Respiratory syncytial virus infection disrupts pulmonary microbiota and immune barriers to induce microglia phenotype shift

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Abstract

Background: The lung-brain-axis is an emerging biological pathway that is being investigated in relation to microbiome medicine. Increasing evidence suggest that pulmonary viral infections can lead to distinct pathological imprints in the brain, thereby the need to explore and understand this mechanism and find possible interventions. Objective: This study used RSV infection in mice as a model to establish the potential lung-brain axis phenomenon. We hypothesized that RSV infection could disrupt the lung microbiota, thereby compromising the immune barriers and thus induces significant shift in microglia phenotype. Methods: Mice were randomized into the Control, Ampicillin, RSV, and RSV+Ampicillin treated groups (n = 6 each). Ampicillin was given intratracheal instillation and seven days after the respective treatments, the mice were anesthetized. Hematoxylin-eosin (HE) staining of lung tissue to detect histopathology. Immunofluorescence label of specific target antigens in both the lung and brain tissues, namely, Malondialdehyde (MDA) and Superoxide dismutase (SOD) were used as markers of cellular damage. RT-qPCR was used to detect viral RNA in both tissues, ELISA to measure IL-1β, iNOS, IL-10 and Arg1 in the supernatant and 16s DNA technology were used to detect the lung microflora. Results: We found out that RSV infection induces elevated oxidative stress, reduced anti-oxidant and caused significant dysbacteriosis in the lungs of the mice. Pulmonary microbes were found affecting Th1-type immunoreactivity induced by RSV infection and eventually, microbiota in lung induced microglia phenotype shift in the brain of the mice. Conclusion. This study was able to establish that RSV infection can disrupt the pulmonary microbiome and immune barriers to induce microglia phenotype shift. Thus, we recommend a large sample size study with robust data analysis for the long-term effects of antibiotics and RSV infection on brain physiology.
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Background: The lung-brain-axis is an emerging biological pathway that is being investigated in relation to microbiome medicine. Increasing evidence suggest that pulmonary viral infections can lead to distinct pathological imprints in the brain, thereby the need to explore and understand this mechanism and find possible interventions. Objective: This study used RSV infection in mice as a model to establish the potential lung-brain axis phenomenon. We hypothesized that RSV infection could disrupt the lung microbiota, thereby compromising the immune barriers and thus induces significant shift in microglia phenotype. Methods: Mice were randomized into the Control, Ampicillin, RSV, and RSV+Ampicillin treated groups (n = 6 each). Ampicillin was given intratracheal instillation and seven days after the respective treatments, the mice were anesthetized. Hematoxylin-eosin (HE) staining of lung tissue to detect histopathology. Immunofluorescence label of specific target antigens in both the lung and brain tissues, namely, Malondialdehyde (MDA) and Superoxide dismutase (SOD) were used as markers of cellular damage. RT-qPCR was used to detect viral RNA in both tissues, ELISA to measure IL-1β, iNOS, IL-10 and Arg1 in the supernatant and 16s DNA technology were used to detect the lung microflora. Results: We found out that RSV infection induces elevated oxidative stress, reduced anti-oxidant and caused significant dysbacteriosis in the lungs of the mice. Pulmonary microbes were found affecting Th1-type immunoreactivity induced by RSV infection and eventually, microbiota in lung induced microglia phenotype shift in the brain of the mice. Conclusion. This study was able to establish that RSV infection can disrupt the pulmonary microbiome and immune barriers to induce microglia phenotype shift. Thus, we recommend a large sample size study with robust data analysis for the long-term effects of antibiotics and RSV infection on brain physiology.

Keywords: RSV infection, pulmonary microbiome, immune barriers, microglia shift, lung-brain-axis.
neurological diseases. For example, an increased risk of Parkinson’s disease (PD) and Alzheimer disease (AD) dementia are both associated with COPD, which is correlated with an altered microbiome [13].

Pulmonary microbes and their metabolites may promote the proliferation of respiratory viruses and enhance their infectivity [15]. Symbiotic flora can also inhibit early replication of influenza virus by increasing the expression of type I interferon receptor and enhancing basal interferon signaling by driving pulmonary interstitial cells [16]. Respiratory viruses can also cause secondary bacterial infections by damaging the host’s mucosal barrier, affecting immune function, and altering the abundance and diversity of the respiratory microbiota, such as RSV infection, which can alter the nasal respiratory microbiota [17].

