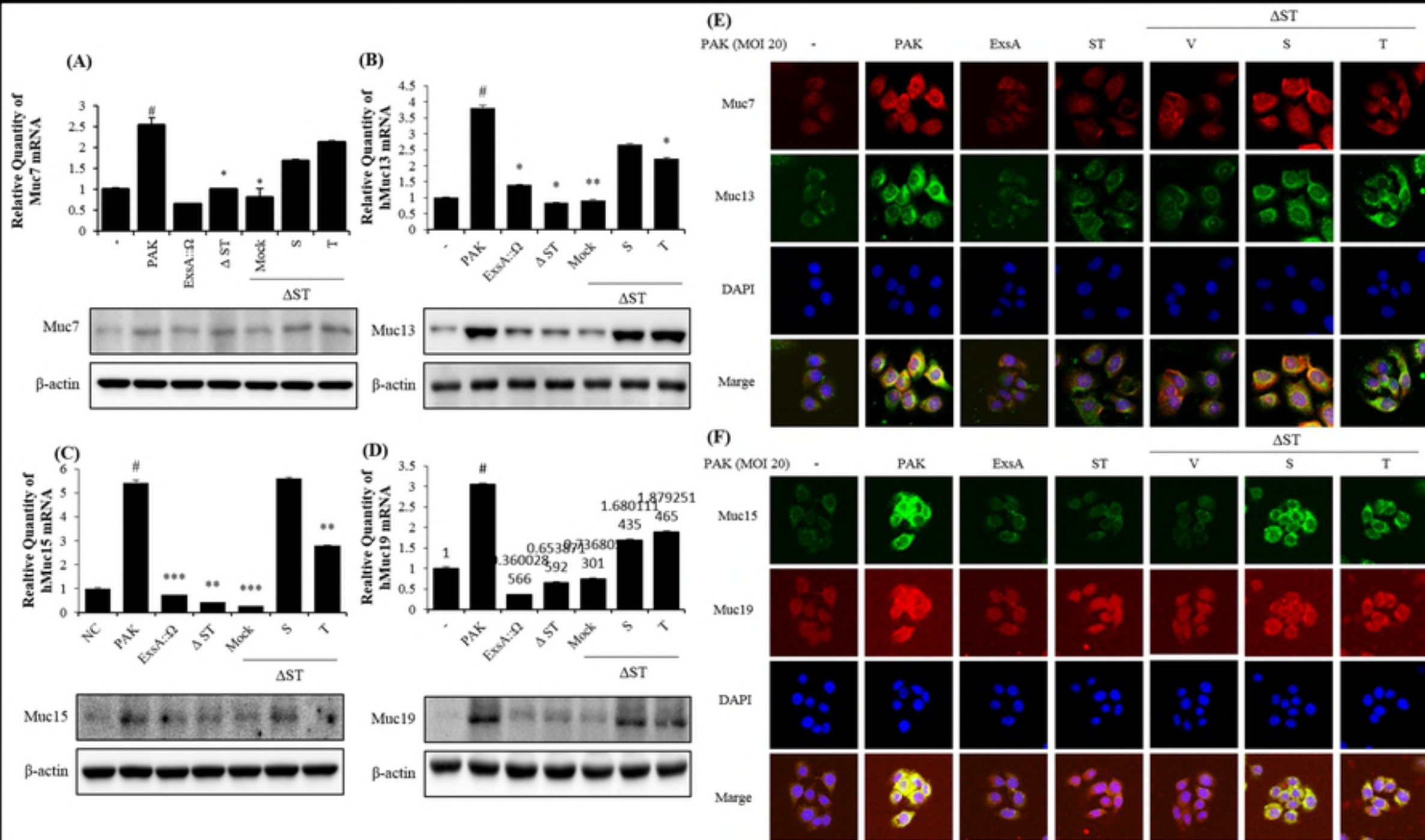




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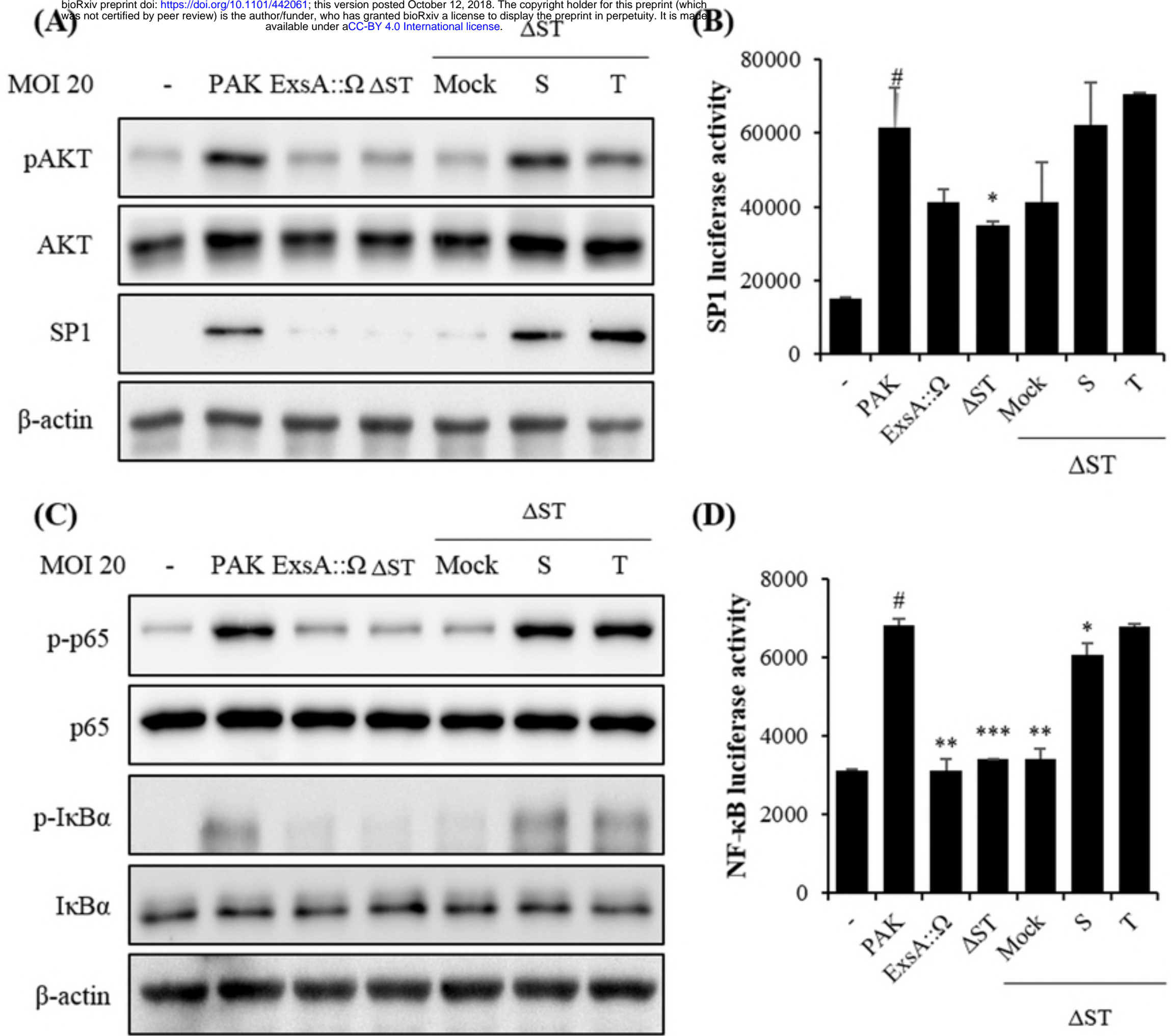


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