Tanshinone IIA inhibits lipopolysaccharide-induced MUC1 overexpression in alveolar epithelial cells

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Tanshinone IIA (TIIA) is a small-molecular-weight natural compound identified as one of the main active ingredients from Chinese herbal medicine Danshen, the dried root of Salvia miltiorrhiza. TIIA has been shown to be effective in protecting organs from acute injury, such as cardiac injury caused by ischemia and reperfusion (10) and acute lung injury by inhalation of LPS or seawater in animals (39–40, 43). These protective effects were attributed at least in part to its antiinflammatory functions, and therefore TIIA is widely used to treat inflammatory conditions associated with various heart and lung diseases in Asian clinics. However, the cellular and molecular mechanisms underlying the anti-inflammatory effects of TIIA are incompletely understood. TIIA could reduce LPS-induced proinflammatory cytokine (IL-1β, IL-6, and TNF-α) release from macrophages during inflammation (2, 4, 7). Lung epithelium represents the front line of host defense against infections. TIIA could protect airway epithelial cells from LPS-induced injury (40); however, the effects of TIIA on lung epithelial cell-mediated inflammation, and the detailed underlying mechanisms remain largely unknown.

Mucin 1 (MUC1 in human, Muc1 in nonhuman species) is a large membrane-tethered glycoprotein, which is normally expressed on the apical surfaces of glandular epithelial cells and on hematopoietic cell surfaces (11). MUC1 consists of a large extracellular (EC) region, which is highly glycosylated on O- and N-linked sites, a single-pass transmembrane region, and an intracytoplasmic tail (CT) (8, 18). The 72-aminocacid carboxy-terminal domain of MUC1 contains seven evolutionally conserved tyrosines, which can be phosphorylated when binding to the kinases and adapter proteins, such as phospha-tidylinositol 3-kinase, Shc, PLC-g, e-Src, and Grb-2, and activates downstream signaling pathway (18, 32). Increased expression and cleavage of MUC1 are commonly seen in various solid carcinomas and in lung tissue with inflammatory lung diseases, such as interstitial lung diseases (13, 21, 42), chronic obstructive pulmonary diseases (COPD) (12, 14), and acute lung injury (5). The functions of MUC1 have been largely recognized as an adhesion, an antiadhesion, and a signaling molecule in normal epithelial and cancer cells, providing a protective layer on epithelial cells against bacterial and enzyme attack and promoting tumor progression (11, 22, 25, 31, 36, 38, 41). Recently, we and others (23–24, 29–30, 35) found that Muc1 is an important negative regulator of airway inflammation induced by microbial infection, enzymatic digestion, and other irritants. Enhanced lung inflammation was found in mice lacking Muc1 inhaled with Pseudomonas aeruginosa (PA), indicated by greater neutrophil recruitment in lung and higher levels of TNF-α and keratinocyte chemotactrant (KC; mouse ortholog of human IL-8) in bronchoalveolar lavage (BAL) fluid when compared with the wild-type littermates, resulting in increased clearance of PA yet impaired resolution of lung inflammation (5, 29). During acute lung inflammation, the level of lung Muc1 was increased, which
was supposed to be critical for controlling the ongoing inflammation through suppressing Toll-like receptor (TLR) signaling, including TLR2, 3, 4, 5, 7, and 9, in a feedback manner via regulating production of TNF-α (5, 17, 35).

This study aimed to evaluate the effects and the underlying molecular mechanisms of TIIA on LPS-induced inflammation in the airway and investigate whether MUC1 is involved in these effects.

**MATERIALS AND METHODS**

*Reagents.* TIIA was gifted from Jiangsu Carefree Pharmaceutical (Nanjing, China). Its purity was ≥98% as assessed by high-performance liquid chromatography. LPS (L3024) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TNF-α protein was purchased from R&D Systems (Minneapolis, MN). All chemicals were from Sigma-Aldrich unless otherwise specified.

