

# Guanosine and uridine alleviate airway inflammation via inhibition of the MAPK and NF- $\kappa$ B signals in OVA-induced asthmatic mice

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## ABSTRACT

Asthma is one of the most common respiratory diseases. Lack of response or poor adherence to corticosteroids demands the development of new drug candidates for asthma. Endogenous nucleosides could be potential options since uridine has been reported to have an anti-inflammatory effect in asthma model. However, its molecular pathways and whether other nucleosides have similar therapeutic effects remain untouched. Thus, we herein report our investigation into the anti-inflammatory effects of guanosine and uridine, and the related inner signaling pathways in asthma model. Present study shows that administration of guanosine or uridine can reduce lung inflammation in OVA-challenged mice. Total cell counts in BALF, cytokines such as IL-4, IL-6, IL-13, OVA-specific IgE and mRNA level of Cxcl1, Cxcl3, IL-17 and Muc5ac were decreased in asthmatic mice after treatment. Besides, the production of IL-6 in LPS/IFN- $\gamma$  induced THP-1 cells was also decreased by both nucleosides. *In vivo* and *in vitro* expressions of key molecules in the MAPK and NF- $\kappa$ B pathways were reduced after the treatment of both compounds. These findings suggest that guanosine has a similar potential therapeutic value in asthma as uridine and they exert anti-inflammatory effects through suppression of the MAPK and NF- $\kappa$ B pathways.

## 1. Introduction

With an overall 4.3% prevalence in adults, asthma is one of the most common chronic, non-communicable respiratory diseases globally, albeit with wide regional variations [1]. It was reported that about 358.2 million people are suffering from asthma in 2015, with an increasing rate of ~12.6% since 1990 [2]. Apart from having devastating effects on select individual patients, asthma also causes enormous societal burdens [2,3]. Inhaled corticosteroids (ICS) are the mainstay maintenance treatment for most patients, but the compliance of ICS is somewhat poor in both children and adults [4] and a certain proportion of patients are glucocorticoid resistant [5]. In some patients' minds, the short-acting  $\beta_2$  agonist (SABA) is safer and cheaper than ICS, and therefore more acceptable to them. For instance, only 5.6% of all patients with asthma had received regular treatment with ICS in China [6]. SABA-only treatment is unfortunately related to increased risk of exacerbations and death according to Global Initiative for Asthma 2019. Furthermore, the use of glucocorticoids also has its own set of disadvantages based on

its side effects [7]. Thus, alternative therapeutic molecular classes in this area are still greatly desired.

Nucleosides are mostly known for their role as fundamental building blocks of genetic materials. They also participate in various cellular processes, including energetic metabolism, and signaling transduction in autocrine and/or paracrine settings [8]. Consequently, nucleoside and nucleotide analogues are widely employed as therapeutic agents against cancer, viral and other infectious microbial diseases [8]. Galmarini et al. systematically reviewed the clinical applications of nucleosides and nucleotide analogues in cancer treatment [9]. Jordheim et al. furthered the advances of nucleoside and nucleotide analogues in the context of viral and cancerous diseases, and delineated the various mechanisms of their actions [10]. Following the surge of immunotherapy against cancer, the immuno-modulating roles of nucleosides, especially adenosine, have drawn greater research interests [11,12]. The immuno-modulating properties of adenosine are involved with adenosine receptors and their downstream signals, which are also entwined with the pathophysiological processes underlying asthma [13]. Currently, there are ongoing efforts towards developing adenosine

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**Abbreviation**

|      |                                 |
|------|---------------------------------|
| AHR  | airway hyper-responsiveness     |
| BALF | bronchoalveolar lavage fluid    |
| H&E  | hematoxylin and eosin           |
| ICS  | inhaled corticosteroids         |
| i.p  | intraperitoneal injection       |
| i.t  | intratracheal injection         |
| MCh  | methacholine                    |
| OVA  | ovalbumin                       |
| Penh | enhanced pause                  |
| PMA  | phorbol 12-myristate 13-acetate |
| SABA | short-acting $\beta_2$ agonist  |
| SAL  | saline                          |
| Th17 | T helper 17                     |
| WBP  | Whole-body Plethysmography      |

related compounds as anti-asthma drugs [14].

Apart from adenosine, there is only sporadic research concerning the anti-asthma effects of other nucleosides. From a systematic perspective, we were intrigued to investigate whether other endogenous nucleosides have the anti-asthmatic properties. Uridine was reported to have anti-inflammatory effects in animal models of asthma, but the specific molecular mechanisms remain unclear [15]. As adenosine's purine counterpart, guanosine was previously investigated concerning its protective effects in central nervous system disorders [16], but so far there has been no corresponding investigations into its effects on asthma. The good safety profiles of both compounds [17,18] prompted us to evaluate their effectiveness as new anti-asthma therapeutic options. Meanwhile we aimed to examine the corresponding molecular pathways to gain deeper insight into further tailoring them as new asthma therapeutics for clinical translation.

Asthma is characterized by bronchial inflammation, airway hyper-responsiveness (AHR), airway remodeling and mucus hypersecretion. Focusing around these characteristics, we evaluated the effect and molecular pathways for guanosine and uridine on both animal and cellular levels based on well-established methods. In our animal model, bronchial inflammation was assessed in ovalbumin (OVA)-induced mice, by histopathological examination and assaying of inflammatory mediators; enhanced pause (Penh) value after inhalation of methacholine (MCh) was used as a surrogate measurement of AHR; mucus secretion was examined by Muc5ac mRNA expression [19]. The classically activated (M1) macrophage, constructed from LPS and IFN- $\gamma$  induced THP-1 cells, was chosen as the investigating model at the cellular level [20] to evaluate effects of nucleosides on inflammation responses. To gain insight into relevant molecular pathways, crucial signaling cascades were investigated in both animal and cellular models. The overall results indicate for the first time that both guanosine and uridine can suppress lung inflammation in mice, and the mechanism of their action is associated with the inhibition of the MAPK and NF- $\kappa$ B pathways.

## 2. Materials and methods

### 2.1. Asthmatic animal model and the administration of agents

Animal studies were approved by the Institutional Animal Care and Use Committee of Sichuan University. Female BALB/c mice of 6–8 weeks old [21–24] were purchased from the Dossy Experimental Animal Company (Chengdu, China), and housed under specific pathogen-free conditions with a 12-h light-dark cycle and standard 4% fat chow. On days 1 and 14, mice were sensitized by intraperitoneal injections of 40  $\mu$ g OVA (Grade V, Sigma-Aldrich) in 100  $\mu$ L PBS (Hyclone) containing 4 mg of aluminum hydroxide (Thermo Scientific Pierce Imject Alum,

Rockford, IL, USA) or with 4 mg aluminum hydroxide in 100  $\mu$ L PBS as normal control. From days 24–26, mice were challenged with 1% OVA (in 0.9% NaCl) aerosols or 0.9% NaCl (SAL, saline) for 30 min each day using an ultrasonic nebulizer (PARI). 1 h before OVA or 0.9% NaCl aerosols, each group received according treatment: normal group (i.p. PBS), disease model group (i.p. PBS), uridine group (i.t. 6.1 mg/kg), guanosine group (i.p. 6.1 mg/kg), dexamethasone (i.p. 1 mg/kg) (Sigma-Aldrich).