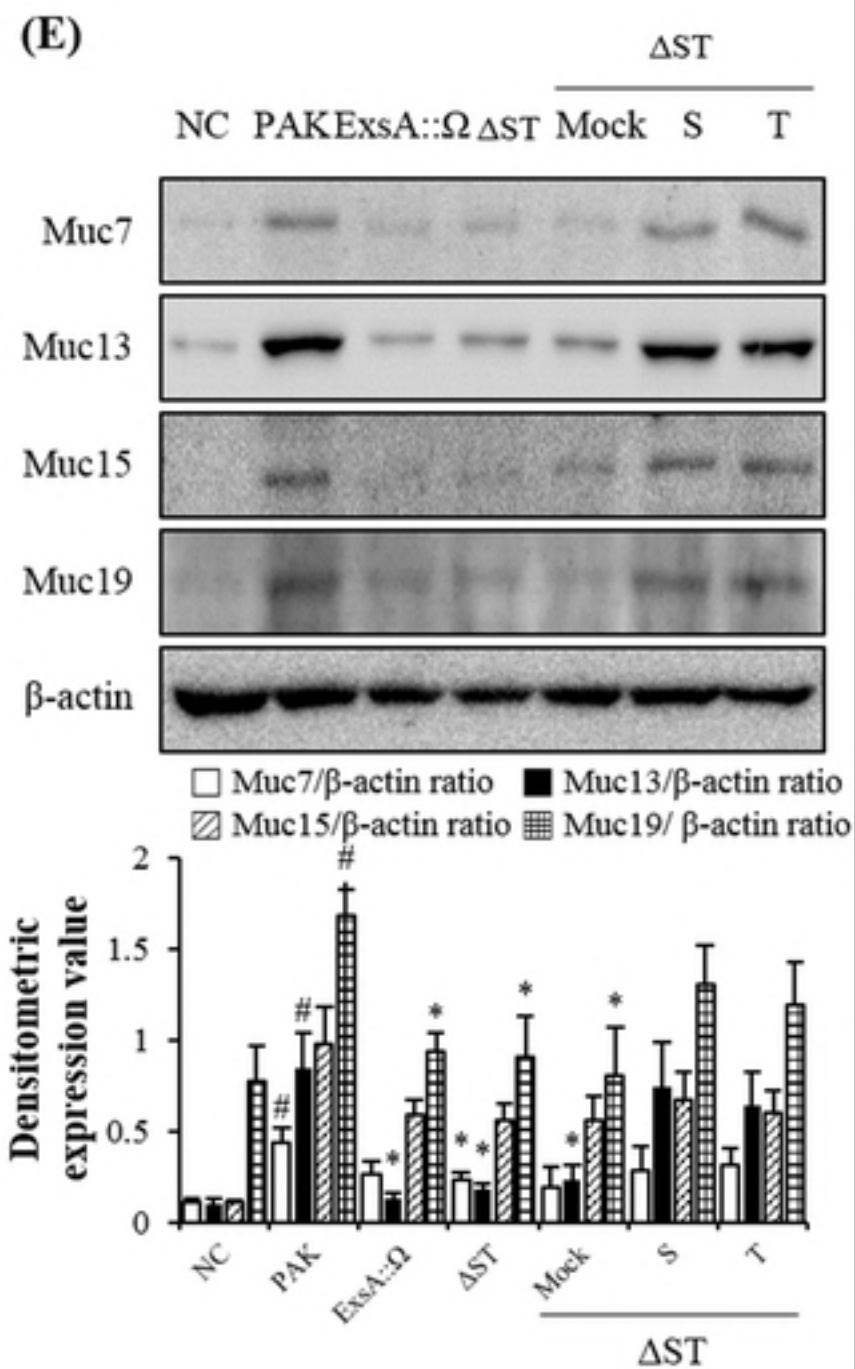
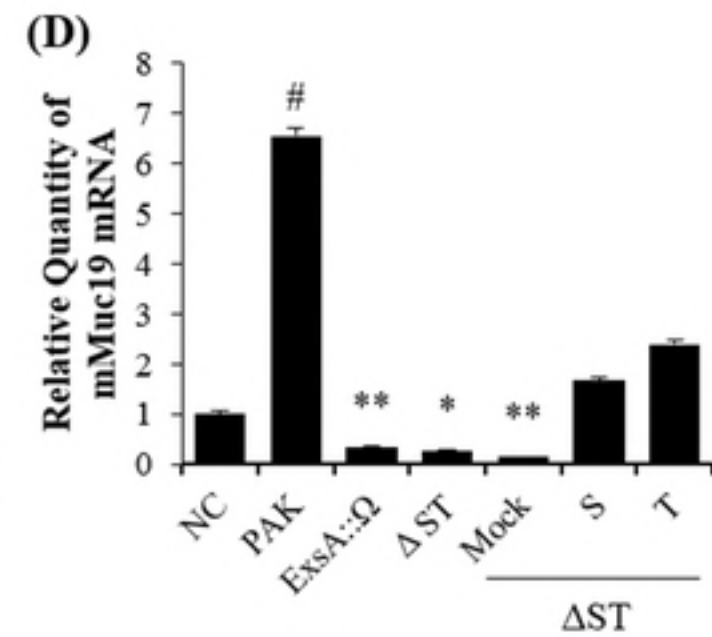
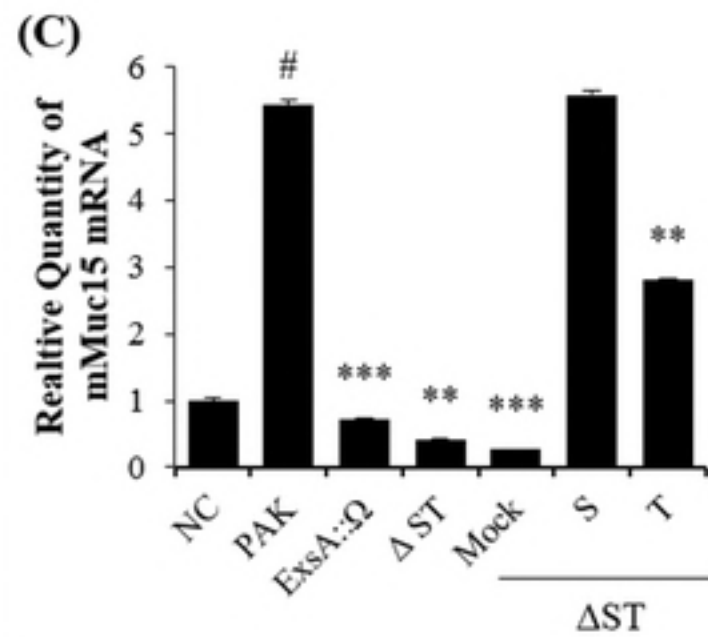
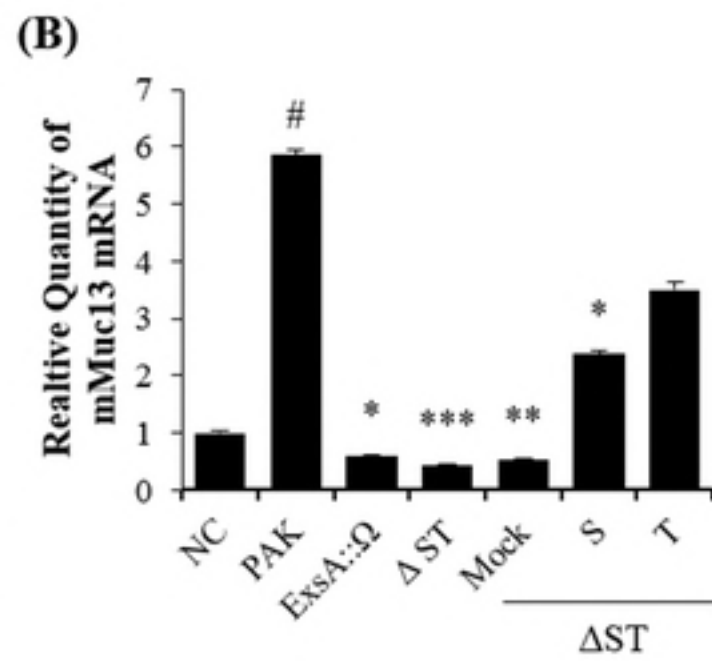
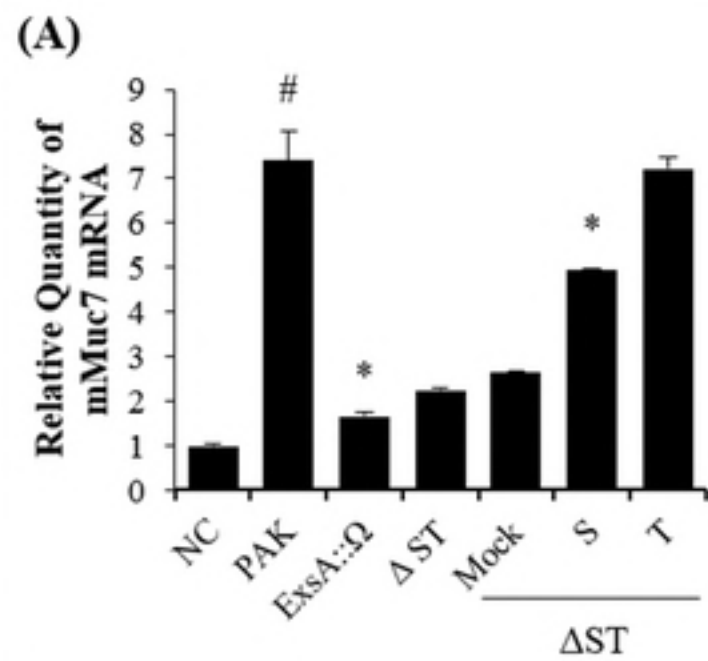