Currently, research on pulmonary microbiota is very limited, and the mechanism of its interaction with viral infection is not entirely clear. Our understanding of the respiratory microbiome is partial. The mechanism and role of intrapulmonary bacteria in lung function is not fully understood. This study aims to identify pulmonary bacterial dysbiosis after RSV infection and the influence of dysbiosis in the lung on the lung-brain axis. In addition, the study sought to identify the mechanisms of lung microbiota on RSV infection by investigating the differentiation of immune cells in the lung and brain during RSV infection.

Materials and methods

Animal models

The study was conducted in accordance with the Helsinki Declaration and approved by the Medical Ethics Committee of Xiangya School of Medicine. A week old male neonatal BALB/c mice (8-15 g) were purchased from Slack Jinda Laboratory Animal Co. Ltd., Changsha, Hunan. The mice were acclimatized for one week in a sterile environment with a 12 hrs. light/dark cycle and a constant temperature of 25°C and fed adaptively for 7 days. The healthy mice were then treated randomly into four groups, namely, the Control (PBS), Ampicillin, RSV, and RSV+Ampicillin groups (n = 6 each). Ampicillin (Sigma, St. Louis, USA) was given intratracheal instillation at 100 mg/kg body weight, while other mice were injected with Phosphate Buffer Saline (PBS) instead. For RSV infection, the mice were anesthetized with isoflurane, $5 \times 10^6$ pfu RSV in 100 μl was intranasally inoculated while mice in the RSV+Ampicillin group were treated with 100 mg/kg/d Ampicillin prior to RSV inoculation and daily after RSV infection. Seven days after the respective treatments, the mice were anesthetized with ether and placed in a supine position. An incision was made from the neck to the bottom of the chest wall, and the left lung was resected, washed with 0.9% sodium chloride, and properly analyzed. Ampicillin treatment was added in order to confirm any altered pathological imprints in the brain for both RSV group alone and the additional Ampicillin.

Hematoxylin-eosin (HE) staining of lung tissue

The resected lung tissue was fixed with a 10% formaldehyde solution for 48 h, embedded in paraffin, and cut into 4 μm thick sections. Tissue sections were heated to 60°C for 2 h, xylene dewaxed (twice, 15 min each), and dehydrated using an alcohol gradient (75%, 95% and 100%) for 5 min each. HE stain was performed as per standard protocols.

Immunofluorescence

Lung and brain tissue sections were treated as described above sections 4 μm thick and boiled with 0.01M citrate buffer for antigen retrieval. After washing twice with PBS (3 min each), sections were fixed with 4% paraformaldehyde for 5 min, washed with PBS, and blocked with normal goat serum for 20 min (Bioss, Beijing, China). Subsequently, sections were incubated overnight with rabbit anti-RSV major surface glycoprotein G monoclonal antibody (Bioss, bs-1264R, Beijing, China, 1:500) at 4°C. iNOS antibody (18985-1-AP) was purchased from Proteintech Group (Rosemont, IL, USA). The following day, TRITC-labeled goat anti-rabbit antibody (Zen-Bioscience, Chengdu, China, 1:300) for RSV determination was added to the cover glass and incubated in darkness at room temperature for 1 hr. Secondary antibodies conjugated with FITC (a0568) and DAPI staining solution (c1006) were from Beyotime (Shanghai, China). After the final three washes with PBS, the sections were counterstained with DAPI (100 ng/ml) for 10 min. After removing
the moisture, the slides were sealed with the anti-fluorescence quenching agent and observed under the fluorescence microscope (Nikon Ti-S, Japan).

**Malondialdehyde (MDA) and Superoxide dismutase (SOD)**

Fresh lung and brain tissue samples prepared were ground in saline solution to make 10% tissue homogenates, followed by centrifugation at 3,500 rpm for 20 min. The resulting supernatant was collected to measure SOD and MDA content using specific kits according to the manufacturer’s instructions. All commercial test kits were obtained from Jiancheng Bioengineering Company (Nanjing, China).

