Andrographolide attenuates RSV-induced inflammation by suppressing apoptosis and promoting pyroptosis after respiratory syncytial virus infection in vitro

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Research Article

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Abstract

Respiratory syncytial virus (RSV), the most common viral pathogen causing acute lower respiratory tract infection among children under five years old worldwide, still lacks specific therapeutic drugs. Andrographolide has been found efficacious in various virus infections, however, its effects on RSV infection remains unknown. Herein, in this study we quantified RSV virus load, IL-6 and IL-8 in A549 cell supernatant, and found andrographolide decreased the viral load and attenuated RSV-induced inflammation. Then, using online databases, we discovered 25 potential targets of andrographolide in the treatment of RSV-infected airway epithelial cells. Subsequently, GO and KEGG enrichment analysis led to the identification of CASP1, CCL5, JAK2, STAT1 as significant players. We verified the mRNA expression change of the potential target genes, which showed that andrographolide noticeably suppressed the increase of CASP1, CCL5, JAK2 and STAT1 post RSV infection. IL-1β, which is downstream of CASP1, was also up-regulated after RSV infection and down-regulated by andrographolide. Furthermore, we conducted Annexin V-FITC/PI apoptosis assay and Western blotting to determine whether andrographolide has an impact on the death pattern of RSV-infected cells. Interestingly, RSV infection decreased the protein levels of caspase-1, cleaved caspase-1, cleaved IL-1β, N terminal of GSDMD and Bcl-2, while andrographolide elevated them. These results suggested that andrographolide might attenuate RSV-induced inflammation by suppressing apoptosis and promoting pyroptosis of infected epithelial cells and thus inducing effective viral clearance.

Introduction

Respiratory syncytial virus (RSV) is a negative-sense single-strand RNA virus and the most common viral pathogen that causes acute lower respiratory tract infection worldwide among children under five years old[42]. Severe RSV infection in early childhood is closely related to recurrent wheezing and abnormal pulmonary function later in life[36]. The efficacy of Palivizumab, the only FDA-approved monoclonal antibody for prophylactic clinical application in RSV disease, remains limited[2]. Ribavirin, an anti-viral drug, used to treat virus infection, also exhibits considerable side effects[33]. Overall, despite its high incidence and economic burden that it imposes on the society, there is currently no specific therapeutic drug or reliable preventive vaccine against the RSV disease[51].

With long history, traditional medicine or alternative medicine has its unique and perspective advantages. Natural products have played pivotal roles in the drug discovery and development process, and have been widely applied to treat a variety of diseases. Andrographis paniculata (Burm.f.) Nees, which belongs to the family Acanthaceae, is an annual herbaceous plant, native to India and Sri Lanka, but widely cultivated in Southeastern Asia[4]. It has long been used in traditional medicine to treat fever, dysentery, malaria, diabetes, snake bites, respiratory diseases, skin infections, urinary tract infections and so on since its discovery in 1951[19] [53] [1] [48] [13]. Diterpene lactones, flavonoids, and polyphenols are main bioactive compounds extracted from Andrographis paniculata (AP)[5]. Diterpene lactones such as andrographolide (AD), 14-deoxy-11,12-didehydroandrographolide (DDAD), and neoandrographolide (NAD) are reported as major components to exert the therapeutic properties of AP[38].
In Asian countries, including China, andrographolide has been successfully used to cure respiratory infection[21]. Among its multi-pharmacological effects, andrographolide has been found to exert antiviral function over a variety of viruses, such as influenza A virus[10] and ZIKA virus[22]. Since 2020, new attention has been drawn to andrographolide and its therapeutic advantage has been re-estimated due to their beneficial role in the combat against COVID-19 [43]. However, despite the wide clinical application of andrographolide in viral infection, there are few relevant research focusing on its effects and mechanism on RSV infection so far.

Traditional or alternative medicines did not become widely accepted due to unclear mechanisms of action. With the rapid development of bioinformatics and systematic biology, network pharmacology has been recognized as a highly effective method that has been successfully used to predict the multiple targets and mechanisms of traditional medicines in numerous diseases[17]. The present study, therefore, designed to firstly identify whether andrographolide has antiviral effect on RSV infection in vitro. Then aimed to analyze the potential targets of andrographolide for the treatment of RSV infection using online databases, and conducted in vitro experiments to uncover the underlying mechanisms.