*Animals.* Male C57BL/6 mice (20–25 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). Muc1 knockout mice (Muc1−/−) in C57BL/6 background were kindly gifted by Professor Sandra J. Gendler at Mayo Clinic (Scottsdale, AZ). All mice were housed under specific pathogen-free environmental conditions with 12-h:12-h light/dark cycles in a humidified atmosphere containing 50% relative humidity.

The human alveolar adenocarcinoma cells A549 (obtained from ATCC, Rockville, MD) were seeded into six-well plates at a density of 1×10⁶ cells per well and were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown until 70% confluence and changed with serum-free medium for 12 h to reach a growth arrest. Then the cells were treated with LPS (10 μg/ml) for 48 h or TNF-α (50 ng/ml) for 12, 24, or 48 h. TIIA (25 μg/ml) was given to the cells 2 h before LPS or TNF-α administration. The cells and cell culture medium were collected for various measurements.

*Knockdown of TNF-α receptor 1 with siRNA.* For the transient knockdown of TNF-α receptor 1 (TNFR1) expression, short-interfering RNA (siRNA) targeted to TNFR1 (siTNFR1; forward, 5'-ACC TGA GTG GAG TGG AAT GG-3'; reverse, 5'-CTA GTG GAG TGG AGT AGG-3') and nontargeting control small-interfering RNA (siNT; forward, 5'-UUA CUA CAG GTT-3'; reverse, 5'-GGA GTG AGT CCA AGT TTC TAT T-3') were transfected into A549 cells using Hiperfect Transfection Reagent (Qiagen, Düsseldorf, Germany) for 1 h at room temperature. The cell pellets were resuspended in 1 ml saline for total cell counting. Cells in BAL were cytospun, stained with hematoxylin and eosin (H and E) and then subjected to differential cell counting.

**Lung histology.** After LPS, TIIA, or saline treatment, the right lung lobes were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were sectioned at 4-μm thickness and stained with H and E.

**RNA extraction and real-time quantitative PCR.** Total RNA from lung tissue or cultured cells was extracted with TRIZOL reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Reverse transcription into cDNA was performed with 1,000 ng of total RNA. Real-time PCR was accomplished by using the following primer pairs and the QuantiTec SYBR Green kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol: MUC1 (forward, 5'-AGC CAT TTC TGC CAA CTT GT-3'; reverse, 5'-ACC TGA GTG GAG TGG AGT AGG-3'); TNFR1 (forward, 5'-TTG TTT GCC AGG AGT A-3'; reverse, 5'-AGA CAC ACA CCT TTG CTT CTT C-3'); and Histone H3 (forward, 5'-CCT GCT ACT AAA CCA TCC AA-3'; reverse, 5'-GGT TTG AGC AGG TCT AGG-3').

**Western blotting.** Lung tissues or cultured cells were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 2 mM EGTA, 2 mM EDTA, and 0.1% SDS) containing protease inhibitor cocktail (1:100; Sigma-Aldrich) and centrifuged at 12,000 g for 30 min at 4°C to remove precipitates. Nuclear and cytosol proteins were extracted using a commercial kit obtained from Abcam (Cambridge, MA) or β-tubulin from Sigma-Aldrich. Bound antibodies were probed with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature. Finally, the signals were visualized with enhanced chemiluminescence reagents (ECL; Bio-Rad).

**Collection of BAL fluid.** BAL fluid was obtained by instilling the lung with 0.4 ml sterile saline three times. The recovered BAL fluid was centrifuged at 3,000 revolution/min for 10 min. The cell-free supernatants were collected and stored at −80°C for further analysis. The cell pellets were resuspended in 1 ml saline for total cell counting. Cells in BAL were cytospun, stained with hematoxylin and eosin (H and E) and then subjected to differential cell counting.