**RT-qPCR**

Total RNA was extracted from lung and brain tissues using Trizol reagent (Takara, Japan). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences are shown in Table 1. Each sample was reverse transcribed into cDNA using RR036A PrimeScript RT Master Mix (Perfect Real Time) (Takara, Japan). The cDNA was then synthesized by reverse transcription and amplified using 2× SYBR Green qPCR Master Mix (Bimake, USA) according to the manufacturer’s instructions. RT-qPCR was performed at 95°C for 3 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. GAPDH was the internal reference for the target genes. Relative expression levels of mRNA were calculated using the 2^(-CT) method.

**Table 3.1 The primer sequences used in the study.**

<table>
<thead>
<tr>
<th>Names</th>
<th>Sequences(5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>GTGACGTTGACATCCGTAAAGA</td>
</tr>
<tr>
<td></td>
<td>GTAACAGTCCGCCTAGAAGCAC</td>
</tr>
<tr>
<td>RORgamma</td>
<td>GCAGGAGCAATGGAAGTCGTC</td>
</tr>
<tr>
<td></td>
<td>AGGACCGTTGCGCATTTGATGAG</td>
</tr>
<tr>
<td>GATA-3</td>
<td>CCGGGTTCGGGATGTTAAGTCG</td>
</tr>
<tr>
<td></td>
<td>GCCTTGCCTTGGGCTTGAT</td>
</tr>
<tr>
<td>T-bet</td>
<td>ATCATCCTAGCAAGGACGACG</td>
</tr>
<tr>
<td></td>
<td>CCAAGACCACATCCACAAACATC</td>
</tr>
<tr>
<td>Foxp3</td>
<td>CCTGTTTGTGAGAAGGTCTTCCG</td>
</tr>
<tr>
<td></td>
<td>TGCTCCAGAGACTGCACCACCTT</td>
</tr>
<tr>
<td>iNOS</td>
<td>GAGACAGGGAAGTCTGAAGCAC</td>
</tr>
<tr>
<td></td>
<td>CACAGCAGTGTCCTCCTCCCG</td>
</tr>
<tr>
<td>IL-10</td>
<td>CGGGAAGACAAAATAACTGCCACC</td>
</tr>
<tr>
<td></td>
<td>CGTTACAGTATCTGGTCCAGC</td>
</tr>
<tr>
<td>Arg-1</td>
<td>CATTGGCTTGCCGAGACGTCAG</td>
</tr>
<tr>
<td></td>
<td>GCTGAAGGTCTCTTCCACACC</td>
</tr>
</tbody>
</table>

**Enzyme Linked Immunosorbtent Assay (ELISA)**

The concentrations of IL-13, iNOS, IL-10 and Arg1 in the supernatant of each treatment group were measured using ELISA according to the manufacturer’s instructions (Fankew, Shanghai, China). Briefly, the supernatant collected was diluted 5 times and added to a 96-well plate with 50 µL per well. Fifty µL of biotin-binding antibodies were added to each well and incubated at 37 for 1 hour. After washing five times with PBS, 100 µL of chromogenic agent was added to each well and incubated in darkness at 37 for 15 min. The optical density (OD) of each well was detected at a wavelength of 450 nm. Fifty µL of the stop solution was added to each well to stop the reactions. The OD value of each well was measured at 450 nm by a microplate reader. The standard curve was plotted according to the standard OD value. The concentration of the target protein in the sample was calculated according to the standard curve. All ELISA results were expressed as cytokine concentrations (ng/mL or pg/mL) and performed in triplicates.
Construction of 16sRNA library

16s DNA technology was used to detect lung microflora. Portions of lung tissue were frozen flash after resection and stored at -80°C. Total genomic DNA was extracted using a DNA extraction kit (TIANGEN Biotech Co. Ltd, China) according to the manufacturer’s instructions, and its purity and concentration were determined by the nucleic acid analyzer (Hangzhou Aosheng Instrument Co. Ltd, China). PCR was performed using double index fusion primers containing sequencing joints, and the amplicons were separated by magnetic beads. A qualified library was used for cluster preparation and sequencing, and after filtering out low-quality reads, high-quality clean data was analyzed (Illumina, USA).