**Materials And Methods**

**Reagent, Cell Lines, and Virus preparation**

Andrographolide was purchased from APExBIO (N1855, APExBIO, USA, purity 99.67%), with High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) reported quality control. Dimethyl sulfoxide (DMSO) was purchased (Solarbio, China). The Human lung carcinoma epithelial cell line A549 (CCL-185), and the human laryngeal cancer epithelial cell line HEp-2 (CCL-23) was obtained from the American Type Culture Collection (ATCC, USA), with Short Tandem Repeat (STR) authentication. Cells were screened to exclude from Mycoplasma contamination using a Mycoplasma Detection Kit (Yise Med, China). RSV-A2 strain (VR-1540, ATCC, USA) was grown in HEp-2 cells and purified by density gradient[14]. The virus titer of used RSV was 1.5 *10^8 PFU/ml, determined by serial dilution plaque assay as previously described[30].

**Cell Counting Kit-8**

To investigate whether andrographolide induces cytotoxicity, A549 cells were seeded in 96-well plates at a density of 3×10^4/mL and pre-incubated with Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (AusgeneX, Australia) at 37°C and 5% CO₂ overnight. Then cells were incubated with medium containing a serial concentration of andrographolide (5, 10, 20, 40, 80 µM) for 36 h. Wells without cells were set as blank, while cells incubated with 10% FBS culture medium were employed as control. After incubation, each well was replaced with 100µL medium supplemented with 10% CCK-8 reagent (Dojindo, Japan) and incubated for 2 h. Absorbance at 450 nm were detected using microplate reader and cell viability was calculated.

**Cell culture and RSV infection**
A549 was cultured and passaged in DMEM supplemented with 10% FBS at 37°C and 5% CO₂. For RSV infection, the cells were seeded in 12- or 6-well plates at a density of 3×10⁵/mL overnight. When the monolayer cell density reached ~80%, RSV A2 was added at a multiplicity of infection of 1. After incubation for 2 h, the culture medium was replaced with DMEM containing 2% FBS. Where used, 5 µM or 10 µM andrographolide or the same DMSO were added to the culture medium.

**RNA isolation and RT-qPCR**

After incubation for 24 h and 36 h, total A549 cells infected with RSV or not in 12-well plates were washed twice with phosphate buffer saline (PBS). RNA was extracted using RNAiso Plus reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentration and A260/A280 ratio were detected using NanoDrop™ One/OneC (Thermo Scientific, USA), and 1.0 µg of the RNA was reversely transcribed using a TaKaRa kit (TaKaRa, Japan). RSV N gene load was also quantified using TaqMan (TaqMan, USA) qPCR as previously described[9], and the qPCR cycle conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The mRNA levels of target genes on each sample were measured and calculated according to the 2⁻ΔΔCt method. GAPDH expression was quantified in all samples as reference. The primer sequences are shown in Table 1. The reaction conditions of qPCR were as follows: 95°C for 5 min, then a total of 40 cycles of 95°C for 10 s, 59°C for 15 s, and 72°C for 1 min.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5'-3')</th>
<th>Reverse (3'-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>GCTGAGGTGGACATCACAGGCA</td>
<td>TGCTGTCAAGGGTTTGGCTC</td>
</tr>
<tr>
<td>CCL-5</td>
<td>CCTGCTGCTTTGGGCTACATTGC</td>
<td>ACACACTGGGCGGTTCCTTCGG</td>
</tr>
<tr>
<td>JAK2</td>
<td>CCAGATGGGAACGTTCGCTCAG</td>
<td>GAGGTTGGTACATCAGAACC</td>
</tr>
<tr>
<td>STAT1</td>
<td>TGTATGCCATCCTCGAGACGC</td>
<td>AGACATCCTGCCACCTTGTG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAATACCTGTGGCCCTTGGGC</td>
<td>TTTGGGATCTACACTCTCCAGCT</td>
</tr>
<tr>
<td>RSV-N</td>
<td>AGATCAACTTCTGTGCTCACCGA</td>
<td>TTCTGACATCATAATTGAGATATCAAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ATCAAGAAGGTGGTGAACGAGGC</td>
<td>TCAAGGTTGGAGATGGGTGTC</td>
</tr>
</tbody>
</table>

**Cell supernatant collection and Cytokine levels measurement**

After incubation for 36 h, cell supernatants were collected after centrifuging at 3000 rpm for 5 min at 4°C. Human IL-6 and IL-8 enzyme-linked immunosorbent assay (ELISA) (NEOBIOSCIENCE, China) was used to detect cytokine levels.