**Fig. 1.** Tanshinone IIA (TIIA) relieved acute lung inflammation induced by LPS. Mice received LPS (10 μg/mouse) via intranasal inhalation. TIIA (25 mg/kg) was given to the mice by intraperitoneal injection 2 h before the LPS administration. The mice were killed 24 h after LPS or saline inhalation, and then lung inflammation was assessed. A: numbers of total leukocytes and neutrophils in bronchoalveolar lavage (BAL) fluid from vehicle (saline + PBS) control (Ctrl), TIIA, LPS, or LPS + TIIA groups were counted. Values are means ± SE (n = 6 mice per group). *P < 0.05 for LPS vs. respective Ctrl groups; **P < 0.05 for LPS + TIIA group vs. respective LPS-alone-treated animals. B: representative images of hematoxylin and eosin-stained lung sections from Ctrl, LPS, or LPS + TIIA groups (magnification, ×400; n = 5).
ELISA. The Muc1 levels in BAL fluid were measured by direct ELISA. Briefly, BAL fluid was diluted in 0.05 mM bicarbonate buffer (1:1, pH 9.5) and coated onto 96-well ELISA plates (100 μl/well) overnight at 4°C. The coated plates were then blocked, incubated with an anti-Muc1 (EC domain) antibody (1:100, F-19; Santa Cruz Biotechnology, Santa Cruz, CA), followed by an HRP-conjugated antibody. Finally, the signal was developed with tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories). The concentration of Muc1 was assessed from a standard curve using serial dilutions of a pooled BAL sample and expressed as arbitrary units. In addition, the TNF-α and KC levels were measured by ELISA with the kit for TNF-α (eBioscience, San Diego, CA) or commercial antibodies for KC (biotinylated rabbit anti-mouse KC as capture antibody, Cell Science, Canton, MA; anti-mouse KC MAB453 as detection antibody, R&D Systems). Recombinant mouse KC (R&D Systems) was used to prepare standard curves when running ELISA for KC measurements.

Statistical analysis. Data were statistically analyzed using ANOVA and Student’s t-test. If significant F ratios were obtained with ANOVA, pairwise comparison of means was performed with two-tailed t-tests. When necessary, a Bonferroni correction was used to adjust level of significance for multiple comparisons. Otherwise, P values ≤0.05 were considered significantly different. Data values were presented as means ± SE, and n equals the number of animals or cell culture wells in each experiment group.

Fig. 2. TIIA decreased levels of inflammatory cytokines and Mucin 1 (Muc1) in BAL fluid and Muc1 expression in lung from LPS-inhaled mice. Mice were inhaled with LPS or saline with or without pretreatment of TIIA. BAL fluid was collected, and the levels of TNF-α (A), keratinocyte chemoattractant (KC) (B), and Muc1 (extracellular domain) (C) in BAL fluid from vehicle (saline and PBS), TIIA, LPS, or LPS + TIIA groups were measured by ELISA. Values are means ± SE (n = 6 mice per group). *P < 0.05 for LPS group vs. respective vehicle control-treated groups. **P < 0.05 for LPS + TIIA group vs. respective LPS-treated groups. D: representative Western blots of Muc1 in lung extracts from 2 randomly chosen mice in each group. Muc1 was recognized with an antibody against Muc1 carboxy-terminal domain. Experiments were repeated 3 times.

Fig. 3. TIIA relieved acute lung inflammation induced by LPS in Muc1−/− mice. Similar to wild-type mice, Muc1 homogenous knockout (Muc1−/−) mice received LPS (10 μg/mouse) via intranasal inhalation. TIIA (25 mg/kg) was given to the mice by intraperitoneal injection 2 h before LPS administration. The mice were killed 24 h after LPS or saline inhalation, and then lung inflammation was assessed. A: numbers of total leucocytes and neutrophils in BAL fluid from vehicle (saline + PBS) control, TIIA, LPS, or LPS + TIIA groups were counted. Values are means ± SE (n = 6 mice per group). *P < 0.05 for LPS vs. respective Ctrl groups; **P < 0.05 for LPS + TIIA group vs. respective LPS-alone-treated animals. B: representative images of hematoxylin and eosin-stained lung sections from Ctrl, LPS, or LPS + TIIA groups (magnification, ×400; n = 5).

