

A retro-inverso modified peptide alleviated ovalbumin-induced asthma model by affecting glycerophospholipid and purine metabolism of immune cells

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ARTICLE INFO

Keywords:

Asthma
Airway hyperresponsiveness
Retro-inverso peptide
Single-cell transcriptome sequencing
Glycerophospholipid metabolism
Purine metabolism

ABSTRACT

Allergic asthma is a heterogeneous disease involving a variety of inflammatory cells. Immune imbalance or changes in the immune microenvironment are the essential causes that promote inflammation in allergic asthma. Tetraspanin CD81 can be used as a platform for receptor clustering and signal transmission owing to its special transmembrane structure and is known to participate in the physiological processes of cell proliferation, differentiation, adhesion, and migration. Previous studies have shown that CD81-targeting peptidomimetics exhibit anti-allergic lung inflammation. However, due to the low metabolic stability of peptide drugs, their druggability is limited. Here, we aimed to generate a metabolically stable anti-CD81 peptide, evaluate its anti-inflammatory action and establish its mechanism of action. Based on previous reports, we applied retro-inverse peptide modification to obtain a new compound, PD00 (NH₂-D-Gly-D-Ser-D-Thr-D-Tyr-D-Thr-D-Gln-D-Gly-COOH), with high metabolic stability. Enhanced ultraperformance liquid chromatography–tandem mass spectrometry was used to investigate the in vitro and in vivo metabolic stabilities of PD00. The affinities of PD00 and CD81 were studied using molecular docking and surface plasmon resonance techniques. An ovalbumin (OVA)-induced asthma model was used to evaluate the effects of PD00 in vivo. Mice were treated with different concentrations of PD00 (175 and 350 µg/kg) for 10 days. Airway hyperresponsiveness (AHR) to acetyl-β-methacholine (Mch), inflammatory cell counts in the bronchoalveolar lavage fluid, and serum OVA-specific IgE levels were detected in the mice at the end of the experiment. Lung tissues were collected for haematoxylin and eosin staining, untargeted metabolomic analysis, and single-cell transcriptome sequencing. PD00 has a high affinity for CD81; therefore, administration of PD00 markedly ameliorated AHR and airway inflammation in mice after OVA sensitisation and exposure. Serum OVA-specific IgE levels decreased considerably. In addition, PD00 treatment increased glycerophospholipid and purine metabolism in immune cells. Collectively, PD00 may regulate the glycerophospholipid and purine metabolism pathways to ameliorate the pathophysiological features of asthma. These findings suggest that PD00 is a potential compound for the treatment of asthma.

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1. Introduction

Asthma is a chronic inflammatory disorder caused by heterogeneous

cells. Glycerophospholipids are important components of cell membranes and metabolised by phospholipase to hydrolyse fatty chains to produce lysophospholipids. Lysophospholipids bind to G

Abbreviations

AHR	Airway hyperresponsiveness
OVA	Ovalbumin
Mch	acetyl- β -methacholine
BALF	bronchoalveolar lavage fluid
H&E	haematoxylin and eosin
GWASs	genome-wide association studies
Th2 cells	T helper 2 cells
UPLC-MS/MS	ultraperformance liquid chromatography–tandem mass spectrometry
EDC	ethyl (dimethylamino propyl) carbodiimide
Dex	dexamethasone
QC	quality control
TIMS	Trapped Ion Mobility Spectrometry
Q-TOF	quadrupole time-of-flight
PASEF	parallel accumulation serial fragmentation