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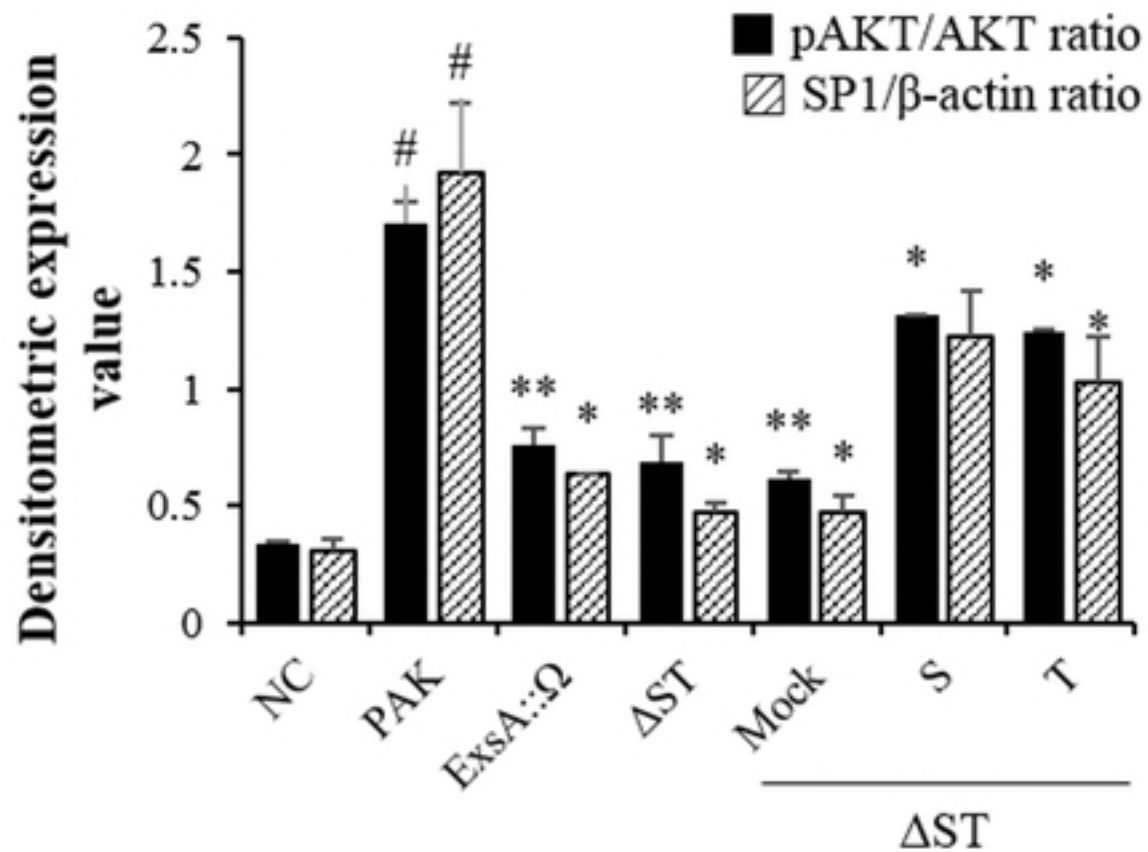
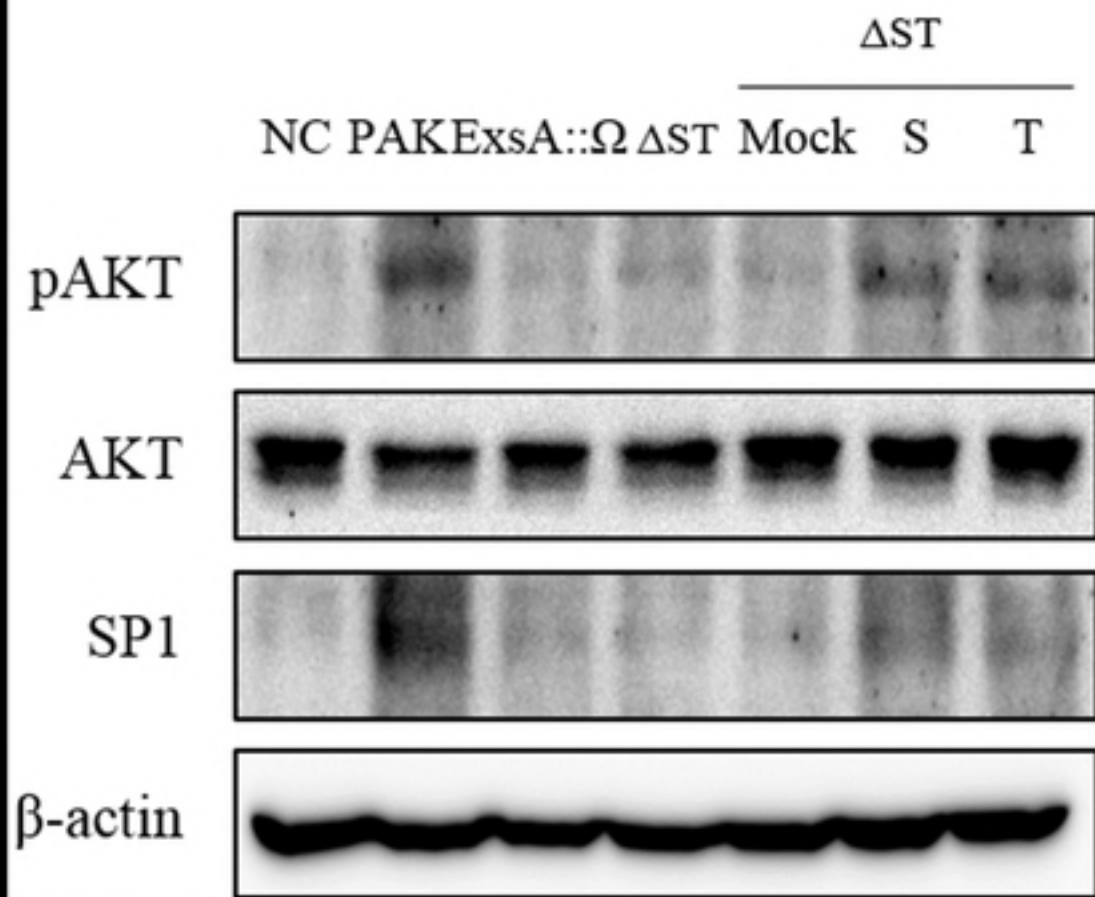




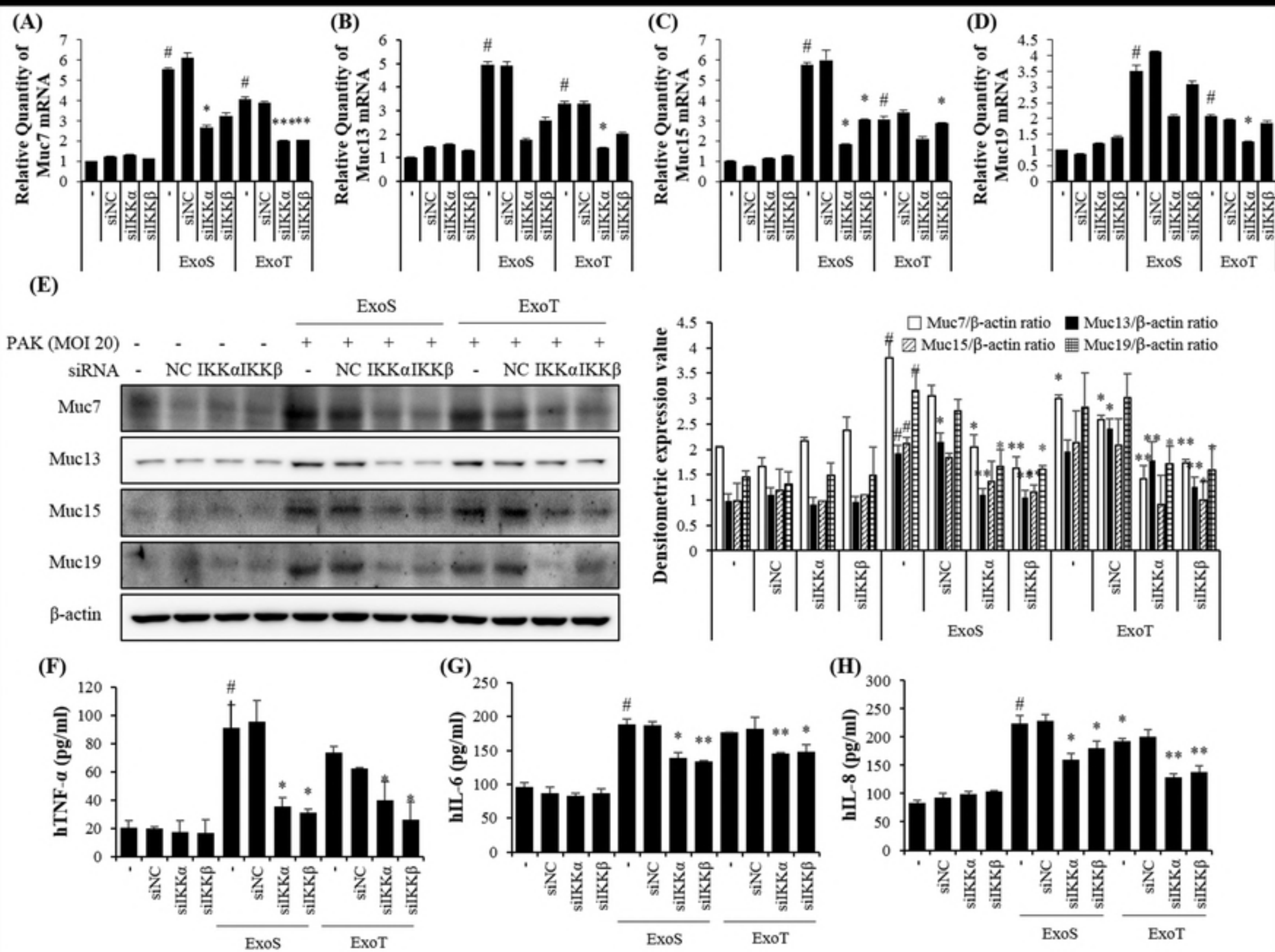