**Identification of RSV infection-related genes**
RNAseq dataset GSE32139 was downloaded from the Gene Expression Omnibus (GEO) database(https://www.ncbi.nlm.nih.gov/geo/). Human primary airway epithelial cells were harvested and cultured in a polarized system, and then infected with RSV. For the gene expression profile in GEO, the raw data were downloaded and normalized by the robust multiarray averaging method. Missing values were detected and omitted. The probes were then converted into the corresponding gene symbols according to the platform of mRNA dataset. The log value of each expression was adjusted by its mean and auto-scaled by its standard deviation. We defined $P < 0.05$ and $|\log \text{FC}| > 0.05$ as cutoff values for screening the differentially expressed genes (DEGs). The “DESeq2” package of R software was used to analyze differentially expressed gene sets.

Identification of andrographolide-related genes

The chemical structure of andrographolide was downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/), and imported to the PharmMapper Database (http://www.lilab-ecust.cn/pharmmapper/) to retrieve the predicted target proteins. PharmMapper Database is a free online reverse-docking database for potential target identification of small molecules using the pharmacophore mapping approach[49]. The gene names of target proteins were extracted from the website of UniProt Knowledgebase (http://www.uniprot.org).

GO and KEGG pathway enrichment analysis

The RSV infection-related and the andrographolide-related genes were merged and uploaded to the online Venn diagram package (http://bioinfogp.cnb.csic.es/tools/venny/index.html) for mapping. The intersection of the two parts was considered as the potential targets of andrographolide for the treatment of RSV infection. The “cluster Profiler” package of R software was used to conduct GO, KEEG analysis of the intersected genes.

Flow cytometry analysis

Following RSV infection in 6-well plates, A549 cells were incubated with culture medium or andrographolide (5µM, 10µM) for 36 h. Cell death was measured using the Annexin V-FITC/PI apoptosis assay kit (NEOBIOSCIENCE, China) according to the manufacturer’s instructions. Viable and apoptotic cells were differentiated and characterized by quadrant separation. The Annexin V+, PI- cells were considered as early apoptosis, and the Annexin V+, PI+ cells as late apoptosis /necrosis[46]. The results were presented as mean percentage ratio of five biological replicates.

Western blot (Immunoblot) analysis

Following RSV infection for 36 h, total A549 cells in 6-well plates were washed twice with PBS, then harvested and lysed using RIPA buffer (CST, USA) containing PMSF (CST, USA) on ice. Lysates were centrifuged at 14,000 g for 10 min at 4°C to obtain the supernatants. Samples containing equal quantities of protein were resolved in 10% SDS-PAGE and then transferred onto PVDF membranes
(MERCK MILLIPORE, Germany). The membranes were blocked with 5% BSA for 1 h and probed with primary antibodies against Caspase-1 (1:1000; Zen-bio, China), cleaved Caspase-1 (1:1000; Abcam, UK), cleaved IL-1β (1:1000; Affinity, China), N terminal of GSDMD (1:1000; Abcam, UK), Bcl-2 (1:1000; Proteintech Group, USA) at 4°C overnight. The GADPH (1:5000; Proteintech Group, USA) was probed as internal reference. Alkaline phosphatase-conjugated goat anti-mouse antibody (1:5000; Proteintech Group, USA) and goat anti-rabbit antibody (1:5000; Proteintech Group, USA) were used as secondary antibodies, and incubated at room temperature for 1 h. The protein bands were detected by using an ECL kit (MERCK MILLIPORE, Germany).

**Statistical analysis**

Statistical analysis related to bioinformatics was conducted as described above. Besides, all statistical tests were performed with Prism GraphPad Software (La Jolla, CA). Quantitative data are expressed as mean values ± standard deviation of three independent experiments. At least five biological replicates were used for statistical analysis. The unpaired Student t test or ANOVA were used to determine differences among all groups. Differences were considered significant when P values were less than 0.05.