PLS-DA	partial least squares discriminant analysis
DEGs	differentially expressed genes
SEM	standard error of mean
AUC _{0-t}	area under the plasma concentration-time curve
T _{1/2}	half-life
K _D	affinity constant
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PA	phosphatidic acid
Ribose 5P	D-ribose 5-phosphate
AIR	5-aminoimidazole ribonucleotide
GMP	guanosine monophosphate
dGMP	guanosine, deoxyguanosine monophosphate
AMP	adenosine monophosphate
dAMP	deoxyadenosine monophosphate
GPCRs	G protein-coupled receptors

gene–environment interactions that are still partially undiscovered [1]. Its pathogenesis involves multiple immune cells and signalling pathways. Numerous genome-wide association studies (GWASs) have identified over 160 asthma-related target genes enriched in 47 signalling pathways [2]. Researchers have focused on the inflammatory response driven by the T helper 2 (Th2) pathway. Several monoclonal antibodies targeting Th2-pathway cytokines or their receptors have been licenced for the treatment of severe eosinophilic asthma [3]. However, targeting a single protein of the type 2 inflammatory pathway cannot bring the most benefit to patients because the lung immune microenvironment is affected by the state of various immune cells.

CD81 (TAPA-1) is a widely expressed cell-surface protein involved in a variety of biological responses, such as adhesion, morphology, activation, proliferation, and differentiation of B lymphocytes, T lymphocytes, and other cells [4]. CD81 knockout mice showed an impaired Th2 response and that CD81 plays a role in developing airway hyperresponsiveness (AHR) [5]. These results suggest that CD81 may be an important target for the treatment of high airway responsiveness and inflammation in asthma. Furthermore, a peptidomimetic GQTYTSG (referred to as SP in this study) designed to target CD81 has been shown to have pharmacological effects against allergic asthma-related inflammation [6,7]. However, the metabolic instability of SP necessitates subcutaneously implanted osmotic minipumps [6] to achieve the desired effect in animal experiments, and metabolic instability limits its druggability. Retro-inverse peptides with reversed peptide bonds can introduce stability in proteolysis and simultaneously stereochemically preserve the topological surface of the side chains in the original L-amino acid sequence [8–10]. We designed and synthesised a series of SP derivatives with different modification sites for amino acid residues that are easily metabolised in SP, of which a retro-inverse peptide (referred to as PD00 in this study) with high metabolic stability was selected for further investigation in this study.

Studies have shown that CD81 is involved in the regulation of lipid metabolism [11], subcellular localisation of the dNTPase SAMHD1 and allows degradation of subcellular localisation of dNTPase SAMHD1 by the proteasome, thereby regulating intracellular dNTP levels [12]. Substance and energy metabolism are indispensable during the maintenance of self and body homeostasis in various types of immune cells. Different metabolites have different biological effects on immune cells, and an imbalance in the metabolome causes dysfunction of immune

protein-coupled receptors (GPCRs) on the membrane and regulate intracellular signal transduction pathways involved in the pathogenesis of various diseases, such as inflammation and immunity in the human body [13].

In this study, we hypothesised that PD00 regulates the metabolism in immune cells through CD81, thereby affecting the function of immune cells to exert anti-asthma effects. To investigate the pharmacological effect of PD00 and its mechanism of action, we studied the effects of PD00 in an in vivo mouse model of OVA-induced allergic asthma. We applied metabolomic techniques to analyse the molecular mechanisms underlying its anti-allergic inflammatory effects.

2. Materials and methods

2.1. Animals

Six-to-eight-week-old female BALB/c mice and male Sprague–Dawley rats were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). They were raised under specific pathogen-free conditions (temperature: 20–26 °C; humidity: 30–70%) with a 12-h light/dark cycle. All animal experiments were performed in accordance with the National Research Council's grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf" title="http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf">Guide for the Care and Use of Laboratory Animals and approved by the Animal Studies Committee of the Shanghai Institute of Pharmaceutical Industry.