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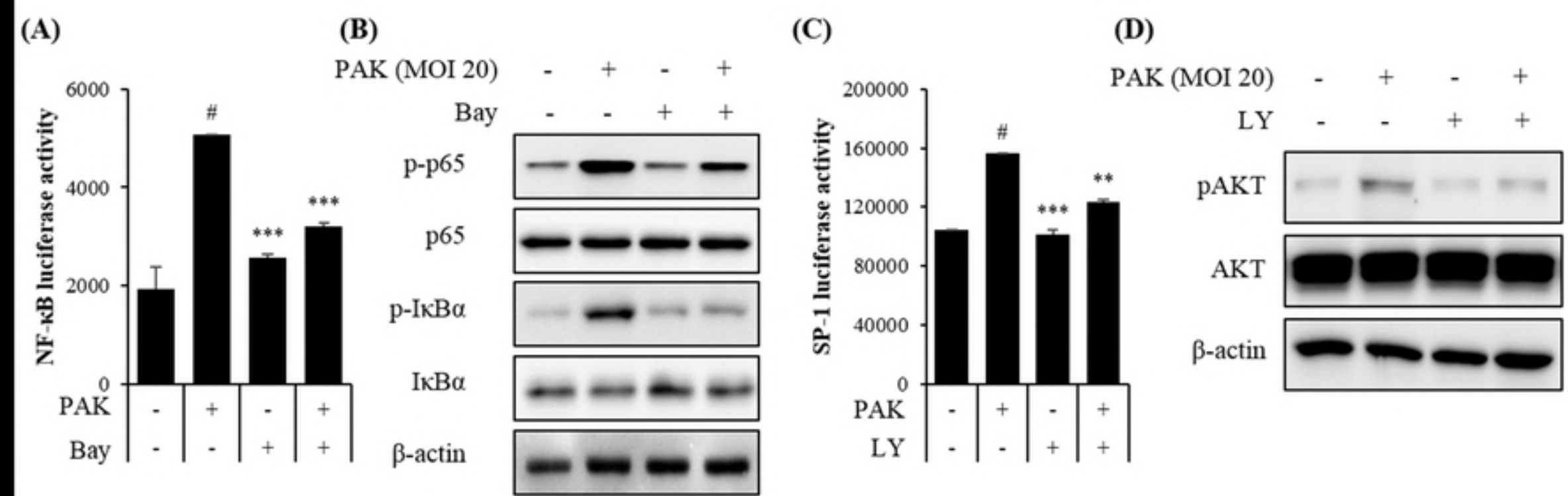
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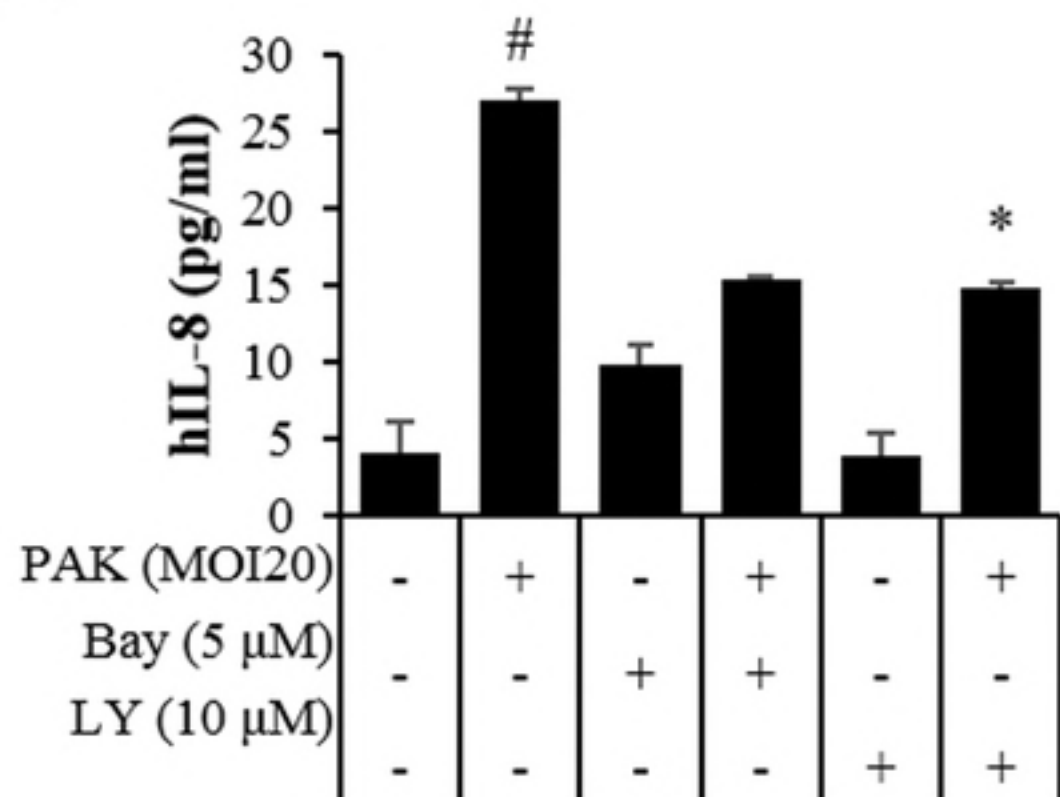
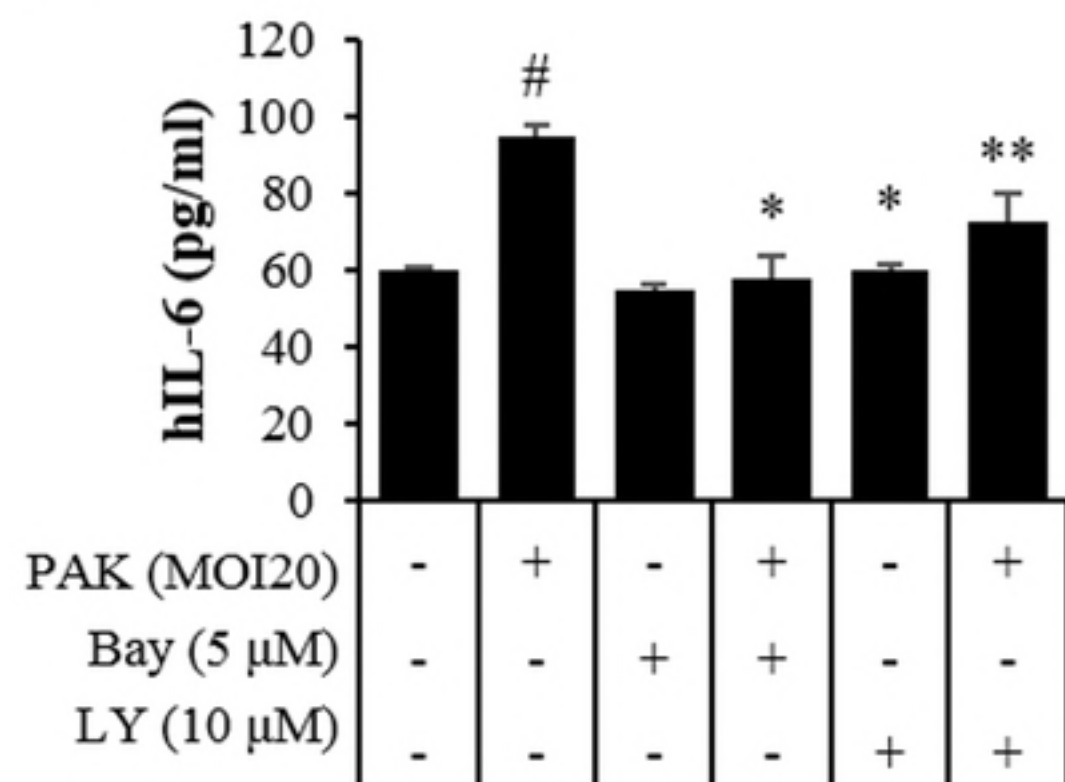