Cell culture and RSV Infection

Human lung epithelial Beas-2b and mouse nerve cells HT-22 cells were cultured in DMEM or RPMI 1640 plus 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B solution at 37°C and 5% CO₂. RSV (Long strain/A2 type) was stored at the Department of Medical Microbiology of the Central South University and later propagated in Hela cells with RPMI 1640 containing 2% FBS. Viral titers were determined using the plaque assay and RSV (1 MOI) was added to collect cell samples for the next experiment.

Statistical Analysis

SPSS 21.0 software was used for statistical analysis. Significant fold changes were analyzed by online features of MetaboAnalyst 2.0. Intergroup differences were analyzed by t-test and ANOVA as appropriate, and LSD pairwise comparison was used to analyze intragroup differences. P < 0.05 was considered statistically significant.

Results

RSV infection induced elevated oxidative stress and reduced antioxidant in the lung

While Ampicillin alone had no effect on normal lung morphology, RSV infection promoted epithelial shedding and infiltration of inflammatory cells (Fig. 1A). In addition, RSV immunofluorescence and titers were evident in the lung tissue, while there was no significant difference between RSV and RSV+Amp (Fig. 1B, C). We then detected plasma MDA and SOD activity in the lung to determine oxidative stress markers and antioxidant enzymes, respectively. No significant differences were found between the RSV and RSV+Amp groups.
Figure 1. **Effects of microbiota on lung injury induced by RSV infection.** A) HE stained lung sections indicating morphological features. B) RSV titers in the lung were assayed using real-time PCR. C) RSV infection was assayed by immunofluorescence (X400). D, MDA and SOD in the lung were assayed by biochemical kits, (n = 6), **** $p < 0.0001$, *** $p < 0.001$.

**Pulmonary microbes affect Th1-type immunoreactivity induced by RSV infection**

With the detection of inflammatory mediators and releases, we found that IL-1 (pro-inflammatory cytokines), IFN-γ (induce Th1 differentiation), IL-4 (induce Th2 differentiation) and IL-6 (induce Th17 differentiation and inhibit Treg differentiation) were significantly elevated. However, IFN-γ significantly decreased in RSV+Amp treated group when compared with RSV group (Fig. 2A). We further examined the expression of T cell differentiation transcription factors. The results showed that transcription factors RORrt, FoxP3, and T-bet significantly increased, with T-bet significantly decreasing in the RSV+Amp group when compared with the RSV group, correlating to the above observation (Fig. 2A).
Figure 2. Effects of lung microbiota on Th differentiation after RSV infection (n = 6). A) lung cytokines were assayed using ELISA. B) Th differentiation was assayed using real-time RT-qPCR. **** p < 0.0001, ** p < 0.01 and * p < 0.05.

Microbiota in lung induce microglia phenotype shift

Microglia influence the immune microenvironment of neurons mainly through the release of effector factors from different phenotypes after activation, thus affecting neuronal survival. Therefore, we first examined the effect of RSV and Amp on the microglia M1/M2 phenotype in vivo. Real-time qPCR results showed that IL-1β and iNOS expression levels were significantly higher after RSV infection than those in the control group. In addition, IL-1β and iNOS were further increased after Amp pretreatment prior to RSV infection (Figure 3A). However, mRNA levels of the Arg 1, M2 phenotype marker were significantly reduced after RSV infection and there was no difference after Amp pretreatment (Figure 3A). In addition, we examined the secretion levels of M1 and M2 phenotypic markers using ELISA. The results showed that the protein expression levels of IL-1β and iNOS, M1 phenotypic markers were significantly increased after RSV infection (p < 0.01, Figure 3B) and further increased after Amp pretreatment prior to RSV infection. However, IL-10 and Arg1 expression levels were slightly increased after RSV infection and not significantly different after Amp pretreatment prior to RSV infection (p > 0.05, Figure 3B). The levels of iNOS in microglia were detected by confocal immunofluorescence. Expression of iNOS was significantly increased after RSV infection and further elevated after Amp pretreatment prior to RSV infection (Figure 3C). These results suggest that lung microbiota may inhibit microglia activation and polarization towards the M1 phenotype. We also assessed brain injury by detecting MDA and SOD activity and found that MDA was significantly increased, and SOD was reduced in the brain tissue. And Amp pretreatment further promoted the secretion of MDA and inhibited SOD in the brain tissues.