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RESULTS

**TIIA inhibited LPS-induced acute inflammation in mouse lung.** Inhalation of LPS caused typical changes of acute inflammation in mouse lung. These changes include remarkable increases of leukocytes, herein the majority of which were neutrophils in BAL fluid (Fig. 1A), and massive inflammatory cell infiltration observed in lung from LPS-inhaled control mice (Fig. 1B). Treatment with TIIA inhibited these lung inflammatory responses to LPS, resulting in reduced total leukocyte and neutrophil counts in BAL fluid (Fig. 1A) and alleviation in lung inflammation (Fig. 1B). In contrast to the massive infiltration of leukocytes in perivascular and bronchial area in LPS-alone-treated mice, only scattered inflammatory cells were seen in lungs from those mice that received LPS plus TIIA (Fig. 1B). TIIA administration alone did not impact mice either on BAL fluid cell counts (Fig. 1A) or on lung texture (data not shown).

In association with reduced leukocyte infiltration in lung, TIIA treatment inhibited the elevation of proinflammatory cytokine production in LPS-inhaled mice; compared with LPS treatment alone, both TNF-α (Fig. 2A) and KC (Fig. 2B) levels were significantly reduced in BAL fluid from mice treated with TIIA plus LPS. TIIA alone did not affect levels of either of these two cytokines (Fig. 2, A and B).

**TIIA inhibited upregulation of Muc1 in lung from LPS-treated mice.** Consistent with what was previously reported that levels of Muc1 in the lung were increased during PA infection (5), in this study, we found that the levels of Muc1 release (Fig. 2C) in BAL fluid and cellular Muc1 in lung tissue (Fig. 2D) were greater in LPS-treated mice. These increments of Muc1 release (Fig. 2C) and cellular Muc1 (Fig. 2D) induced by LPS were essentially blocked in mice pretreated with TIIA.

**TIIA inhibited LPS-induced acute lung inflammation in mice absent of Muc1.** Similar to what we observed in Muc1+/+ mice, TIIA relieved LPS-induced acute lung inflammation in Muc1−/− mice as well; total leukocyte and neutrophil number in BAL fluid (Fig. 3A), the inflammatory cell infiltration in lung (Fig. 3B), and the TNF-α (Fig. 3C) and KC (Fig. 3D) levels in BAL fluid from LPS-inhaled mice were all reduced by TIIA treatment. Again, TIIA did not affect lung inflammation in control Muc1+/− mice. These results indicate that the anti-inflammatory effects of TIIA were independent of the presence of Muc1.

**TIIA inhibited LPS-induced increases of TNF-α in A549 cells.** Airway epithelial cells represent the first line of defense mainly via producing a plethora of proinflammatory cytokines, i.e., TNF-α in response to airborne intrusion (29). Therefore, we next wanted to see whether the production of TNF-α on LPS exposure in epithelial cells could be influenced by TIIA in the same manner as in mouse lung in vivo. As shown in Fig. 4A, LPS greatly increased the level of TNF-α in the culture medium of A549 cells; this increase was reduced in cells treated with TIIA (Fig. 4A). Treatment of TIIA alone did not change the level of TNF-α in A549 cells (Fig. 4A).

**TIIA inhibited LPS-induced upregulation of MUC1 in A549 cells via inhibiting TNF-α-mediated signaling.** In conjunction with the change of TNF-α induced by LPS, both mRNA (Fig. 4B) and protein (Fig. 4C) expression of MUC1 were dramatically increased in cells stimulated with LPS; these increases were essentially blocked by cotreatment with TIIA (Fig. 4, B and C). TIIA alone did not affect the basal levels of MUC1 in A549 cells.

To examine the relationship of TNF-α and MUC1 in response to LPS and TIIA, we further investigated the effects of TIIA on TNF-α-induced MUC1 production in A549 cells. Similar to what we observed previously (19), treatment of TNF-α (50 ng/ml) time dependently upregulated MUC1 protein expression in A549 cells; the maximum upregulation was reached at 24 h of TNF-α treatment (Fig. 5A). Preincubation with TIIA completely eliminated the upregulation of MUC1 mRNA (Fig. 5B) and protein (Fig. 5C) induced by TNF-α. In addition, we found that exposure to specific siRNA targeted to TNFR1 resulted in an ~93% reduction of TNFR1 mRNA (Fig. 5D) and 83% reduction in protein (Fig. 5E) in A549 cells; the knockdown of TNFR1 abolished LPS-induced upregulation of MUC1 protein expression in A549 cells (Fig. 5F), suggesting the dependence of TNF-α-mediated signaling in upregulation of MUC1 on LPS stimulation.