### 2.2. Determination of average Penh values

Murine average Penh values were measured in response to increasing doses of MCh by Whole-body Plethysmography (WBP) (Buxco Research Systems) and enhanced pause (Penh) was used here as a surrogate measurement of airway responsiveness [25]. 24 h after the final OVA-aerosol challenge, mice were acclimatized within the WBP chamber for 10 min and baseline readings were recorded for 3 min. Aerosolized PBS and increasing doses of MCh (12.5, 25, 50, 100 mg/mL) were nebulized for 2 min. After each nebulization, Penh values were recorded for 6 min.

### 2.3. Analysis of BALF, lung histology and immunohistochemistry

24 h after the last OVA challenge, bronchoalveolar lavage fluid BALF samples were collected by 3 gentle, repeated infusing and withdrawing of 0.6 mL cold PBS into the right lung. The collected BALF was centrifuged at 3500 rpm for 15 min at 4 °C. Then, the cell pellet was softly resuspended in PBS and counted by automatic cell counter (countstar® BioTec). Murine left lung lobes were removed and fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were cut into 4  $\mu$ m sections, following deparaffinization for 10 min in xylene, rehydrate for 5 min in each decreased gradient ethanol (100%, 95%, 80%, 60%). For hematoxylin and eosin (H&E) stain, sections were stained with hematoxylin and eosin. For Masson stain, slices were stained in sequence in Weigert's iron-hematoxylin solution, ponceau acid fuchsin solution, aniline blue solution. For immunohistochemistry, slices were immersed in citrate buffer and heated in microwave to boiling for antigen retrieval, and then incubated in hydrogen peroxide (3%) to abolish the endogenous peroxidase activity. After blocking in 5% BSA, primary antibody was added into slices for incubation at 4 °C overnight. After washing slices with PBS three times, secondary antibody was added for incubation at 37 °C for 30 min, followed by incubation with diaminobenzidine, and then counterstained in hematoxylin. After staining, all slices were dehydrated in increasing gradient ethanol and xylene and sealed by neutral resin. Images were captured using microscopy (AX10 imager A2/AX10 cam HRC, Zeiss).

### 2.4. Measurement of OVA-specific IgE and cytokines by ELISA

24 h after the last OVA challenge, the plasma samples were obtained from blood collected by cardiac puncture for measurement of OVA-specific IgE. Lung tissues were grinded in liquid nitrogen and lysed by RIPA lysis buffer (Beyotime). Lysates were centrifuged at 13000 g for 10 min at 4 °C, the supernatants were collected and stored at –80 °C until assay. Protein concentrations were measured by BCA Protein Assay Kit (Beyotime). Levels of IL-4, IL-6, IL-13 in lung protein extracts [26] and OVA-specific IgE were analyzed by ELISA (Neobioscience) according to the manufacturer's recommendations. Equal amount of protein aliquots was added in antigen-coating plates and sequentially incubated at 37 °C with biotin-conjugated secondary antibody for 1 h, peroxidase-labeled streptavidin for 0.5 h, 3,3',5,5'-tetramethylbenzidine substrate for 15 min. Stop-reaction solution was added and the absorbance was read at 450 nm.

## 2.5. Cell culture and treatments

THP-1 cells were purchased from Cell Bank of Chinese Academy of Sciences in Shanghai. Cells were maintained in 1640 RPMI medium (Hyclone) supplemented with 10% fetal bovine serum in the presence of streptomycin (100 µg/mL), penicillin (100U/mL), 0.05 mM β-mercaptoethanol (Gibco) and incubated in an incubator with 5% CO<sub>2</sub> at 37 °C. The cells were seeded on 6 well plates at a density of ~10<sup>6</sup> cells/well and treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). After incubation for 24 h, the supernatants were discarded and the cells were washed twice with PBS, and then co-cultured with different doses of uridine, guanosine and dexamethasone in the presence of 100 ng/mL LPS (Sigma-Aldrich) and 20 ng/mL IFN-γ (Proteintech) for 24 h (5% CO<sub>2</sub>, 37 °C). Later, the supernatants were collected to assess the level of the IL-6 by ELISA kits and the cells were collected for western blot.

## 2.6. Western blot

Cells or lung tissues grinded in liquid nitrogen were lysed in RIPA (Beyotime) with protease and phosphatase inhibitors (Thermo Fisher Scientific) on ice for 30 min. After ultrasonication, the cell lysates were centrifuged for 10 min at 13000 rpm/min at 4 °C. Protein concentration in supernatants was measured by Protein BCA assay kit, then the mixture of supernatants was boiled with loading buffer for 5 min at 100 °C. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to PVDF membrane (Immobilon) by the wet transfer method (300 mA for 60 min), blocked in 5% skim milk in TBST buffer (25 mM Tris, 150 mM NaCl, pH 7.5, and 0.1% Tween 20) for 1 h at room temperature and incubated with appropriate primary antibody overnight at 4 °C. The blots were washed with TBST three times, and then hybridized with horse radish peroxidase-conjugated secondary antibody in a 1:5000 dilution at room temperature for 1 h, then washed with TBST three times. Finally, visualization was done by using the ECL system (Bio-Rad) after treating with ultra-sensitive ECL chemiluminescence substrate (4A BIOTECH). The following primary antibodies and dilutions were used, p-NF-κB (ser536) (Cell Signaling Technology, #3033; 1:1000 dilution), p-p38 MAPK (Thr180/Tyr182) (Affinity, AF4001; 1:500 dilution), p-STAT3 (ser727) (Affinity, AF3294; 1:1000 dilution), p-IκBα (Ser32/36), (Cell Signaling Technology, #9246; 1:1000 dilution), p-p38 MAPK (Proteintech, 14064-1-AP; 1:1000 dilution), p-65 RELA (Proteintech, 10745-1-AP; 1:1000 dilution), STAT3 (Affinity; AF6294 1:1000 dilution), IκBα (Proteintech, 10268-1-AP; 1:1000 dilution), HIF-1α (Proteintech, 20960-1-AP; 1:1000 dilution), β-actin (Proteintech; 1:1000 dilution).

## 2.7. RNA isolation and quantitative real-time PCR

Lung tissues were grinded in liquid nitrogen and total RNA was extracted with TRIzol Reagents (Introgen) according to the manufacturer's recommendations. Briefly, total RNA (0.5 µg) was reverse-transcribed to cDNA using HiScript II Q RT SuperMix for qPCR (Vazyme) according to the manufacturer's protocols. Real-time PCR was performed on a 10 µL reaction mixture per well containing 2 µL diluted cDNA, 0.3 µL forward primer, 0.3 µL reverse primer, 2.4 µL DEPC water (Beyotime) and 5 µL SYBR Green Supermix (Bio-rad). The thermal cycling conditions were set as 95 °C for 3 min, 95 °C for 10 s followed by 60 °C for 30 s, for 40 cycles, melting curve analysis was then performed. The mRNA abundance of four target genes (Cxcl1, Cxcl3, IL-17, Muc5ac) in murine lung tissues was normalized against the expression of RPLP2 [27].