2.2. Chemicals and reagents

SP (NH₂-Gly-Gln-Thr-Tyr-Thr-Ser-Gly-COOH, purity $\geq 99.37\%$) and PD00 (NH₂-D-Gly-D-Ser-D-Thr-D-Tyr-D-Thr-D-Gln-D-Gly-COOH, purity $\geq 98\%$) were provided by Shanghai Domirui Biotechnology Co. Ltd. (Shanghai, China). Dexamethasone (Dex) was purchased from Chenxin Pharmaceutical Co. Ltd. (Jining, China). Acetyl- β -methacholine (Mch), ovalbumin (OVA, grade II and V), formic acid, and ammonium bicarbonate were purchased from Sigma-Aldrich, (St Louis, MO, USA). Aluminium hydroxide (Alum) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mouse OVA-specific IgE Antibody (OVA-IgE) ELISA Kit (E–26046) was purchased from Aiyuan Biotechnology

Co. Ltd. (Shanghai, China). Wright–Giemsa stain solution was purchased from Solarbio Science & Technology Co. Ltd. (Beijing, China). Recombinant human CD81 protein (14244-HNCH), recombinant rat CD81 protein (80331-RNCH), and recombinant mouse CD81 protein (50743-M07H) were purchased from Yiqiao Shenzhou Technology Co. Ltd. (Beijing, China); Series S Sensor Chip CM5 chip (BR100530), amino coupling kit (BR-1000-50), and 10 × HBS-EP+ (BR-1006-69) were purchased from Cytiva (Singapore, Republic of Singapore). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q water purification system (Merck Millipore, Burlington, MA, USA).

2.3. Metabolic stability analysis

Referring to a previously established method for testing the metabolic stability of peptidomimetic drugs [14], enhanced ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) was used to investigate the in vitro and in vivo metabolic stabilities of PD00. A rat whole blood incubation model was used to study the metabolic stability of PD00 in vitro. A pharmacokinetic study of PD00 was conducted on eight Sprague–Dawley rats weighing around 200 ± 20 g. PD00 solution was injected subcutaneously at a dose of 5 mg/kg. Blood samples were collected through orbital bleeding into pre-acidified EDTA-K2 tubes at 0 (before administration), 0.05, 0.1, 0.167, 0.333, 0.667, 1, 2, 3, 4, 6, 8 and 12 h after administration and placed in an ice-water bath (0 °C) immediately. After centrifugation at 1000×g for 15 min, the plasma samples were analysed using UPLC-MS/MS (Refer to Supplementary Materials for detailed methods).

2.4. Determining affinity between PD00 and CD81 based on Biacore T200 analysis system

2.4.1. Screening of coupling pH

HBS-EP buffer was used as the working buffer. PD00 was diluted with 10 mM sodium acetate buffer (pH 5.5, 5.0, 4.5 and 4.0) to a final concentration of 71.6 µg/mL. The manual sampling method in the control software was used for pH value screening, and the pH with the largest coupling value was selected for subsequent experiments.

2.4.2. Coupling of PD00 on the surface of CM5 sensor sheet

The amino coupling method was used to couple the PD00 to the CM5 chip. The HBS-EP buffer was used as the working buffer, and PD00 was diluted to a final concentration of 71.6 µg/mL using 10 mM sodium acetate buffer with the best pH value obtained from the above screening. The chip surface was activated with a mixture of 0.2 M ethyl (dimethylamino propyl) carbodiimide (EDC) and 50 mM N-hydroxysuccinimide at a ratio of 1:1. The samples were injected at a flow rate of 10 µL/min. After continuously injecting the sample for 7 min, the PD00 solution was injected. Subsequently, 1 M ethanolamine hydrochloric acid (pH = 8.5) blocking solution was injected for 7 min to block the surface of the activated chip.

2.4.3. Kinetic analysis of PD00 and recombinant CD81 proteins

Different species of recombinant CD81 protein were diluted in HBS-EP buffer to prepare 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.90625 nM. Glycine (50 mM; pH = 9.5) was used as a regenerant. Kinetic testing was performed using kinetic and affinity methods of the kinetic analysis wizard template to analyse the interaction between the ligand and receptor. The obtained data were fitted in analysis software, and kinetic constant was calculated using time and response value as the abscissa and ordinate, respectively.