Figure 3. Effects of lung microbiota on microglia phenotype shift after RSV infection. A) IL-1β, iNOS, IL-10 and Arg1 in the brain were assayed using real-time RT-qPCR. B) IL-1β, iNOS, IL-10 and Arg1 in the brain were assayed using real-time ELISA. C) NOS levels in microglia were detected by confocal immunofluorescence. D) MDA, and SOD in the brain were assayed by biochemical kits, n=6. **** p < 0.0001, ** p < 0.01 and * p < 0.05.

RSV infection induced lung dysbacteriosis

The effect of RSV infection on the lung microbiota was studied in 6-8 weeks old BALB/c male mice weighing 16-22 g. The mice were divided into the control group, Ampicillin treated group, RSV infected group and RSV+Ampicillin treated group. Ampicillin was injected into the tail vein once a day, while RSV+Ampicillin
group was injected into the tail vein once a day at the same time of infection. Seven days after infection, 16s DNA technology was used to detect lung microflora. The abundance and species of pulmonary bacteria in mice showed relatively good species richness and uniformity in the lungs of all four groups. Alpha diversity across four groups were not significant ($P > 0.05$), suggesting no significant variation in operational taxonomic unit (OTU) species richness (data not shown). In the beta diversity analysis, it was found that the normal control group had the highest diversity, while the remaining groups showed a significant decrease in pulmonary bacterial diversity (Fig. 4A). The Venn diagram showed no significant difference in bacterial abundance between the different groups (Fig. 4B). Moreover, in the RSV infection group, the number of common clinical pathogens such as *Escherichia, Acinetobacter, Pseudomonas and Treptococcus species* increased significantly, while the number of probiotics such as *Bifidobacterium, Fibrobacter, Selenoselomonas and Butyrivibrio species* decreased significantly. Compared to the RSV infected group, dysbacteriosis in the lung of the RSV+Ampicillin group was more pronounced, and probiotics decreased further (Fig.4C). By analyzing the metabolic function of different groups, the results showed that the difference in metabolic function was mainly manifested in membrane transport function, carbohydrate metabolism and amino acid metabolism (Fig. 5).

![Figure 4. Effect of RSV infection on lung microflora.](image)

A) Beta diversity analysis among the groups showed that the normal control group had the highest bacterial diversity, while the remaining groups showed a significant decrease in bacterial diversity. B) Venn diagram show that there was no significant difference in bacterial abundance between control and RSV infection groups. C) The results of differential microflora heat map showed that RSV infection caused significant dysbacteriosis, and the tendency of dysbacteriosis induced by RSV infection was more obvious after Ampicillin treatment, n= 3.
Figure 5. Changes in metabolic function of microflora in lung after RSV infection. A) Heat maps showing significant changes in metabolic function among the different groups. B) Radar maps showed that the top 10 metabolic differences among groups were mainly the function of cell membrane transport, carbohydrate metabolism, and amino acid metabolism.

Discussion

Our findings established the interaction of lung microbiota and RSV infection. RSV infection can induce bacterial dysbiosis. Although no significant difference was seen in the bacterial abundance between groups, in the RSV infection treated group, the number of common clinical pathogens such as *Escherichia*, *Acinetobacter*, and *Pseudomonas* and *Treptococcus* species increased significantly, while the number of probiotics such as *Bifidobacterium*, *Fibrobacter*, *Selenomonas*, and *Butyrivibrio spp.* decreased significantly. *Fibrobacter* was the most active cellulolytic bacterium known, producing succinic acid as its main fermentation product [18]. Thus, reduction in *Selenomonas* is a marker of asthma severity [19]. *Butyrivibrio spp.* was one of the symbiotic bacteria that can produce short-chain fatty acids, especially butyrate. Following Ampicillin administration, the number of *Bifidobacterium* was further reduced compared to the RSV group. *Bifidobacterium* is the most common probiotic for restoring beneficial flora [20]. These indicated that Ampicillin administration had no benefits in restoring beneficial flora, which may accelerate bacterial dysbiosis. However, abnormal increases in opportunistic pathogens in asthma and COPD patients often require antibiotics to control infections [21, 22]. Our study indicated that antibiotics may accelerate bacterial dysbiosis.