## 2.8. Statistical analysis

Data are represented as the mean ± SEM and analyzed by one-way or two-way ANOVA with post-hoc Tukey test or Games-Howell using SPSS

Statistics 24 software. Differences were considered statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Guanosine and uridine alleviate airway inflammation in OVA-induced mice

In light of their safety profiles and the protective effects in other disorders of guanosine and uridine mentioned in the introduction, we first investigated the anti-inflammatory efficacy of guanosine and uridine on OVA-induced mice asthmatic model [28]. Dexamethasone was chosen as a positive treatment control by a standard intraperitoneal injection. Uridine was administered by intratracheal injection with a compatible dosage (6.1 mg/kg) in accordance with previous report for exact comparison purpose [15]. Owing to the poorer water solubility of guanosine compared with uridine, which may cause inaccurate quantification of adsorption by intratracheal injection, it was administered via an intraperitoneal manner following the same procedures as dexamethasone. Regarding the guanosine's dosage, according to previous study (also i.p.), guanosine at 8 mg/kg has protective effects in central nervous system disorders and at 4 or 8 mg/kg has anti-inflammatory effect in colitis [29–31]. Herein, for comparison with uridine, we therefore chose the same 6.1 mg/kg of guanosine as the dosage of administration, which is also compatible with previous relevant reports.

The overall schematic of experimental setups is shown in Fig. 1A. The mice were sensitized to OVA/alum twice on day 1 and 14, and from day 24–26 they were treated with both compounds once per day (6.1 mg/kg), or PBS following OVA aerosols challenge, respectively.

Following the treatments, lung tissue slices were assessed with H&E and Masson stains. From H&E, the histological analysis of OVA-challenged disease model group (Fig. 1B, OVA + PBS) revealed typical pathologic features of asthma compared to the control group (Fig. 1B, SAL + PBS): numerous inflammatory cells infiltrated around the bronchioles and blood vessels, the airway epithelial cells thickened, epithelial cell debris shed into the lumen of bronchioles. While in SAL + PBS mice, the structure of bronchioles remained intact without these features. This indicated the success in establishment of OVA-induced mice asthmatic model. Mice treated with guanosine, uridine and dexamethasone showed significant reduction in the infiltrating cells around the bronchioles, in the thickening and shedding of epithelial cells (Fig. 1B, OVA + guanosine, uridine, dexamethasone). From Masson staining, the abundant blue-stained collagen deposition around the peribronchiolar and perivascular regions was observed in OVA-challenged mice (Fig. 1C, OVA + PBS) vs. control group (Fig. 1C, SAL + PBS). Such collagen deposition was significantly attenuated by treatment with guanosine, uridine and dexamethasone (Fig. 1C, OVA + guanosine, uridine, dexamethasone).

Apart from the lung section histopathological examination, BALF was collected for total cells count. Consistent with the H&E staining, the infiltrating cells in BALF were significantly increased in OVA-challenged group compared with the control group (Fig. 1D,  $P < 0.05$ ). The increased total cells in BALF in treated groups were markedly reduced by the administrations of guanosine, uridine or dexamethasone (Fig. 1D,  $P < 0.05$ ).

### 3.2. Guanosine and uridine reduce airway hyper-responsiveness

Next, we assessed the impact of guanosine, uridine, dexamethasone on the airway behavioral feature of asthma. This feature can be characterized by AHR to inhaled MCh. Nowadays, both invasive techniques such as forced oscillation [32] and non-invasive technique such as WBP [25] are used to evaluate the AHR. Despite some limitations of the WBP, owing to some environmental complicating influences [33,34], WBP can still objectively reflect the airway resistance [35]. Due to our facility constraints and considering the operative conveniences, herein we chose