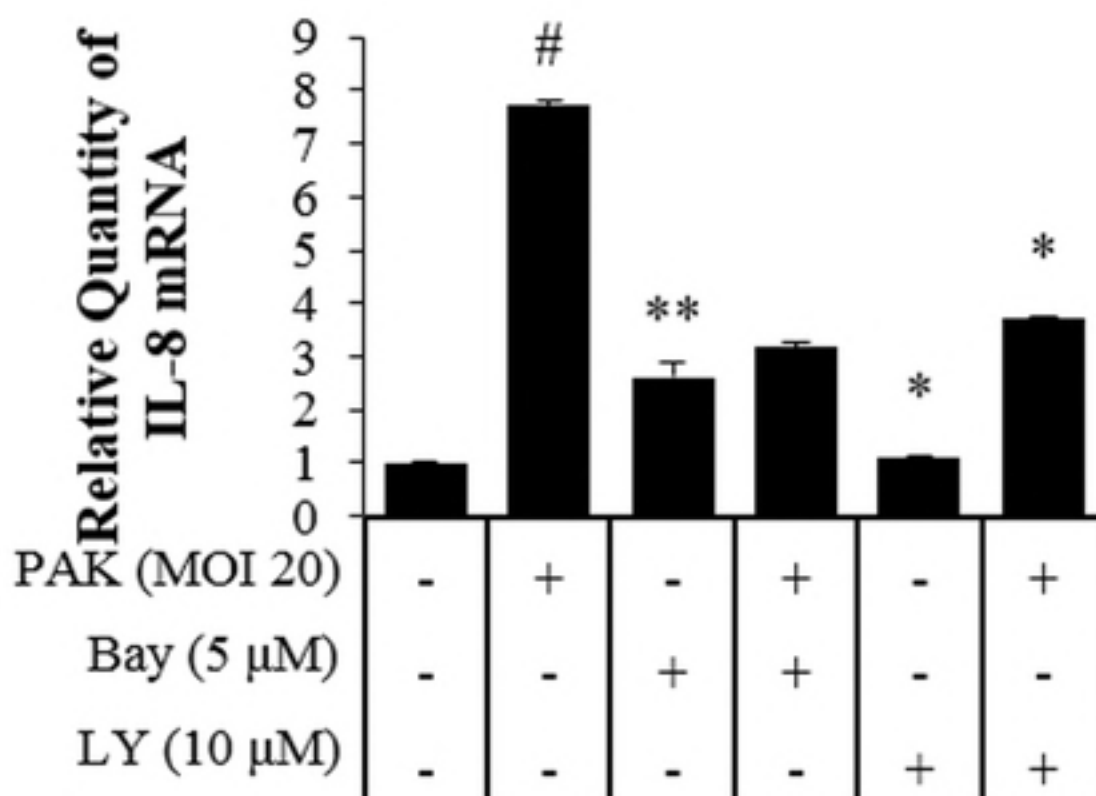
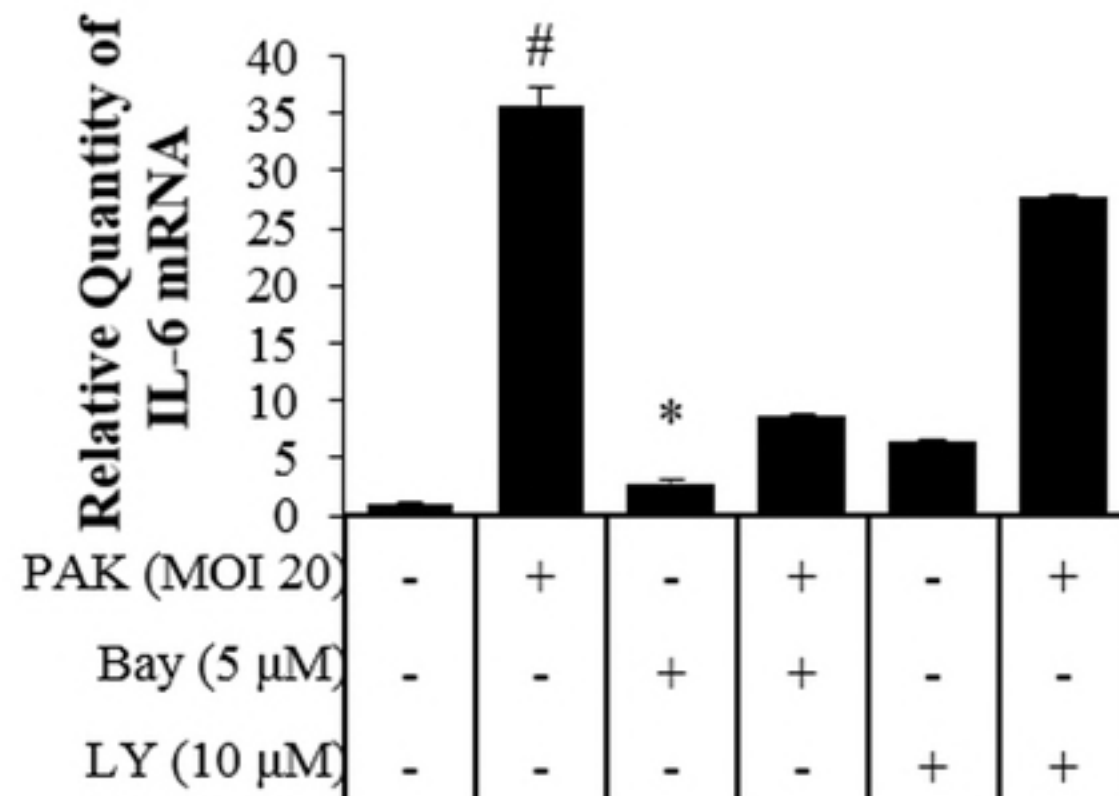


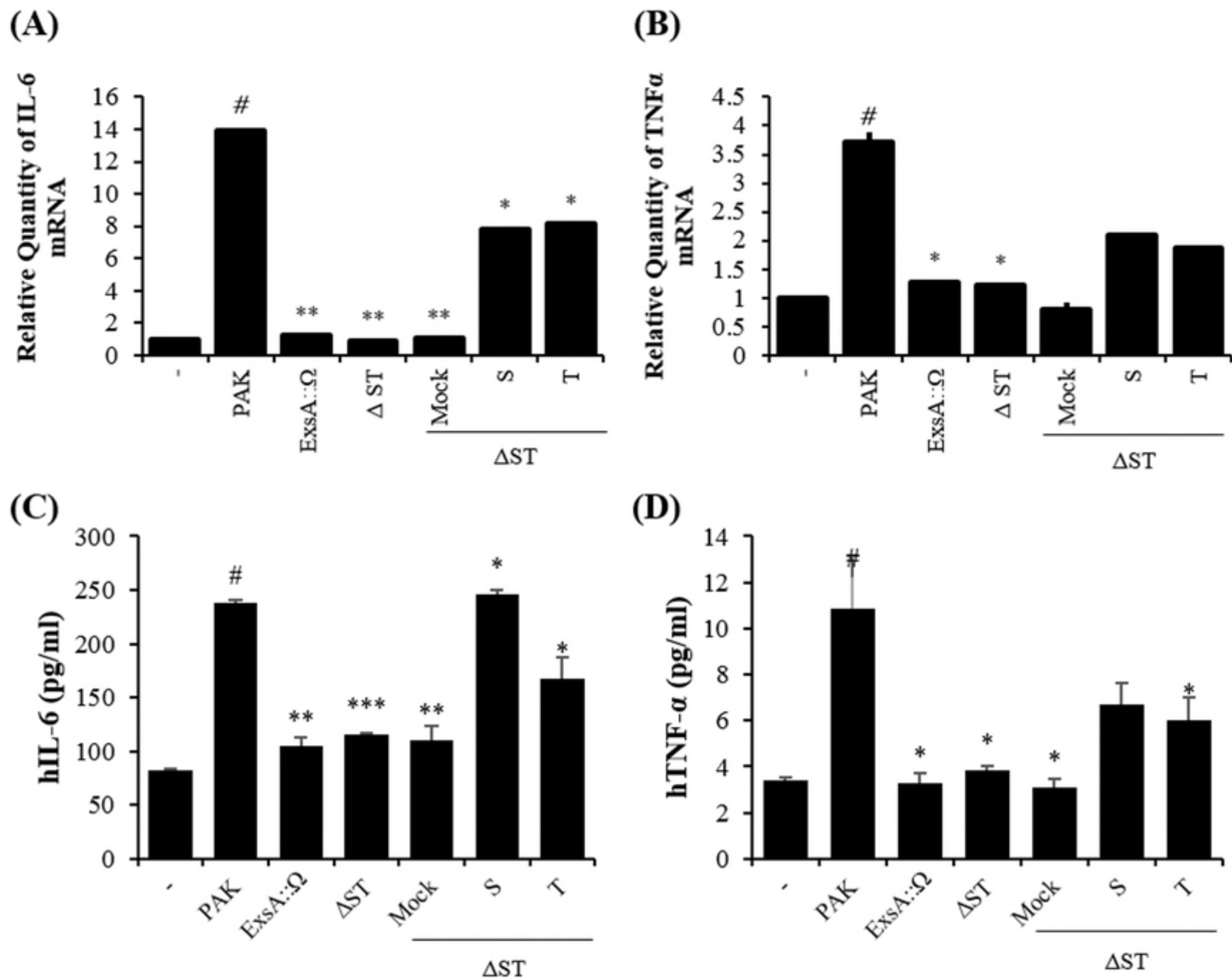
Figure



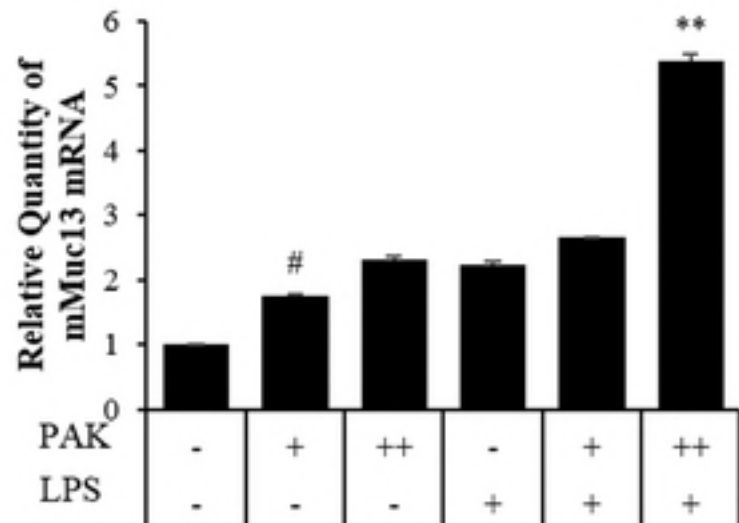
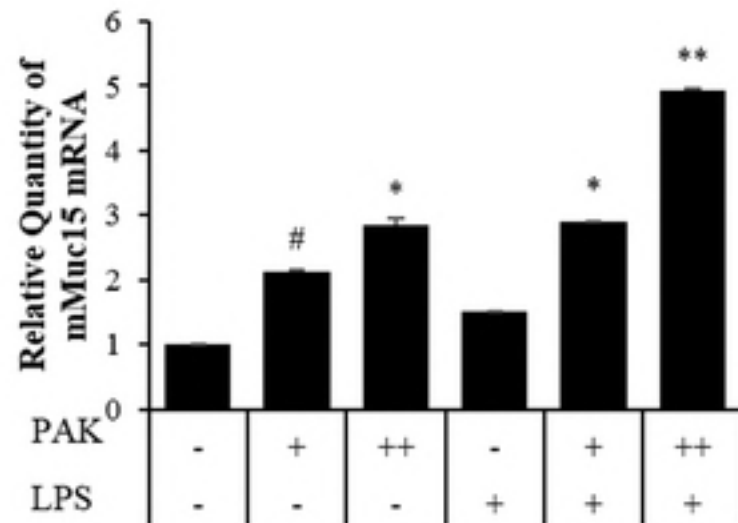