**TIIA inhibited LPS-induced nuclear translocation of NF-κB and upregulation of TLR4 in A549 cells.** TIIA was found to inhibit LPS-induced degradation of IκB and translocation of NF-κB.
NF-κB in macrophages (15). To examine whether this inhibitory effect of TIIA happens to airway epithelial cells, we pretreated A549 cells with TIIA (25 μg/ml) for 30 min before exposure of cells to LPS (10 μg/ml) for 10 min. As seen in Fig. 6A, Western blotting demonstrated that LPS greatly enhanced the translocation of NF-κB into the nucleus; in association with this enhancement, the phosphorylation of IκB was promoted, and the total amount of IκB in the cytosol was significantly reduced in A549 cells. Treatment of TIIA inhibited the increases of nuclear NF-κB, phosphorylated IκB, and deceased total IκB stimulated by LPS (Fig. 6). TLR4 is the receptor responsible for NF-κB recognition. In addition to the above increases of NF-κB translocation, which reflects activation of TLR4, we found that LPS stimulation (10 μg/ml; 48 h) also upregulated TLR4 mRNA (Fig. 6B) and protein (Fig. 6C) in A549 cells; pretreatment of cells with TIIA (25 μg/ml) abolished these upregulations on TLR4 (Fig. 6, B and C).

**DISCUSSION**

Both TIIA and Muc1 are effective in alleviating acute lung inflammation. The present study provided evidence strongly indicating that the effects of compound drug TIIA on attenuating LPS-induced acute lung inflammation were upstream of Muc1 and independent of Muc1 presence. This evidence includes the following: 1) TIIA inhibited LPS-induced neutrophil infiltration and TNF-α and KC production in lung from mice absent of Muc1; 2) the increases of Muc1 in LPS-challenged mouse lung and BAL fluid were reduced by TIIA treatment; 3) TIIA inhibited LPS- and TNF-α-induced Muc1 expression in alveolar epithelial cells. Instead, we found that TIIA prevented the excessive lung inflammation induced by LPS likely via directly inhibiting TLR4-mediated signaling; as a result, NF-κB translocation and upregulation of TLR4-α in lung epithelial cells were reduced, and consequently the increase of Muc1 was blocked.

Consistent with what has been reported before (39–40), our study also demonstrated that injection of TIIA protected LPS-induced acute inflammation in mouse lung. Muc1 was supposed to be upregulated during inflammation by TNF-α, thereby inhibiting release of proinflammatory cytokines, which constitutes a feedback loop on preventing excessive immune response. In mechanism, overexpressed Muc1 binds to IκK-γ, thereby inhibiting NF-κB activation and downregulating its target genes such as IL-8 and TNF-α (6, 9). It is reasonable to speculate whether TIIA exerts anti-inflammatory function for LPS-stimulated inflammation via upregulating Muc1, as the activation of NF-κB is the primary downstream event incurred...
by LPS-AP-1 and TLR4 signaling. In this case, we would expect to see enhanced expression of Muc1 during this inflammation process upon TIIA treatment and a disappearance of the anti-inflammatory effects of TIIA on Muc1−/− mice. Indeed, we observed that TIIA inhibited LPS-induced NF-κB translocation and TLR4 expression in alveolar epithelial cells. However, contrary to our expectation, the enhanced expression of Muc1 was inhibited by TIIA treatment in mouse lung, and the anti-inflammatory function of TIIA on LPS-induced acute lung inflammation maintained in Muc1−/− mice. These results suggest that Muc1 is unlikely responsible for eliciting the anti-inflammatory function of TIIA. In this situation, we speculated that TIIA attenuates inflammatory reaction by pathways independent of Muc1 overexpression in epithelial cells.