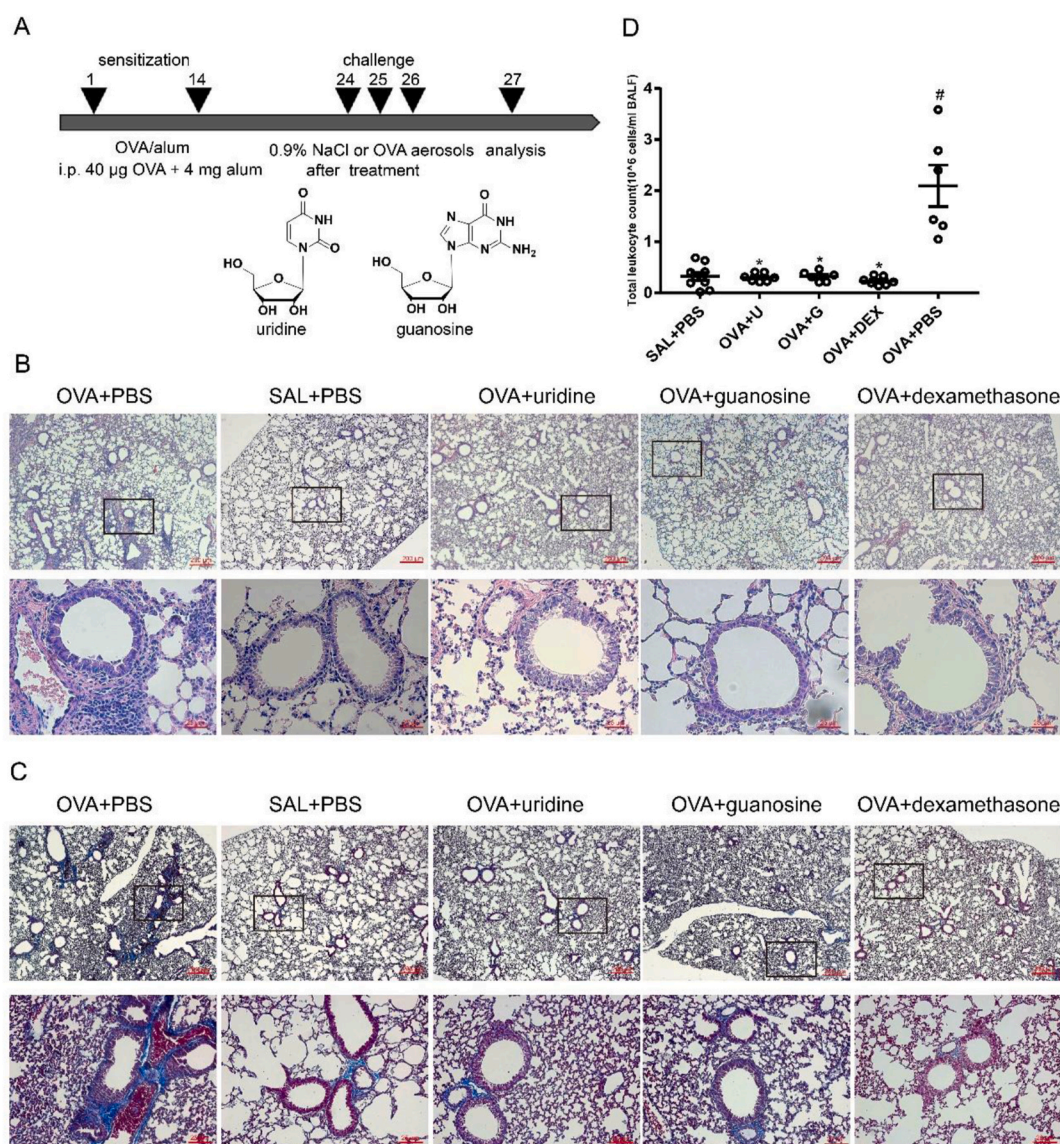


**Fig. 1.** Effects of guanosine and uridine on OVA-induced accumulation of inflammatory cells and collagen deposition. (A) Design of OVA-induced mice asthmatic model. Mice were sensitized by an intraperitoneal injection of OVA in alum on day 1, 14. On day 24–26, mice were treated with intratracheal injection of uridine (6.1 mg/kg), intraperitoneal injection of guanosine (6.1 mg/kg), dexamethasone (1 mg/kg), or PBS daily, then exposed to 1% OVA aerosols for 30 min. 24 h after the last OVA exposure, Penh was measured and then plasma, BALF and lung sections were collected. (B) Lung sections were stained with H&E to measure inflammatory cells. (n = 7–9). Higher-power images are listed in the bottom (top images, magnification: 50, scale bar: 200 µm; bottom images, magnification: 400, scale bar: 25 µm). (C) Lung sections were obtained in each group. Masson's trichrome was assayed to evaluate the collagen deposition (n = 7–9). Higher-power images are listed in the bottom (top images, magnification: 50, scale bar: 200 µm; bottom images, magnification: 200, scale bar: 50 µm). (D) 24 h after the last OVA challenge, BALF samples were collected for total cell counts by automated cell counter (Countstar® BioTech) (n = 6–9). Data are represented as the mean ± SEM. #P < 0.05 vs. the SAL + PBS group; \*P < 0.05 vs. the OVA + PBS group. SAL refers to saline.

the WBP as the surrogate measurements. OVA-challenged mice exhibited significant enhancement in responsiveness to MCh compared to that of control group. As shown in Fig. 2, over the doses from 25 to 100 mg/mL of inhaled MCh, higher average Penh values were observed for OVA + PBS challenged mice ( $P < 0.001$ ). The Penh values were significantly reduced by treatments with dexamethasone ( $P < 0.01$ ), and such Penh values responded to MCh (50–100 mg/mL) were also significantly attenuated by treatments with uridine and guanosine ( $P < 0.05$ ). These phenomena were in accordance with the measurements of IgE (Fig. 2B).

### 3.3. Guanosine and uridine reduce the production of pro-inflammatory cytokines and IgE

AHR and mucus secretion are the two main component mechanisms

of asthma, which are mostly mediated by pro-inflammatory cytokines and IgE. To investigate the inflammatory mediators in the lung, we extracted the total protein of lung tissues and assayed the levels of cytokines by ELISA [26,36]. Compared to the SAL + PBS mice, there were significant rises at IL-4, IL-6, IL-13 levels in the lung tissues of OVA-challenged mice. In contrast, guanosine, uridine and dexamethasone treatment significantly decreased the level of IL-4 (Fig. 3A,  $P < 0.05$ ), IL-6 (Fig. 3B,  $P < 0.001$ ), IL-13 (Fig. 3C,  $P < 0.05$ ). Efficacy of these agents displayed subtle differences. Effects of guanosine for IL-4, IL-6, IL-13 were same as the dexamethasone, whereas the uridine effect on IL-6 was somewhat weaker than dexamethasone (Fig. 3B,  $P < 0.05$ ).

Plasma was collected on the indicated day and the level of OVA-specific IgE was assayed by ELISA. Compared to the OVA-challenged

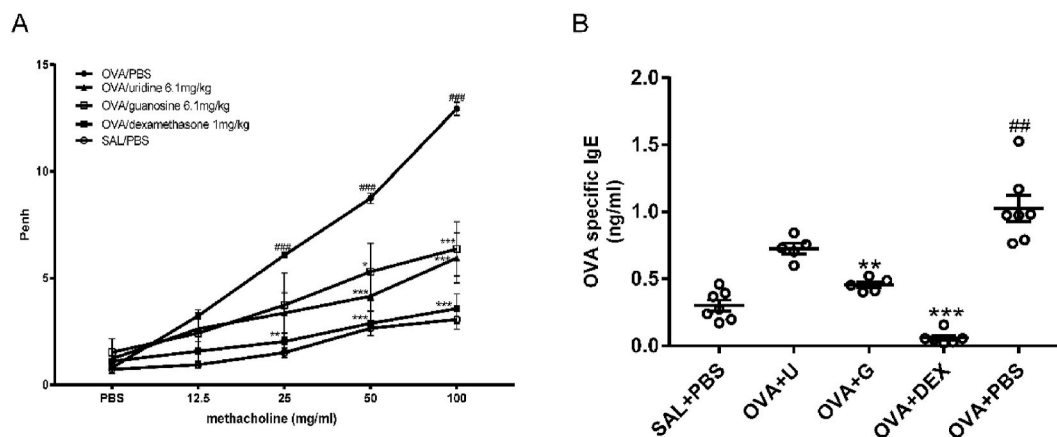


**Fig. 2.** Effects of uridine and guanosine on airway hyper-responsiveness and OVA specific IgE. 24 h after the last OVA aerosol, airway responses of mice (A) to increasing concentration of MCh were measured by WBP ( $n = 4$ ). Concentration of OVA specific IgE (B) in plasma was measured by ELISA ( $n = 5-7$ ). Data are represented as the mean  $\pm$  SEM.  $###P < 0.001$ ,  $##P < 0.01$  vs. the SAL + PBS group;  $**P < 0.01$ ,  $***P < 0.001$  vs. the OVA + PBS group. SAL refers to saline.