**Results**

**Andrographolide decreased RSV viral load and attenuated inflammation in vitro.**

Andrographolide has been found to exert antiviral function over a variety of viruses, but its role on RSV infection has not been reported yet. To clarify whether andrographolide has antiviral effect on RSV, we conducted in vitro experiment using human lung carcinoma epithelial cell line A549. Firstly, we investigated whether andrographolide induced cytotoxicity in A549 cell line and selected appropriate concentrations to conduct in vitro experiment. As shown in Fig. 1A, obvious impact on cell viability was seen in concentration more than 20 µM after 36 h treatment. Therefore, we selected 5µM and 10µM as appropriate concentration to conduct following in vitro experiment. Next, we measured the RSV N gene load both at 24 h and 36 h post-infection. After treatment with andrographolide, RSV viral load was noticeably decreased at 36 h dose-dependently (Fig. 1B). To further elucidate the impact of andrographolide on RSV-induced inflammation in vitro, we measured IL-6 and IL-8 levels in cell supernatants 36 h post-infection. Both cytokine levels were dramatically increased post RSV infection, while andrographolide attenuated them significantly (Fig. 1C and D).

**Identification of RSV infection-related genes**

To identify RSV regulated cellular genes, RNAseq dataset GSE32139 was downloaded from the Gene Expression Omnibus (GEO) database. As presented in Table 2, there were a total of 1519 DEGs between the mock-infected and the RSV group, and among them, 891 were up-regulated, while 628 were down-regulated. The results are presented as heatmap of all DEGs (Fig. 2A), and volcano plot (Fig. 2B). DEGs represented in red were up-regulated, while those in blue were down-regulated. Detail information about
the DEGs of RSV infection was presented as supplementary material (Supplementary Materials Table S1).

### Table 2

<table>
<thead>
<tr>
<th>Compare</th>
<th>All</th>
<th>Up</th>
<th>Down</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV vs Mock</td>
<td>1519</td>
<td>891</td>
<td>628</td>
<td>DESeq2 pvalue&lt;0.05</td>
</tr>
</tbody>
</table>

**Andrographolide-related target identification via PharmMapper database**

PharmMapper Database and PubChem were used as described in the Materials and Methods. The chemical structure of andrographolide (Fig. 3A) and the potential targets of andrographolide along with their gene symbols are presented in Supplementary Table S2.

**GO and KEGG pathway enrichment analysis**

As illustrated in Fig. 3B, RSV infection-related genes and andrographolide target genes were merged, and the intersection of the two sets was considered as the potential targets of andrographolide for the treatment of RSV infection. There were 25 genes in total, as presented in Table 3. To further understand these targets, GO and KEGG enrichment analysis were performed and shown in Fig. 3C and D. The top five significant functions were regulation of cytokine-mediated signaling pathway, regulation of response to cytokine stimulus, cellular response to interferon-gamma (IFN-γ), regulation of response to IFN-γ, and regulation of IFN-γ-mediated signaling pathway. The KEGG pathways involved were influenza A, pyruvate metabolism, necroptosis, steroid hormone biosynthesis, NOD-like receptor signaling pathway, cytosolic DNA-sensing pathway, chemokine signaling pathway, prolactin signaling pathway, Leishmaniasis, lipid and atherosclerosis, Coronavirus disease COVID-19, PD-L1 expression and PD-1 checkpoint pathway in cancer, Th1 and Th2 cell differentiation, Toll-like receptor signaling pathway, C-type lectin receptor signaling pathway, Th17 cell differentiation, toxoplasmosis, growth hormone synthesis secretion and action, and 2-Oxocarboxylic acid metabolism. Target genes related to these pathways are listed in Table 4.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Target protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH1C</td>
<td>Alcohol dehydrogenase 1C</td>
</tr>
<tr>
<td>AMY1A</td>
<td>Alpha-amylase 1A</td>
</tr>
<tr>
<td>ANXA5</td>
<td>Annexin A5</td>
</tr>
<tr>
<td>BCAT2</td>
<td>Branched-chain-amino-acid aminotransferase, mitochondrial</td>
</tr>
<tr>
<td>CASP1</td>
<td>Caspase-1</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine 5</td>
</tr>
<tr>
<td>CFD</td>
<td>Complement factor D</td>
</tr>
<tr>
<td>DUSP6</td>
<td>Dual specificity protein phosphatase 6</td>
</tr>
<tr>
<td>GLO1</td>
<td>Lactoylglutathione lyase</td>
</tr>
<tr>
<td>GMPR</td>
<td>GMP reductase 1</td>
</tr>
<tr>
<td>HNMT</td>
<td>Histamine N-methyltransferase</td>
</tr>
<tr>
<td>JAK2</td>
<td>Tyrosine-protein kinase JAK2</td>
</tr>
<tr>
<td>LTA4H</td>
<td>Leukotriene A-4 hydrolase</td>
</tr>
<tr>
<td>MMP12</td>
<td>Macrophage metalloelastase</td>
</tr>
<tr>
<td>MMP13</td>
<td>Collagenase 3</td>
</tr>
<tr>
<td>NR1H3</td>
<td>Oxysterols receptor LXR-alpha</td>
</tr>
<tr>
<td>SEC14L1</td>
<td>SEC14-like protein 1</td>
</tr>
<tr>
<td>SORD</td>
<td>Sorbitol dehydrogenase</td>
</tr>
<tr>
<td>SPARC</td>
<td>SPARC</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1-alpha/beta</td>
</tr>
<tr>
<td>STS</td>
<td>Steryl-sulfatase</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>Sulphotransferase Family 1E Member 1</td>
</tr>
<tr>
<td>TNNC1</td>
<td>Troponin C, slow skeletal and cardiac muscles</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>TYMS</td>
<td>Thymidylate synthase</td>
</tr>
</tbody>
</table>
Table 4
Target genes involved in KEGG pathways