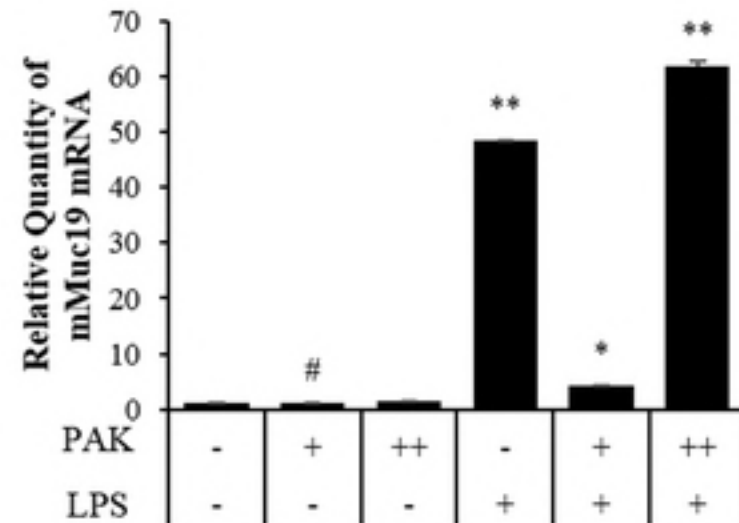
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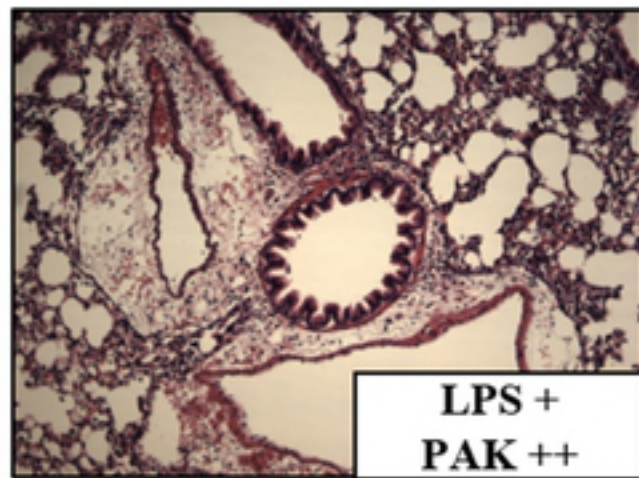
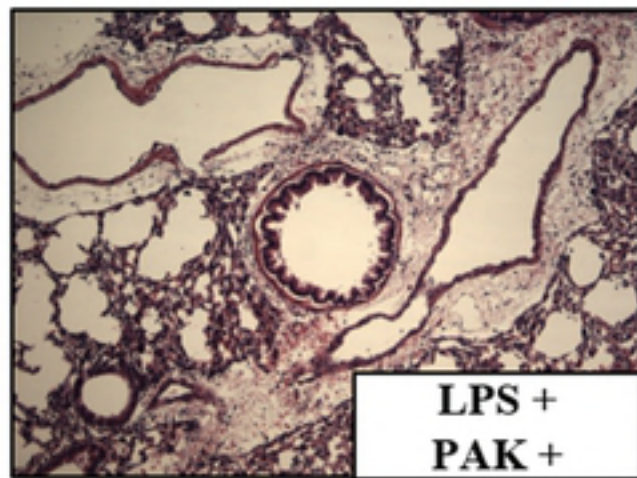