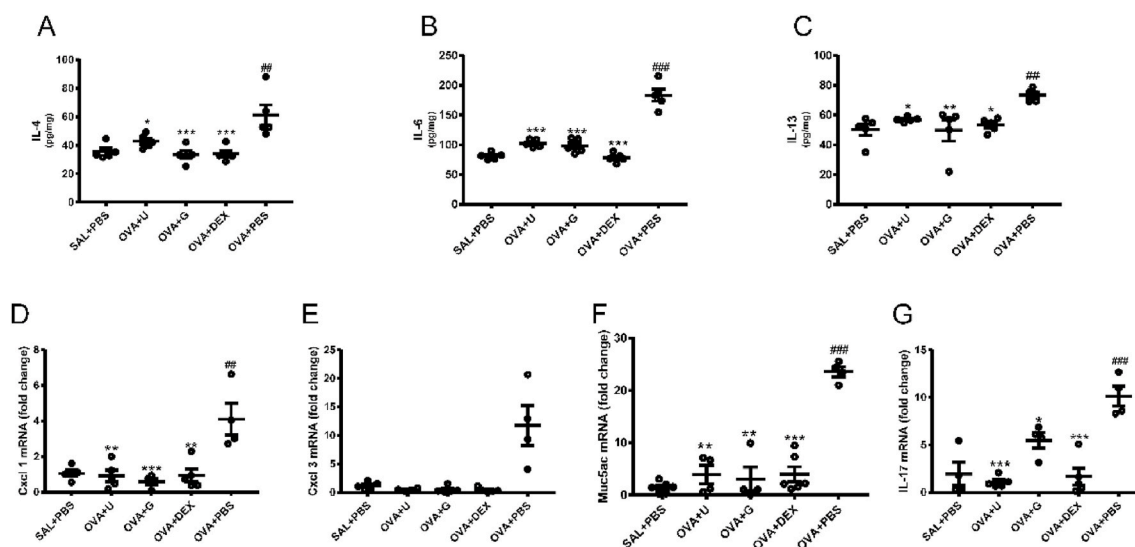


**Fig. 3.** Effects of guanosine and uridine against allergic airway inflammation *in vivo*. 24 h after the last OVA challenge, lung tissues were collected and lysed by lysis buffer for protein extraction. Concentration of IL-4 (A), IL-6 (B), IL-13 (C) in the lungs were measured by ELISA ( $n = 5-7$ ). Total RNA in lung tissues was extracted by TRIzol. After RNA was reverse-transcribed to cDNA, lung tissue gene expression of Cxcl1 (D), Cxcl3 (E), Muc5ac (F) and IL-17 (G) were assessed by qPCR ( $n = 4-6$ ). Data are represented as the mean  $\pm$  SEM.  $\#P < 0.05$ ,  $##P < 0.01$ ,  $###P < 0.001$  vs. the SAL + PBS group;  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs. the OVA + PBS group. SAL refers to saline.

mice, guanosine and dexamethasone treatment significantly decreased OVA-specific IgE ( $P < 0.01$ ), but uridine just slightly decreased it without significant difference (Fig. 2B). Dexamethasone effect on the reduction of OVA-specific IgE was stronger than guanosine ( $P < 0.05$ ).

### 3.4. Effects of guanosine and uridine on NF- $\kappa$ B, MAPK pathways and gene expression *in vivo*

To further investigate the anti-inflammatory molecular pathways, we next examined the effects of guanosine and uridine on OVA-induced activation of two central inflammatory signaling pathways relevant to asthma (NF- $\kappa$ B and p38 MAPK) [37,38] by western blot of lung tissues and immunohistochemistry of lung sections. According to the densitometric analyses of p-I $\kappa$ B $\alpha$  in Fig. 4C, guanosine, uridine and dexamethasone evidently inhibited the phosphorylation of I $\kappa$ B $\alpha$  in OVA-induced asthma mice (Fig. 4A). Guanosine, uridine and dexamethasone also significantly reduced the phosphorylation of NF- $\kappa$ B (ser536) ( $P < 0.01$ ) and p38 MAPK (Thr180/Tyr182) ( $P < 0.05$ ) compared to OVA-challenged group, with reverting extents almost back to the normal

standard (Fig. 4B and C). This tendency was also observed on lung sections and the immunohistochemical stain of p-p38 MAPK (Fig. 4D).

IL-6, together with IL-23 and TGF- $\beta$ , regulates the differentiation of T helper 17 (Th17) cells through the JAK-STAT pathway [39]. Since IL-6 levels were significantly decreased by guanosine and uridine *in vivo* (Fig. 3B), we therefore investigated the status of p-STAT3 (Fig. 4A) and IL-17 mRNA expression. As expected, the level of p-STAT3 (ser727) (Fig. 4C) was decreased by treatments of guanosine and the expression of IL-17 mRNA (Fig. 3G,  $P < 0.05$ ) were decreased by treatments of guanosine, uridine and dexamethasone compared to OVA-challenged mice. Moreover, Th17-inducible chemokines Cxcl1 and Cxcl3, two recruitment factors of neutrophils into the airways, were apparently decreased by guanosine and uridine, to the same extent of dexamethasone (Fig. 3D, E,  $P < 0.05$ ). As mentioned before, besides AHR, overproduction of mucus is also a prominent pathological characteristic of asthma. To check the levels of the essential constituent mucin, the Muc5ac mRNA expression was monitored and was found to be significantly decreased by treatment of guanosine, uridine, dexamethasone (Fig. 3F,  $P < 0.05$  vs. OVA + PBS group).