<table>
<thead>
<tr>
<th>ID</th>
<th>KEGG Pathway</th>
<th>Pvalue</th>
<th>Gene Symbol</th>
</tr>
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<tr>
<td>hsa05164</td>
<td>Influenza A</td>
<td>0.000886596</td>
<td>CASP1/CCL5/JAK2/STAT1</td>
</tr>
<tr>
<td>hsa00620</td>
<td>Pyruvate metabolism</td>
<td>0.006454215</td>
<td>ADH1C/GLO1</td>
</tr>
<tr>
<td>hsa04217</td>
<td>Necroptosis</td>
<td>0.007619001</td>
<td>CASP1/JAK2/STAT1</td>
</tr>
<tr>
<td>hsa00140</td>
<td>Steroid hormone biosynthesis</td>
<td>0.010690573</td>
<td>STS/SULT1E1</td>
</tr>
<tr>
<td>hsa04621</td>
<td>NOD-like receptor signaling pathway</td>
<td>0.011355888</td>
<td>CASP1/CCL5/STAT1</td>
</tr>
<tr>
<td>hsa04623</td>
<td>Cytosolic DNA-sensing pathway</td>
<td>0.011373674</td>
<td>CASP1/CCL5</td>
</tr>
<tr>
<td>hsa04062</td>
<td>Chemokine signaling pathway</td>
<td>0.01274064</td>
<td>CCL5/JAK2/STAT1</td>
</tr>
<tr>
<td>hsa04917</td>
<td>Prolactin signaling pathway</td>
<td>0.013911961</td>
<td>JAK2/STAT1</td>
</tr>
<tr>
<td>hsa05140</td>
<td>Leishmaniasis</td>
<td>0.016673315</td>
<td>JAK2/STAT1</td>
</tr>
<tr>
<td>hsa05417</td>
<td>Lipid and atherosclerosis</td>
<td>0.017247702</td>
<td>CASP1/CCL5/JAK2</td>
</tr>
<tr>
<td>hsa05171</td>
<td>Coronavirus disease - COVID-19</td>
<td>0.021089305</td>
<td>CASP1/CFD/STAT1</td>
</tr>
<tr>
<td>hsa05235</td>
<td>PD-L1 expression and PD-1 checkpoint pathway in cancer</td>
<td>0.021902894</td>
<td>JAK2/STAT1</td>
</tr>
<tr>
<td>hsa04658</td>
<td>Th1 and Th2 cell differentiation</td>
<td>0.023304382</td>
<td>JAK2/STAT1</td>
</tr>
<tr>
<td>hsa04933</td>
<td>AGE-RAGE signaling pathway in diabetic complications</td>
<td>0.027218185</td>
<td>JAK2/STAT1</td>
</tr>
<tr>
<td>hsa04620</td>
<td>Toll-like receptor signaling pathway</td>
<td>0.029268752</td>
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<tr>
<td>hsa04625</td>
<td>C-type lectin receptor signaling pathway</td>
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<tr>
<td>hsa04935</td>
<td>Growth hormone synthesis secretion and action</td>
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<td>hsa01210</td>
<td>2-Oxocarboxylic acid metabolism</td>
<td>0.048190537</td>
<td>BCAT2</td>
</tr>
</tbody>
</table>