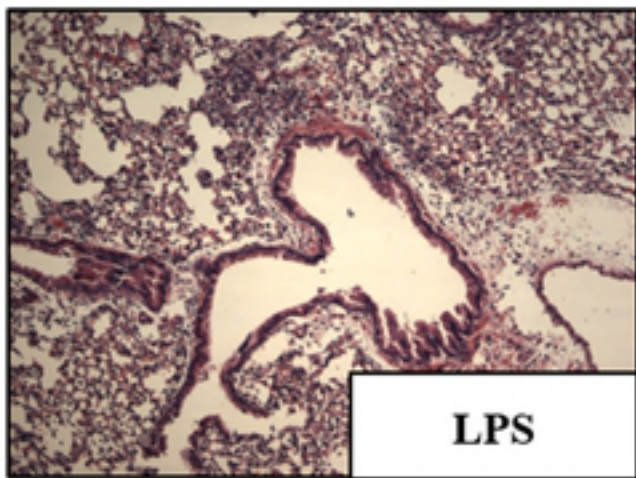
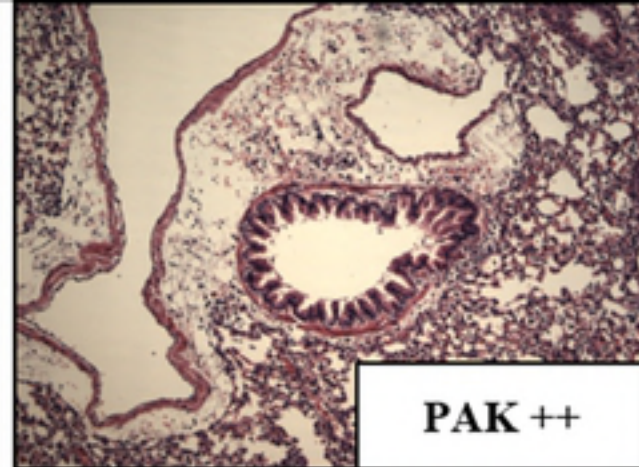
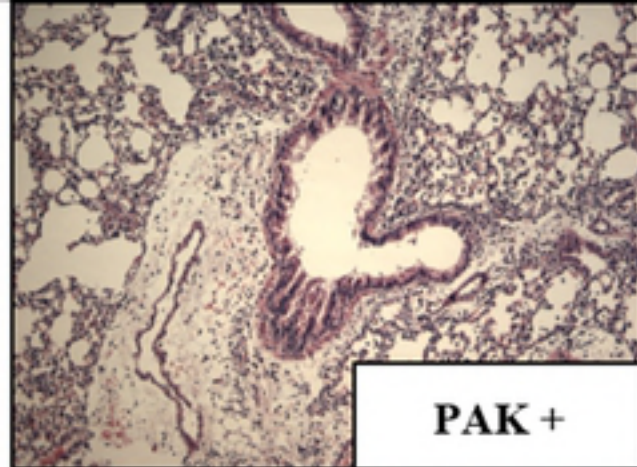
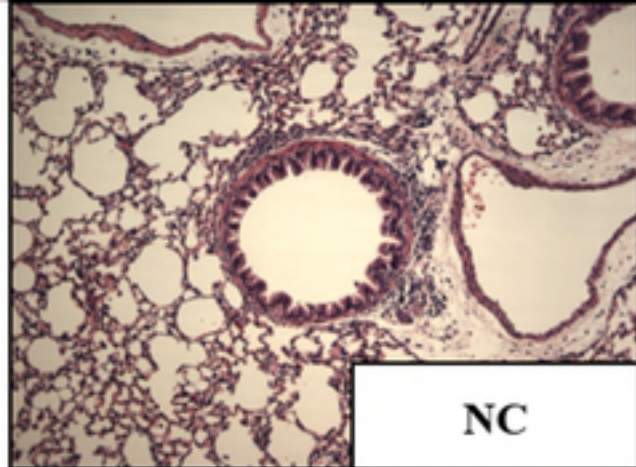
**(A)****(B)****(C)****(D)**



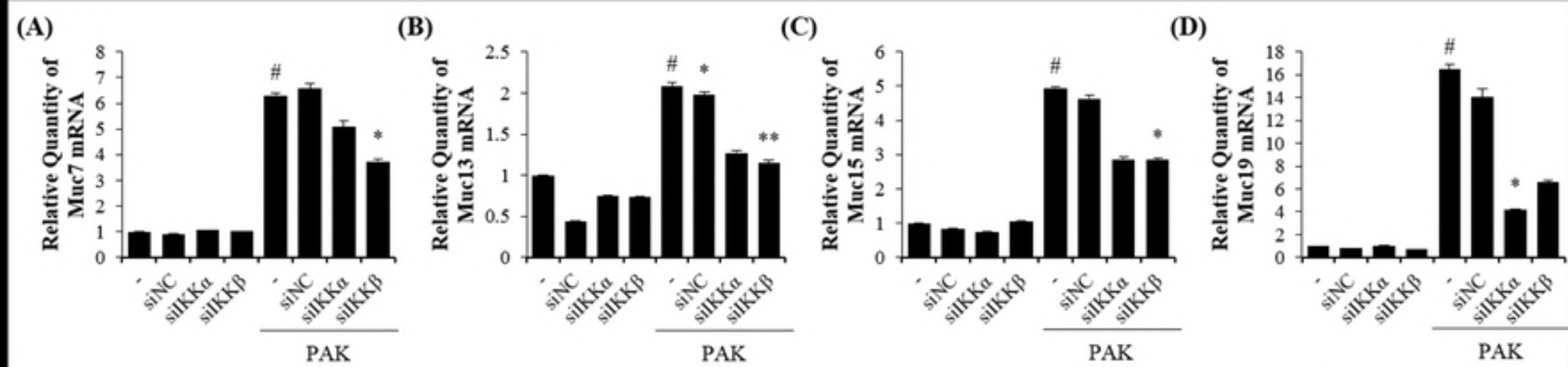