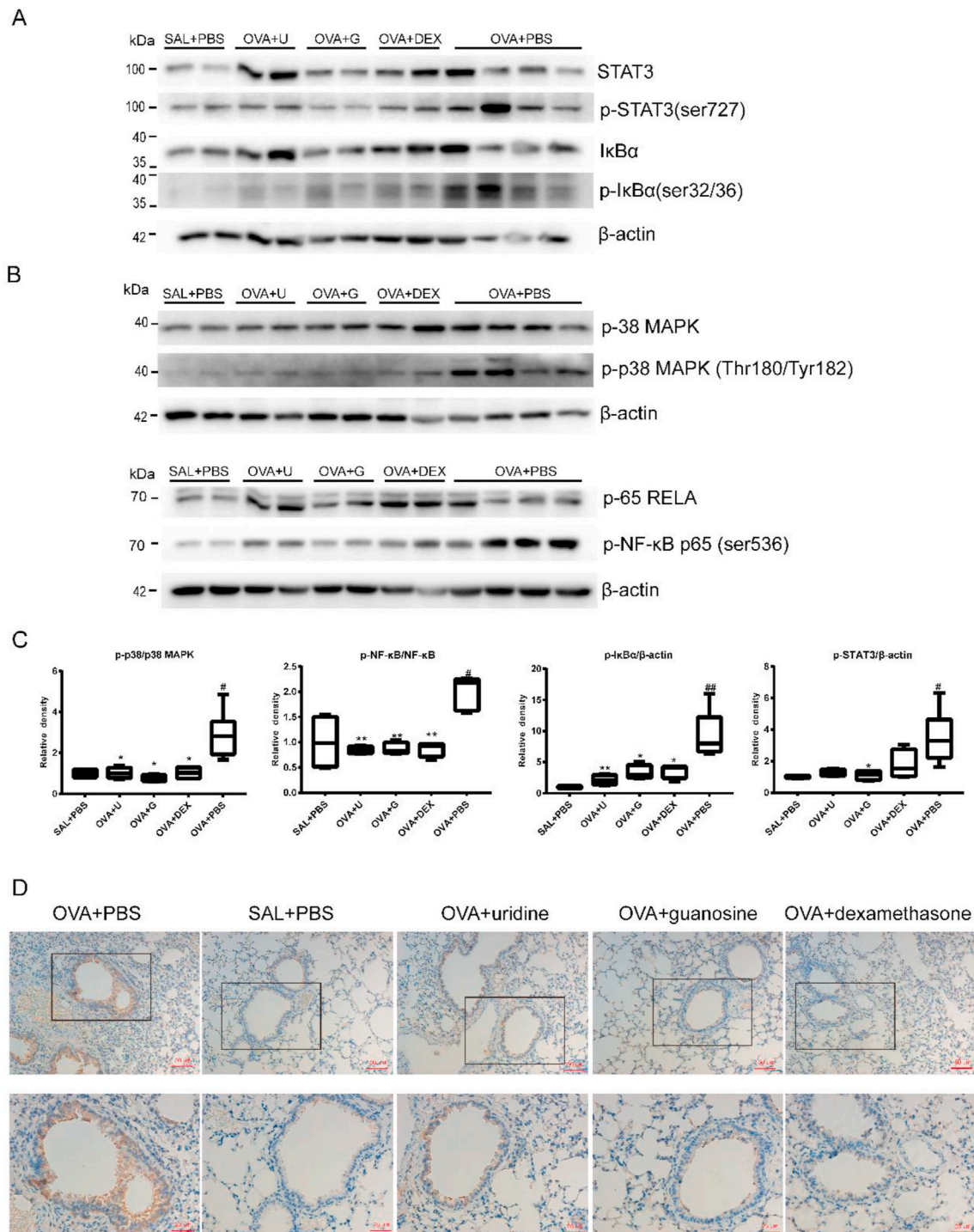


**Fig. 4.** Guanosine and uridine decrease the activation of MAPK and NF-κB *in vivo*. 24 h after the last challenge, lung tissues were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Expression of STAT3, p-STAT3 (ser727), IκBα, p-IκBα (ser32/36) (A) in lung tissues was examined by western blot and β-actin served as the standard and densitometric analyses (n = 4) are presented as the relative ratio of p-IκBα (ser32/36) to β-actin, p-STAT3 to β-actin (C). Expression of p-p38 (Thr180/Tyr182) MAPK and p38 MAPK; p-p65 NF-κB (ser536) and NF-κB in lung tissues (B) and densitometric analyses are presented as the relative ratio of p-p38 MAPK to p38 MAPK, p-p65 NF-κB to NF-κB (C). Immunohistochemistry of p-p38 MAPK (D) in lung sections. Higher-power images are listed in the bottom (top images, magnification: 200, scale bar: 50 μm; bottom images, magnification: 400, scale bar: 25 μm). Data are represented as the mean ± SEM. #P < 0.05; ##P < 0.01 compared with SAL + PBS mice. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 compared with OVA + PBS mice. SAL refers to saline.

### 3.5. Anti-inflammation effects of guanosine and uridine and mechanisms *in vitro*

Since the effectiveness of guanosine and uridine was confirmed on the *in vivo* model, it was desirable to identify the source of their effects at the cellular level with the *in vitro* model. Macrophages are the most

abundant immunocytes in the lungs and the primary source of several pro-inflammatory cytokines, and macrophage polarization (particular classically activated, M1) has been firmly associated with the development of asthma [40]. To investigate whether the lessening of pathological features of asthma generated by guanosine and uridine is related to macrophages, we examined the effects of guanosine, uridine on the

production of IL-6 and the corresponding molecular pathways in M1 macrophages. M1 macrophage polarization was achieved by a well-established method of inducing THP-1 cells with LPS and IFN- $\gamma$  [20]. The experimental results indicated that guanosine and uridine treatments indeed significantly suppressed the LPS/IFN- $\gamma$ -induced release of IL-6 in M1 macrophages (Fig. 5A,  $P < 0.05$ ). Regarding the relevant molecular pathways, key corresponding molecules were assayed. Consistent with the results *in vivo*, phosphorylation of NF- $\kappa$ B, STAT3 (Fig. 5B) and p38 MAPK (Fig. 5C) were reduced in the treatment group of guanosine and uridine compared to negative control. HIF-1 $\alpha$

was also reported to play a critical role in immune and inflammatory responses and regulated by these pathways [41,42]. In agreement with this, we also found that the accumulation of HIF-1 $\alpha$  was reduced by both guanosine and uridine treatments as well (Fig. 5B).

#### 4. Discussion

In addition to fundamental units of genetic information in DNA and RNA, nucleosides are also endowed with various pharmacological activities at the monomeric level. Due to their participation in many