RT-qPCR verification of potential target genes

To verify change of gene mRNA expression analyzed by network pharmacology, we extracted total RNA at 24 and 36 h post RSV infection on A594 cells. The expression level of target genes (CASP1/CCL5/JAK2/STAT1) and the downstream gene of CASP1 (which also functions as IL-1β-converting enzyme) were measured using RT-qPCR. As shown in Fig. 4, RSV infection dramatically elevated the expression of CASP1, CCL5, JAK2, STAT1 and IL-1β both at 24 and 36 h. Furthermore,
andrographolide clearly mitigated the increase of CASP1, CCL5, JAK2, STAT1 and IL-1β mRNA levels at
36 h.

**Andrographolide inhibited apoptosis of RSV-infected epithelial cells**

There are three major programmed cell death pathways, namely, apoptosis, pyroptosis and necroptosis, which have been documented to be involved in airway epithelial cells infected with viruses[35]. Airway epithelial cell death caused by viral infection has been considered an important defense mechanism that restricts virus replication and spread[18]. Since CASP1 is an important gene involved in pyroptosis signal pathway[41], we conducted flow cytometry analysis using Annexin V-FITC/PI dual staining to figure out whether andrographolide have effect on cell death after RSV infection. A549 cells, intervened or not with
andrographolide, were collected after 36h infection with RSV, and was stained with Annexin V-FITC/PI according to the manufacturer’s instruction. We defined Annexin V positive, PI negative quadrant as early apoptotic cells, while dual Annexin V and PI positive quadrant as late apoptotic or necrotic cells[46]. After 36 h of infection, RSV noticeably increased the ratio of apoptosis of A549 cells. Andrographolide obviously inhibited apoptotic ratio of RSV-infected cells, which was dose-dependent responsive. Similar effect was not seen in uninfected cells (Fig. 5).

**Andrographolide elevated protein levels of caspase-1, cleaved caspase-1, N terminal of GSDMD and Bcl-2 to suppress apoptosis and promote pyroptosis of RSV infected cells**

As the results of RT-qPCR and flow cytometry had indicated, andrographolide may have impact on death pattern of airway epithelial cells infected with RSV. In the process of pyroptosis, formation of inflammasome cleaves pro-caspase-1 into its active form, which then cleaves Gasdermin-D (GSDMD), pro-IL-1β, pro-IL-18, and pro-IL-33 into biologically active forms[15], resulting the production of IL-1β, IL-33, IL-18 and pyroptosis[28, 40]. Therefore, we measured the protein levels of pro-caspase-1, cleaved caspase-1, cleaved IL-1β and N terminal of GSDMD at 36 h post RSV infection to further elucidate the effects of andrographolide on pyroptosis. The results showed that RSV infection decreased the protein levels of all four, but andrographolide elevated them (Fig. 6). Since B-cell lymphoma-2 (Bcl-2) family play a pivotal role in regulating cell apoptosis, we also assessed the expression level of antiapoptotic protein Bcl-2 to identify the effects of andrographolide on apoptosis. RSV suppressed the expression of antiapoptotic protein Bcl-2 at 36 h post infection, while andrographolide increased it (Fig. 6). Based on the above results, we hypothesized that andrographolide elevated protein levels of caspase-1, cleaved caspase-1, N terminal of GSDMD and Bcl-2 to suppress apoptosis and promote pyroptosis of RSV infected cells.

**Discussion**

In this study, we discovered that andrographolide decreased RSV viral load and attenuated inflammation in vitro. By using online databases to reveal the RSV infection-related genes and the potential targets of andrographolide, we then verified the change of potential target genes in infection studies in vitro. RSV infection strongly elevated the mRNA level of CASP1, CCL5, JAK2 and STAT1, while andrographolide suppressed the increase of them. Moreover, IL-1β, which is activated by CASP1-mediated cleavage, was
also enormously up-regulated after RSV infection and conspicuously down-regulated by andrographolide at 36 h. Next, we conducted Annexin V-FITC/PI dual staining and Western blot analysis of pivotal proteins involved in apoptosis and pyroptosis to figure out whether andrographolide has influence on death pattern of RSV-infected A549 cells. We found that andrographolide inhibited apoptosis but promoted pyroptosis of infected cells via elevating the protein levels of caspase-1, cleaved caspase-1, N terminal of GSDMD and Bcl-2, indicating that andrographolide might switch the death pattern of RSV-infected cells from apoptosis to pyroptosis. Therefore, we speculated that andrographolide may decreased RSV viral load and attenuated inflammation due to the suppression of apoptosis and promotion of pyroptosis. As far as we known, this is the first time andrographolide was proved to have effect on cell death pattern after RSV infection.