Figure

**(A)****(B)****(C)****Figure**

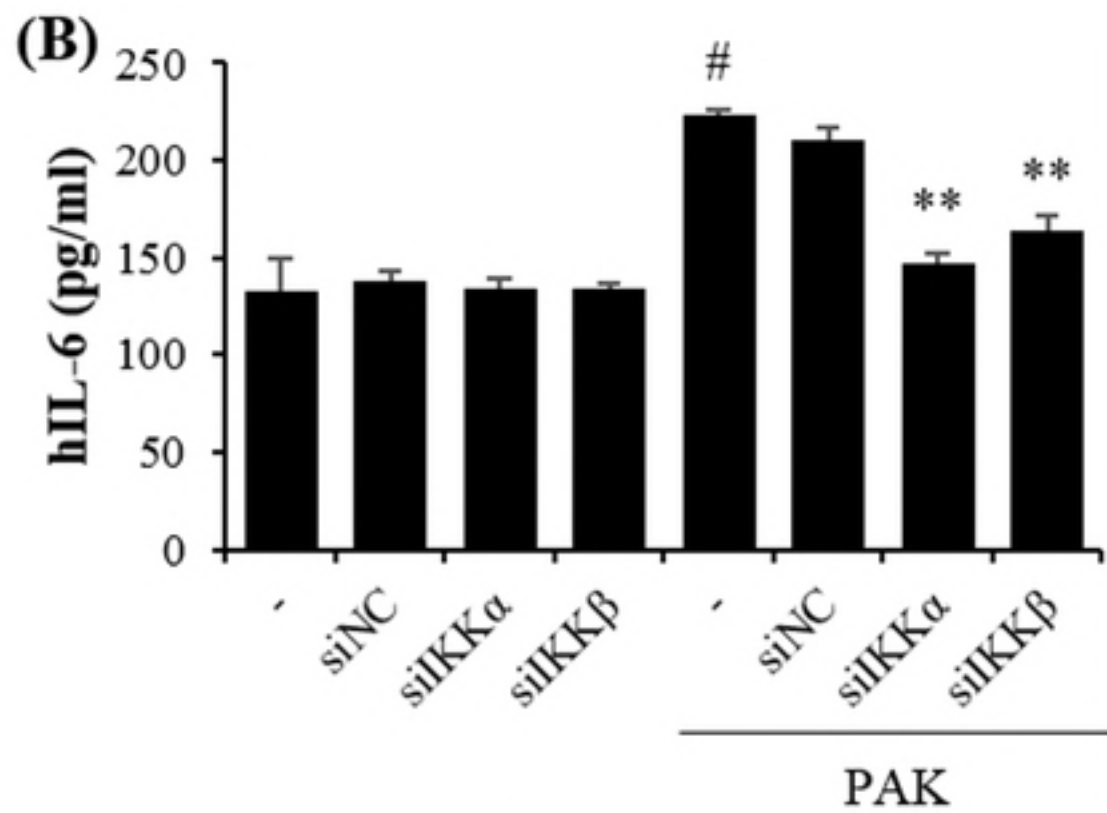
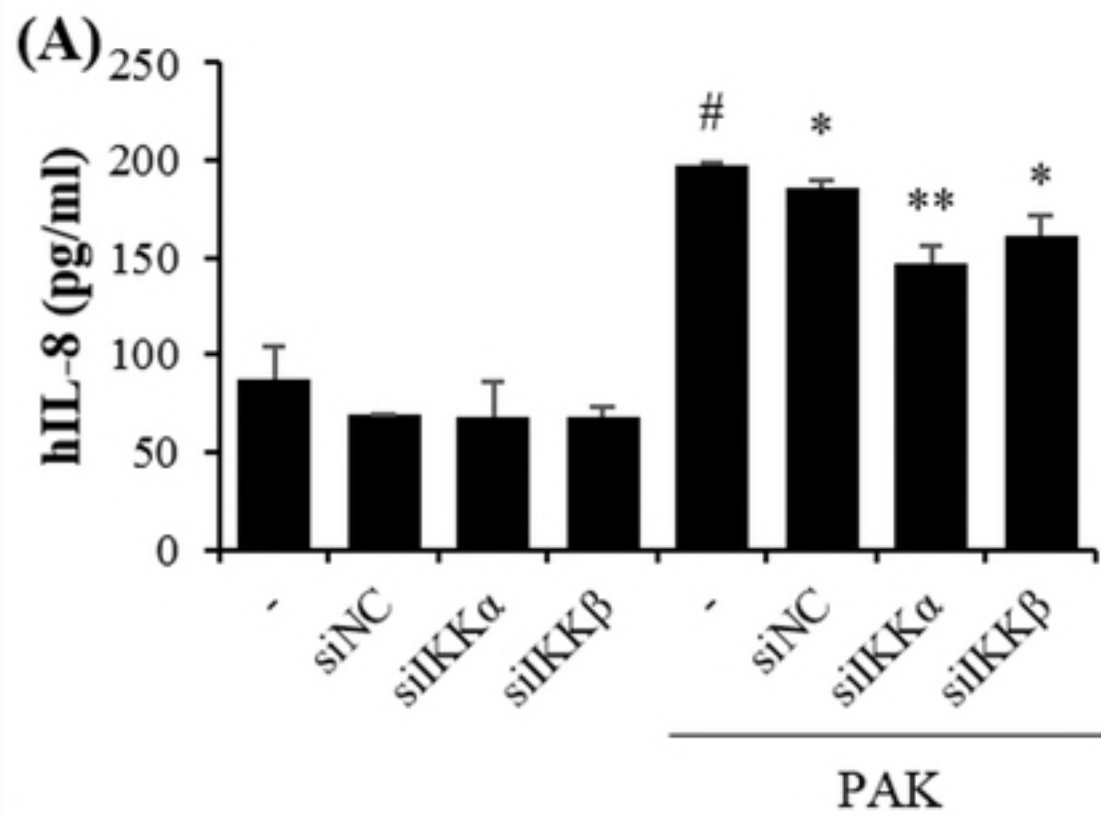
**Peribronchial  
lesion**



**Figure**



Figure



Figure