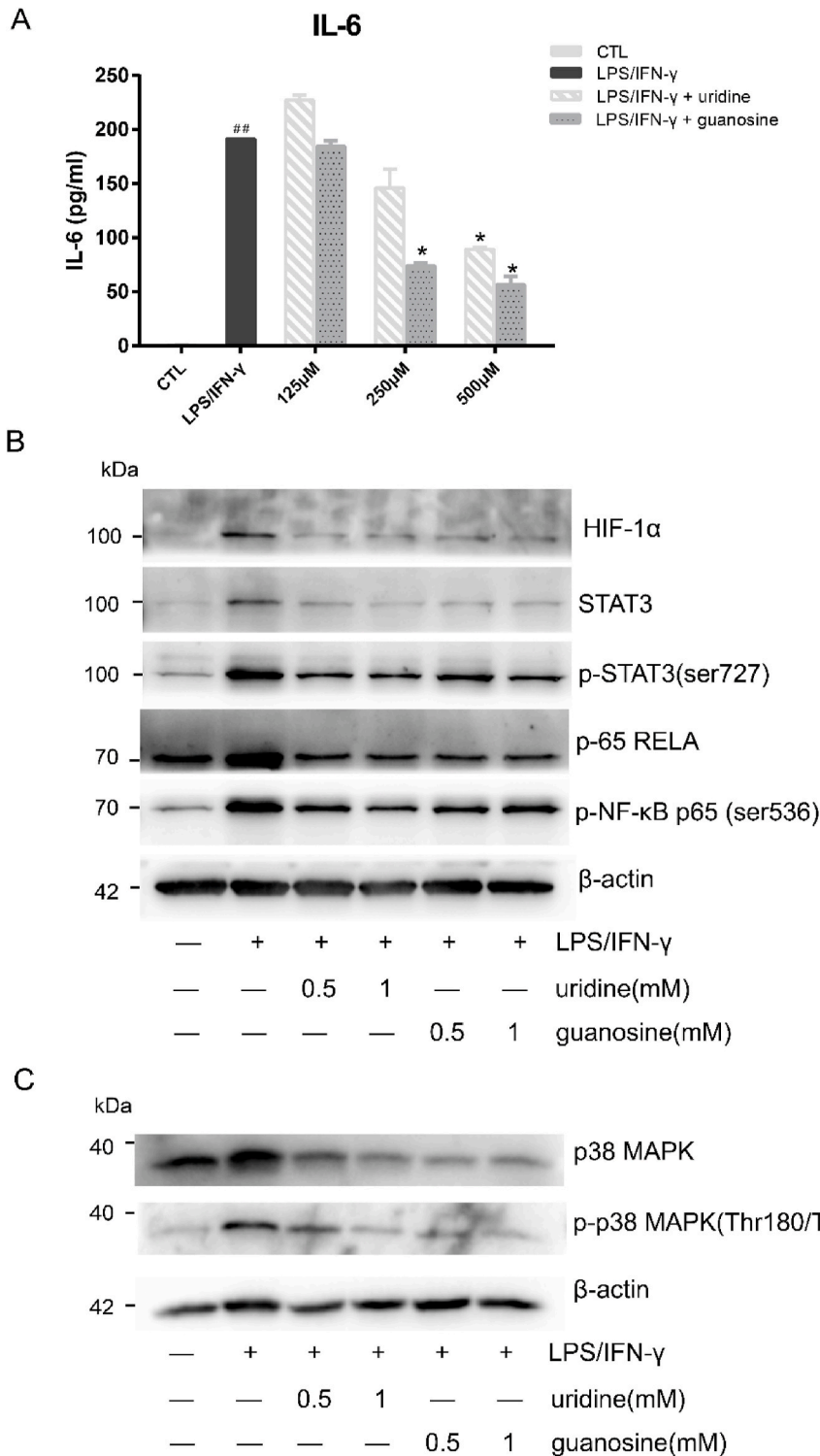


**Fig. 5.** Guanosine and uridine decrease the production of IL-6 and the levels of phosphorylation of p38 MAPK and p65 NF- $\kappa$ B in LPS/IFN- $\gamma$  induced THP-1 cells. THP-1 cells were stimulated with PMA (100 ng/mL) for 24 h, then stimulated with LPS (100 ng/mL) and IFN- $\gamma$  (20 ng/mL) followed by pretreatment with indicated concentration of guanosine and uridine for 1 h. 24 h later, supernatants were collected to determine the concentration of IL-6 (A) by ELISA ( $n = 4$ ), cells were collected for western blot. Expression of total and phosphorylated of STAT3, NF- $\kappa$ B p65, p38 MAPK and HIF-1 $\alpha$  in cell lysis (B), (C) was tested by western blot.  $\beta$ -actin served as the standard. Data are represented as the mean  $\pm$  SEM. <sup>##</sup> $P < 0.01$  vs. the control (CTL) group; \* $P < 0.05$  vs. the LPS/IFN- $\gamma$  group.

important physiological processes, modified nucleoside analogues have been developed into efficient antiviral or anticancer drugs [8]. However, their utility as anti-inflammatory agents, especially in asthmatic area, remains at the infancy stage. Adenosine is a prominent nucleoside with diverse physiological and pharmaceutical effects under ongoing intensive investigation [43]. Consequently, some adenosine receptor ligands entered clinical studies for treating asthma [44]. Other endogenous nucleosides still remain underdeveloped in this area. It is desirable to know whether other nucleosides can act as anti-asthmatic agents. As a part of a systematic evaluation of endogenous nucleobases, nucleosides and deoxynucleosides, we chose guanosine and uridine as initial tested molecules based on the following reasons. For uridine, previous reports identified its protective properties in pulmonary fibrosis [45], hypoxia-induced lung injury [46] and acute coronary insufficiency through activating the mitochondrial ATP-dependent potassium transport [47,48]. Recently, uridine was also reported to have anti-asthmatic efficacy in mice models induced by OVA or house dust mite [15]. The molecular pathways of uridine under such conditions are still unknown. As for guanosine, it has been reported to have neurotrophic and neuroprotective effects by preventing oxidative damage, mitochondrial dysfunction and inflammatory responses [49–52]. However, no study has yet addressed the effect of guanosine in the context of asthma. In present study the efficacy of guanosine in inhibiting inflammation in asthma has been demonstrated for the first time, and the molecular pathways of such activities for both compounds have been also inspected.

IL-6 plays an important role in the development of inflammation related responses in asthma [53]. We observed that IL-6 production was significantly reduced by both guanosine and uridine. Therefore, we were prompted to clarify how the production of IL-6 is influenced through the upstream molecular pathway, and how some of the downstream related cytokines and biological effects of IL-6 were affected, which in turn led to the alleviation pathologic features of asthma in the mouse model.

NF- $\kappa$ B and MAPK are among the most important upstream pathways regulating the production of IL-6 [37,54,55]. To activate NF- $\kappa$ B pathway, I $\kappa$ B needs to be phosphorylated by kinases upon its regulatory domain, causing its degradation by proteasomes, and then the detached NF- $\kappa$ B will be phosphorylated and translocated from the cytoplasm to the nucleus, where it promotes the expression of multiple target genes including IL-6 [38]. Within this signal axis the phosphorylation of p65 NF- $\kappa$ B is the key molecular event used for identifying NF- $\kappa$ B pathway status [56]. The results from the LPS and IFN- $\gamma$  induced M1 macrophage indicated that both guanosine and uridine inhibited the phosphorylation of p65 NF- $\kappa$ B *in vitro*, demonstrating the blockade of NF- $\kappa$ B under these treatments (Fig. 6A). Apart from NF- $\kappa$ B, the expression of IL-6 is also enhanced by activated p38 MAPK which is activated via a sequential phosphorylation cascade: the active MAPK kinases (i.e. MKK3, MKK6) - activated by MAPK kinase kinases (i.e. TAK) - phosphorylate p38 MAPK at the threonine and tyrosine residues. As the final effector in this route, the phosphorylated p38 MAPK (like NF- $\kappa$ B) translocates to the nucleus to phosphorylate various transcription factors (i.e. ternary complex factor, activating transcription factor 2) [37]. Decreased phosphorylation of p38 MAPK was also observed by the treatments of guanosine and uridine *in vitro*. Thus, the blockade of both NF- $\kappa$ B and MAPK pathways collectively contributed to reduction in IL-6 levels upon treatments by guanosine and uridine (Fig. 6A).

On the downstream side, once the IL-6 level is decreased, the expression of HIF-1 $\alpha$  (an enhancer of many proinflammatory cytokines) is also decreased via reduced STAT3 phosphorylation [41]. In accordance with this autocrine regulation, it was found that the status of p-STAT3 and the expression of HIF-1 $\alpha$  were both decreased, confirming that downstream signal of IL-6 was indeed influenced by guanosine and uridine as well in the cellular model (Fig. 6A).

To confirm the aforementioned molecular pathways are still effective at the *in vivo* level, we measured several proinflammatory cytokines in OVA-challenged asthmatic mice. Firstly, compared to negative controls,

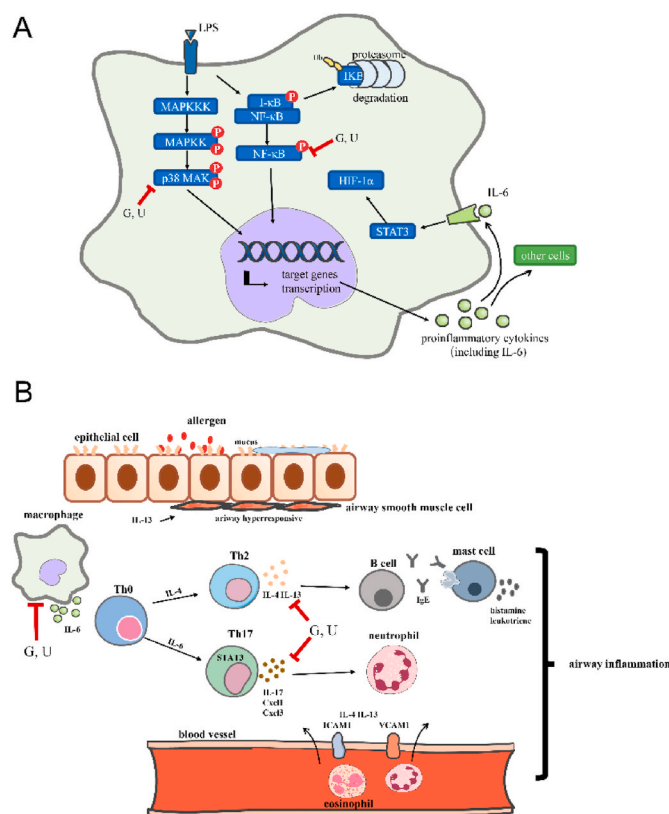


**Fig. 6.** Guanosine and uridine inhibit inflammation in macrophage and attenuate airway inflammation. (A) Guanosine and uridine inhibit production of proinflammatory cytokines (including IL-6) via p-38 MAPK and NF- $\kappa$ B pathways in macrophage. (B) Guanosine and uridine attenuate asthma by inhibiting the activation of macrophage and reducing production of inflammatory mediators. These inflammatory mediators lead to AHR and overproduction of mucus, control the secretion of IgE and extravasation of eosinophils and neutrophils via ICAM1 and VCAM1.