As indicated earlier, andrographolide has attracted much attention and been extensively studied due to its antivirus property. Plenty of studies have shown potential therapeutic properties against numerous viruses[20]. One study indicated that andrographolide's anti-H1N1 virus effects might be related to its inhibition of viral-induced activation of the RLRs signaling pathway[52]. As for EV-D68, Andrographolide inhibits acidification of the virus-containing endocytic vesicle and significantly decreases viral RNA replication and protein synthesis[47]. Despite the wide clinical application of andrographolide in viral infection, there is no relevant research on its effects on RSV infection so far. We conducted in vitro RSV infection model using A549 cell line, assessed andrographolide's antiviral effect via detection of RSV N gene load and pro-inflammatory cytokine levels in cell supernatant, and identified andrographolide decreased RSV viral load and attenuated inflammation in vitro.

By using a network pharmacology approach, we speculated that cell death pattern might be participated in the underlying anti-RSV mechanism of andrographolide. Airway epithelial cell death caused by viral infection has been considered an important defense mechanism that restricts virus replication and spread[18]. There are three major programmed cell death pathways, namely, apoptosis, pyroptosis and necroptosis, which have been documented to be involved in airway epithelial cells infected with viruses[35]. Studies have shown that RSV non-structural proteins (NS1 and NS2) can inhibit pro-apoptotic proteins and promote anti-apoptotic proteins synthesis, which interferes with host cell apoptosis at the early phase of infection, allowing the virus to complete replication[44]. However, at the late phase of infection, the expression of RSV F protein can activate the phosphorylation of p53, leading to transcription of pro-apoptotic proteins and caspase activation, which results in apoptosis of epithelial cells and spread of virus [11]. It has been proposed that apoptosis of infected airway epithelial cells cannot effectively restrict the replication of RSV and may help the virus spread into extrapulmonary organs of the host [12]. Pyroptosis, another major programmed cell death pathway, is a primary cellular response following the sensing of potentially damaging insults, including pathogen ligands, DAMPs, altered levels of host metabolites and environmental irritants[31], and results in the lysis of the affected cells. Recent studies have provided evidence that pyroptosis occurs in vivo, and that it is a potent mechanism used to clear intracellular pathogens[29, 32] and Shigella flexneri was reported to inhibit pyroptosis to evade anti-Shigella humoral immunity[24].
In our study, RSV mitigated the antiapoptotic protein Bcl-2 expression to promote apoptosis, and decreased the expression of caspase-1, cleaved caspase-1 and N terminal of GSDMD to inhibit pyroptosis in A549 cells at 36 h post infection. While andrographolide elevated protein levels of those four to suppress apoptosis and promote pyroptosis of RSV infected cells. Several studies have reported that RSV infection induced the expression of antiapoptotic proteins Bcl-2, which is consistent with our finding, indicating the suppression effect of RSV infection on cell apoptosis. Andrographolide was reported to exhibit anti-apoptotic potential through the activation of the Akt-BAD pathway in HUVECs during growth factor (GF) deprivation-induced apoptosis. It was also shown to inhibit renal tubular cell apoptosis, cardiac apoptosis, and regulate the apoptosis-NETosis balance of neutrophils to ameliorate Rheumatoid Arthritis. Recently, study on Enterovirus 71 (EV71) infection had shown similar effect that it inhibited EV71-induced RD cells apoptosis.

Although pyroptosis is highly important for an effective antiviral response, it can also contribute to the immunopathology and excessive inflammation. The main pathological effect of RSV infection is excessive inflammatory response and tissue damage. Among many pro-inflammatory cytokines released after RSV infection, such as those of the IL-1 family, IL-6, and tumor necrosis factor (TNF-α), IL-1β acts as a predominant pro-inflammatory factor. However, in our study, the mRNA expression levels of caspase-1 and IL-1β decreased after andrographolide treatment. We speculated that andrographolide may exert a negative feedback regulation after inducing pyroptosis to promote virus clearance and restricts the replication of the virus, so as to avoid the excessive synthesis of caspase-1 and IL-1β that would otherwise cause excessive inflammation.