2.5. Protein-drug docking study

AutoDock CrankPep approach (ADCP) [15,16] was applied to dock the drugs with the CD81 protein by orienting the centre of the

peptide-binding pocket close to the biologically active site of CD81. The crystal structures used for docking and the residues used to locate the pocket centre are listed in Table 1. During docking via ADCP, the number of replicas was set to 100, and the number of steps in the Monte Carlo trajectory per replica was set to one million steps per amino acid in the peptides. The grid box size was set to the length of the peptide to be docked × 3.8 Å, where 3.8 is the distance between the two adjacent α-carbon atoms in the peptide backbone. The helix ratio in the initial conformation of the peptide was set at 30%. Notably, due to limitations of the applied programme, L-type amino acids were processed as D-type amino acids during docking. Considering that the differences in the final binding modes and poses between L-type and D-type amino acids are insignificant, we believe that the results obtained after this processing should be superior to that of the reference value.

2.6. Induction of allergic asthma and experimental protocol

Seventy BALB/c mice (18–20 g) were randomly divided into seven groups: a control group; OVA group; group with a low-dose of PD00 (175 mg/kg); group with a high-dose of PD00 (350 mg/kg); group with a low-dose of SP (175 mg/kg); group with a high-dose of SP (350 mg/kg); and a Dex group (2 mg/kg). Except for the control group, the mice were sensitised by intraperitoneal injection of OVA (20 µg in phosphate buffer solution (PBS) and alum, Grade V) with a total volume of 200 µL on days 0, 7 and 14. Mice were treated with vehicle, PD00, SP subcutaneously, and Dex intraperitoneally from days 19–28. Mice were challenged with OVA (1%, Grade II) aerosolised in an airtight box for 30 min from days 21–28 (Fig. 3A). On day 29, mice were euthanised by intraperitoneal injection of 1% pentobarbital sodium (0.1 mL/20 g body weight) for tissue collection. At the experimental endpoint, AHR, total IgE and OVA-specific IgE in serum, inflammatory cell [17,18] accumulation in bronchoalveolar lavage fluid (BALF) gene expression of cytokines in lung tissue and pathophysiological features of asthma were assessed. Furthermore, untargeted metabolomics and single-cell transcriptome analyses of lung tissues were conducted.

2.7. Assessment of allergen-induced airway responsiveness

Mice were anaesthetised with 1% pentobarbital sodium 24 h after the last OVA challenge and tracheotomies were performed. A whole-body plethysmography chamber (Buxco Electronics, Sharon, CT, USA) was used to assess airway responsiveness to Mch. Mice were placed supine inside the body plethysmograph chamber and induced to breathe PBS, and the concentration of Mch was allowed to increase gradually (0.0125, 0.025, 0.05 and 0.1 mg/mL) through a jet nebuliser. Lung resistance (R_L) and respiratory dynamic compliance (C_{dyn}) in response to Mch were recorded and expressed as changes from the baseline value [17].

2.8. Determination of total cells and eosinophils in the BALF

The BALF of mice was collected according to a previously established

Table 1

Crystal structures used in CD81 virtual screening and the amino acid information used to locate the centre of the pocket.

PDB number ^a	Chain number ^b	Positioning amino acids ^c
6U9S	C	E188
6U9S	F	N180
5TCX	A	F94
6EJM	A	E188
7JIC	B	F186

^a PDB number of crystal structures used for the docking study.

^b Chain number of CD81 in the PDB structure.

^c Residues used to locate the centre of the potential peptide-binding pocket in the docking study. Protein Data Bank.

protocol [19]. The mice were euthanised with an overdose of 1% pentobarbital sodium. BALF was collected by flushing the lungs three times with 1 mL of cold PBS, and 10 μ L of the recovered PBS was secured for counting total cells using a cell counter (DeNovix CellDrop BF Cell Counters). The results are expressed as the number of total cells ($\times 10^6$ /mL) for each group.

BALF was then centrifuged at $500\times g$ for 10 min at 4 °C, and the cell pellet was resuspended in 100 μ L of PBS. Slides were prepared for eosinophil count by adding 20 μ L of resuspension and stained with Wright–Giemsa stain solution (Solarbio Science & Technology Co. Ltd.). The number of eosinophils was determined using an optical microscope (1000 $\times$ magnification) by counting at least 200 cells per slide, and the percentage of eosinophils was reported.