both guanosine and uridine reduced the levels of IL-6 in lung tissues. To investigate whether this phenomenon was also regulated through NF- $\kappa$ B and p38 MAPK *in vivo*, we assayed the phosphorylation of p65 NF- $\kappa$ B and p38 MAPK in lung tissues. Confirming the *in vitro* results, the western blot of lung tissues and immunohistochemistry of lung slices showed that, guanosine and uridine indeed inhibited the phosphorylation of these two key molecules. Like the *in vitro* experiments, in addition to looking at the upstream signaling pathways involved in IL-6 production, we also examined how IL-6 affected the process of asthma in the animal model. After decreasing the levels of IL-6, the differentiation of Th17 cells will be disrupted by the STAT3. Once the differentiation of Th17 cell is inhibited, cytokines and chemokines secreted by Th17 cell naturally declined, and hence neutrophil recruitment to the airways also lessened [39,57]. In the process of Th17 differentiation and function, STAT3 is the key molecule in the differentiation of Th17 cell; IL-17, Cxcl1 and Cxcl3 are the important effector molecules. The results from the OVA-challenged asthmatic mice showed that both guanosine and uridine reduced the phosphorylation of STAT3, the mRNA levels of IL-17, Cxcl1 and Cxcl3 in lung tissues, and reduced inflammatory cell infiltration in H&E stained slices, confirming the downstream signals of IL-6 were indeed influenced by guanosine and uridine in the mouse model (Fig. 6B).

Asthma is a disease with complex pathophysiology. Apart from IL-6, other cytokines, for instance IL-4 and IL-13 secreted by T helper 2 cell, also participate its physiologic process [58]. The present study results showed that guanosine and uridine treatment also reduced the levels of IL-4 and IL-13 in lung tissues (Fig. 6B). Downstream to IL-13 [59],

guanosine and uridine treatment also reduced the expression of Muc5ac mRNA in lung tissues via the direct effect of IL-13 on airway epithelial cells (Fig. 6B). Furthermore, IL-13, together with IL-4, will indirectly promote the expression of vascular cell adhesion molecules on endothelial cells, which helps adhesion of eosinophils, basophils, monocytes and T lymphocytes to the vascular endothelial cells, thus boosts their migration to inflammatory sites. Furthermore, IL-4 and IL-13 stimulate B cells to secrete IgE, which binds to the high-affinity IgE receptor in mast cells triggering the exocytosis of preformed granules (histamine) and release of newly synthesized mediators, leading to the AHR and recruitment and activation of eosinophils [60]. As a whole, consistent with the decreasing levels of IL-4 and IL-13 *in vivo*, in OVA-challenged asthmatic mice, guanosine and uridine treatment reduced the production of IgE in plasma, alleviating airway hyper-responsiveness upon MCh administrations, decreasing total cells in BALF and the infiltrating inflammatory cells around bronchioles according to H&E slices (Fig. 6B).

This is the first demonstration of nucleoside-mediated mitigation of lung inflammation, Penh, mucus hypersecretion in the OVA-challenged asthmatic mouse model, and the molecular pathways of these actions by guanosine and uridine *in vivo* and *in vitro*. Nevertheless, the *bona fide* receptors or molecules that directly mediate the actions of guanosine and uridine deserve further investigation. Though guanosine and uridine displayed similar effects on the NF- $\kappa$ B and p38 MAPK pathways in current models, there were some precedent studies implying that they might have different direct targets upstream of these two pathways under different contexts. On one hand, in central nervous system, guanosine exhibited antioxidant properties and protection by activating heme oxygenase 1 [61,62]. In C6 astroglial cells, guanosine' inhibition of NF- $\kappa$ B activation can be abrogated by zinc protoporphyrin IX, a heme oxygenase 1 inhibitor [49,52]. On the other hand, the neuroprotection of guanosine was reported to be associated with the activation of adenosine A<sub>1</sub> and A<sub>2a</sub> receptors [62]. In contrast, the anti-inflammatory effects of uridine were reported not via activating A<sub>1</sub> and A<sub>2a</sub> receptors on A549 cell models [15]. So, additional sophisticated investigations are needed to fully elucidate the genuine targets/mediators upstreams of NF- $\kappa$ B and p38 MAPK pathways. However, the effectiveness revealed in current studies offers helpful information to address the basic and clinical research demands.

Additionally, the good safety profiles of both molecules are advantageous for their further clinical development. The LD<sub>50</sub> of intraperitoneal guanosine was over 960 mg/kg, and up to 120 mg/kg guanosine (i. p. and p.o.) did not induce any apparent behavior disorders [17]. Vistogard (uridine triacetate) and Xuriden (uridine triacetate) have been approved for marketing by FDA for the treatment of capecitabine overdose and hereditary orotic aciduria, signifying uridine's great translational potential. Consequently, guanosine and uridine may serve as a promising avenue of development for new alternative anti-asthmatic agents.

## 5. Conclusion

Guanosine has a similar potential therapeutic value in asthma as uridine and they exert anti-inflammatory effects *in vivo* and *in vitro* through suppression of the MAPK and NF- $\kappa$ B pathways.

## Author statement

Yujiao Luo: Investigation, Methodology, Formal analysis, Writing-original draft, Visualization. Hai Chen: Investigation, Data Curation, Writing-original draft. Ridong Huang: Investigation, Formal analysis, Resources. Qiong Wu: Formal analysis, Resources. Ying Li: Formal analysis, Validation, Data Curation. Yang He: Conceptualization, Methodology, Funding acquisition, Supervision, Writing-original draft, Writing-review and editing, Project Administration.

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## Declaration of competing interest

The authors declare no conflict of interest.

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Not applicable.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pupt.2021.102049>.

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**Supplementary Material**  
**Guanosine and uridine alleviate airway inflammation via inhibition of the MAPK and NF- $\kappa$ B signals in OVA-induced asthmatic mice**

All the antibodies used in the experiments are specificity to the target protein, the validation studies are listed as follows, p-NF- $\kappa$ B (ser536) [1], p-p38 MAPK [2], p-STAT3 (ser727) [3], p-I $\kappa$ B $\alpha$  (Ser32/36) [4], p-65 RELA [5], p-38 MAPK [6], STAT3 [7], I $\kappa$ B $\alpha$  [8], HIF-1 $\alpha$  [9].

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