Nevertheless, there were some obvious limitations in our study, which still need improvement. First, our experiments were performed in vitro, and therefore, the effects require in vivo confirmation. Second, a detailed molecular mechanism of how andrographolide affects the apoptosis and pyroptosis in RSV infection, remains to be fully elucidated. Clearly, further experiments should be conducted to investigate how andrographolide influences the death pattern of RSV-infected airway epithelial cell and how it translates into ameliorating the pathology in an animal model for eventual clinical applications.

Declarations

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Author contributions

All authors contributed to the study conception and design. SC performed the experiments, data analysis and manuscript draft. JL performed the bioinformatics data analysis. EL and JX critically revised the manuscript. All authors read and approved the final manuscript.
Conflict of interest

The authors declare they have no conflict of interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Andrographolide decreased RSV viral load and attenuated inflammation in vitro. (A) Cytotoxicity of andrographolide on A549 cells were tested using CCK-8 kit. After RSV infection (MOI=1), cells were treated with andrographolide (5μM and 10μM) or not for 36 h. (B) Total RNA was extracted to quantify the RSV N gene load. (C) (D) IL-6 and IL-8 levels of cell supernatants were detected. Data were shown as Mean ± SD of three independent experiments (n≥5). Data were analyzed using one-way ANOVA. ***P<0.005.
Figure 2

Identification of RSV infection-related genes. RNAseq dataset GSE32139 was downloaded from the Gene Expression Omnibus (GEO) database(https://www.ncbi.nlm.nih.gov/geo/). (A) Heatmap of RSV infection-associated differentially expressed genes (DEGs) analyzed in GSE32139. (B) Volcano plot of RSV infection-associated differentially expressed genes (DEGs) analyzed in GSE32139.
Figure 3

Potential targets of andrographolide for the treatment of RSV infection. PharmMapper Database (http://www.lilab-ecust.cn/pharmmapper/) were used to retrieve the predicted target proteins of Andrographolide. The RSV infection-related and the andrographolide-related genes were merged and the intersection of the two parts was considered as the potential targets of andrographolide for the treatment of RSV infection. (A) Chemical structure of Andrographolide. (B) Venn diagram of potential targets of Andrographolide for RSV infection. (C) GO enrichment analysis of 25 potential target genes. (D) KEGG pathway analysis of 25 potential target genes.
Figure 4

Quantitative real-time polymerase chain reaction verification. Total RNA of A549 cells treated with andrographolide (5μM and 10μM) or not for 24 h and 36 h post RSV infection (MOI=1) were extracted to determine the mRNA expression level of Caspase-1(CASP1), CCL5, JAK2, STAT1 and IL-1β by quantitative real-time PCR. (A) The mRNA expression levels of CASP1. (B) The mRNA expression levels of CCL5. (C) The mRNA expression levels of JAK2. (D) The mRNA expression levels of STAT1. (E) The mRNA expression levels of IL-1β. Data were shown as Mean ± SD of three independent experiments (n≥ 4). Data were analyzed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.005.
Figure 5

Andrographolide inhibited apoptosis of RSV-infected epithelial cell. (A) A549 cells treated with andrographolide (5μM and 10μM) or not at 36 h post RSV infection (MOI=1) were stained with Annexin V-FITC/PI. (B) Percentage of apoptotic cells were shown (n=6). Data were analyzed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.005.
Andrographolide suppressed apoptosis and promoted pyroptosis of RSV infected cells. A549 cells treated with andrographolide (10\(\mu\)M) or not at 36 h post RSV infection (MOI=1) were collected to extract whole protein. Protein levels of caspase-1, cleaved caspase-1 P20, cleaved-IL-1\(\beta\), Bcl-2, N terminal of GSDMD were determined by Western blotting. GAPDH was determined as reference protein. Fold change of caspase-1, cleaved caspase-1 P20, cleaved IL-1\(\beta\) Bcl-2, N terminal of GSDMD over GAPDH was calculated. Three independent experiments were conducted.

**Supplementary Files**

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