An increased MDA is a biomarker of injury, while SOD is a biomarker of anti-injury. The imbalance between this elevated MDA (oxidative stress) and SOD (reduced antioxidant) was found to increase the risk of complications in a study conducted on pregnant women [23]. This can be suggested since increased oxidative stress (the imbalance between free radicals and stabilizing antioxidant enzymes) can react with DNA, lipids or protein and lead to degenerative disorders. Moreover, since SOD as an antioxidant is reduced, it would not lower oxidants with electron donation and thus non-stabilization of free radicals, which leads to harmful effects in the cell [24]. We found that, while Ampicillin alone had no effect on MDA and SOD secretions after RSV infection, MDA was significantly increased and SOD decreased in the brain tissues after Amp treatment prior to RSV infection, indicating that lung microbiota can promote neuronal survival and inhibit microglia activation and also polarization towards the M1 phenotype. While Ampicillin alone had no effect on normal lung morphology, RSV infection promoted epithelial shedding and inflammation infiltration. With the detection of inflammatory mediator releases, IL-1, IFN-γ, IL-4 and IL-6 showed increased levels of which IFN-γ significantly decreased in RSV+Amp when compared with RSV. Reduced IFN-γ was seen with elevated TGF-β signaling, which is a biomarker for damage in connective tissue [25]. Significant increases in...
T cell differentiation transcription factors; RORγt (for Th17 development), FoxP3 (for Treg cell development) and T-bet (as a regulator of type 1 inflammatory responses) [26] are critical in immune homeostasis [27]. However, the significant decreases of IFN-γ and T-bet in the RSV+Amp group when compared with the RSV group indicates a decreased Th1-type immune response in the lung [28].

In correlating RSV-induced dysbiosis in lung tissue with specific CNS and brain, we detected microglia that affected the neuronal survival. From our study, Ampicillin alone was seen to increase in IL-1β and iNOS in brain tissues. Inducible nitric oxide synthase (iNOS), one of the enzymes that produces nitric oxide (NO) from the amino acid l-arginine confers some anti-inflammatory properties that regulate human asthma while IL-1β antibody has some protective effects on the brain from neuropathology of hypoperfusion [29]. An increased phenotypic marker of M1, which are IL-1β and iNOS during RSV infection can cause neurotoxicity and DNA damage respectively [30].

Although we were able to explore a lot about short-term intrapulmonary bacteria alleviating RSV-induced inflammation in the lung, the main limitations of the study were the limited sample size, and the lack of data analysis for the long-term effects of antibiotics and RSV infection in these BALB/c mice. In addition, metabolomics and metagenomics will confer a clear revelation as to the further mechanism.

**Conclusion**

From our study, we found that pulmonary microbes were found to affect Th1-type immunoreactivity induced by RSV infection, and ultimately, the lung microbiota induced microglia phenotype shift in the mouse brain tissues. We were able to add scholarly information on the lung-brain axis through RSV infection. The disruption of the pulmonary microbiome and immune barriers that induce microglia phenotype shift in the brain is related to the mechanism and role of intrapulmonary bacteria on RSV infection. Furthermore, the focus on connecting the lung to the brain in the gut-brain axis biological pathway has associative effects of dysbiotic pulmonary microbes and some alterations in the brain. With these novel microbiome findings and their impact on human physiology, we recommend a large sample size study with robust data analysis for the long-term effects of antibiotics and RSV infection on brain tissue physiology.

**Declarations**

**Funding:** Not applicable.

**Conflicts of interest:** The authors declare that they have no conflict of interest.

**Ethical approval:** The study was approved by the Institutional Ethic Committee of Xiangya School of Medicine, Central South University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Authors’ contributions:** OB and ZT designed the study, OB, ZT, YM and XQ conduct the experiments, YT evaluate the study, OB, LB, RK and TY wrote the manuscript

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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