LPS is known to induce signaling via both MyD88-dependent and -independent pathways in acute immunity. Recruitment of MyD88 is an essential event for production of proinflammatory cytokines, i.e., TNF-α, post-TLR activation. The attenuation on NF-κB activation is critical for the protective functions of TIIA on lung injury. TIIA inhibits NF-κB activation and proinflammatory factor release, including IL-6 and TNF-α in macrophages, thereby attenuating seawater aspiration-induced lung injury (43). In LPS-induced acute lung inflammation, TIIA protects epithelial cells by inhibiting NF-κB activation as well (40). The effects of TIIA on blocking TLR signaling might contribute to the inhibition of NF-κB activation and therefore the decrease of proinflammatory cytokine expression (26, 37).

In addition to NF-κB, activator protein-1 (AP-1) could also be activated by LPS stimulation via MyD88-dependent pathway (34). TIIA inhibited AP-1 activation by means of inhibiting phosphorylation of c-Jun, which is one of the components of AP-1, and then impairing AP-1 DNA binding (16), thus attenuating inflammatory reactions. Inhibition of another AP-1 component, c-Fos, in alveolar epithelial cells contributed to decreased nitric oxide synthase 2 levels, which triggers the innate host response to a wide range of microbial products (1). Although it is not known how TIIA affects c-Fos in alveolar epithelial cells, TIIA incubation and c-Fos knockdown elicit similar effects on various physiological processes in different cell types (3, 28). It is also possible that TIIA prevents excessive inflammatory response by inhibiting LPS-induced NF-κB translocation, via a crosstalk between AP-1 and NF-κB pathway, because the inhibition of either AP-1 or NF-κB was followed by the inhibition of both AP-1 and NF-κB. This effect was inflicted by inhibition of a common ERK1/2-dependent pathway for activation of AP-1 and NF-κB (20). Other possible effects of TIIA on NF-κB include the inactivation on cytokine or signaling protein like connective tissue growth factor or Akt, which consequently inhibits NF-κB pathway by inhibiting IKK phosphorylation and nuclear translocation of NF-κB p65 subunit (16, 27). Lastly, TIIA could protect the lung from LPS-induced injury by alleviating inflammatory cell migration (33, 43).

In regards to Mcu1 decrease upon TIIA administration, we propose that it might be due to the inactivation of elements such as TNF-α, which upregulates Mcu1 expression in alveolar epithelial cells. TNF-α stimulated MUC1 transcription in A549 cells (19). TNF-α was further found to be critical for upregulation of Mcu1 in inflammatory lung, and mice lacking TNFR1 were absent of Mcu1 increase and impaired in resolving acute lung inflammation caused by PA infection (5). Similarly, we found that exposure to LPS led to overexpression of MUC1 and TNF-α in alveolar epithelial cells; the stimulated MUC1 expression induced by both LPS and TNF-α were inhibited by TIIA treatment. Moreover, the LPS-induced MUC1 expression was prevented when TNFR1 was knocked down in A549 cells treated with TNFR1-specific siRNA. These results suggest that the downregulated effects of TIIA on LPS-induced MUC1 overexpression were via inhibiting TNF-α release. The property that TIIA inhibits inflammation without upregulating MUC1 might be particularly useful for treating chronic inflammatory lung diseases such as COPD, in which MUC1 is constantly overexpressed (our unpublished data). Persistent overexpression of MUC1 might be harmful by promoting epithelial mesenchymal transformation, thereby contributing to the pathology of airway remodeling and carcinogenesis associated with COPD. It will be our future interest to investigate the effects of TIIA in COPD development.

In summary, this study demonstrated that TIIA relieved LPS-induced acute inflammation and Muc1 expression in mouse lung and human alveolar epithelial cells. The relief of TIIA on inflammation was via pathways other than regulating Muc1 expression, whereas the inhibitory effects of TIIA are likely due to the consequence of reduction of TNF-α release. TLR4-mediated signaling is demonstrated to be involved in the anti-inflammatory effects of TIIA; however, the underlying detailed mechanisms remain intriguing and warrant further investigation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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