2.9. Measurement of serum OVA-specific-IgE

After inducing anaesthesia, blood from each mouse was collected from the retro-orbital venous plexus and centrifuged at $1000\times g$ for 15 min at 4 °C. Serum samples were used to determine the total IgE concentration using ELISA, following the manufacturer's instructions.

2.10. Histopathological analysis

After BALF collection, the lung tissues of the mice from each group were removed for histological examination. The lung tissues were previously perfused with PBS (pH = 7.4) and then fixed in 10% formalin for 24 h. Paraffin-embedded lungs were cut at a thickness of 3 μ m and mounted on slides, then stained with haematoxylin and eosin (H&E). Slides were examined and photographed under a light microscope (Eclipse E200, Nikon, Tokyo, Japan). Pathological assessment was conducted blindly based on a scoring system as follows and inflammatory changes were scored on a semiquantitative scale of 0–4 for cellular infiltration around the airways: 0, no infiltration; 1, few inflammatory cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells greater than four cells deep [17].

2.11. Metabolomics analysis

2.11.1. Sample preparation

Mice were anaesthetised 24 h after the last aerosol challenge, and after BALF collection, the lung tissues of mice from each group were removed for metabolomic analysis. Lung tissue samples were accurately weighed 20 ± 0.2 mg and homogenised with 200 μ L of water using a Scientz-48 high-throughput tissue grinder (Ningbo Scientz Biotechnology, Zhejiang, China). After homogenisation at 40 Hz for 2 min at 4 °C, 600 μ L of a mixture of methanol and acetonitrile (50:50, v/v) was added to the homogenised tissue samples. The tissue samples were further vortexed for 40 s and sonicated for 10 min at 4 °C. An overnight incubation of samples at -20 °C was carried out to facilitate protein precipitation. After centrifugation at $14,000\times g$ for 15 min at 4 °C, 700 μ L of the supernatant was removed and evaporated to dryness at 25 °C using a Labconco vacuum concentrator (Labconco, Kansas City, MO, USA). The residues were reconstituted in 100 μ L of acetonitrile-water (50:50, v/v) and centrifuged at $14,000\times g$ for 10 min at 4 °C before 4D-metabolomics analysis. In addition, an equal volume of the individual samples was used to prepare the pooled tissue samples, which were used as quality control (QC) samples to guarantee high-quality data collection in batches.

2.11.2. 4D-Metabolomics analysis

The 4D-metabolomics analysis was performed on a Bruker Elute UPLC coupled to a hybrid quadrupole time-of-flight MS (Q-TOF-MS) equipped with a trapped ion mobility spectrometer (TIMS; Bruker, Billerica, MA, USA). A Waters ACQUITY UPLC BEH T3 column (100 mm $\times$ 2.1 mm, 1.7 μ m; Waters, Milford, MA, USA) was used for the liquid

chromatography separation at a flow rate of 0.35 mL/min, and the column temperature was maintained at 35 °C. The mobile phases were water (mobile phase A) and acetonitrile (mobile phase B), both containing 0.1% formic acid in the positive ion mode and 5 mM ammonium bicarbonate in the negative ion mode. The gradient was set as follows: 0–2 min: 2% B; 2–16 min: 2% B to 100% B; 16–18 min: 100% B; 18–18.2 min: 100% B to 2% B; and 18.2–22 min: 2% B. The injection volume was 2 μ L. All samples were randomly injected during data acquisition. Data acquisition of mass spectrometer was performed in data-dependent acquisition mode. The source parameters were set as follows: capillary voltage, 4500 V; end-plate offset, 500 V; nebuliser flow, 2.2 bar; dry gas flow, 10 L/min; and dry temperature, 220 °C. The TIMS-Q-TOF-MS scan parameters were set as follows: TIMS, enable; scan mode, parallel accumulation serial fragmentation (PASEF); mass scan range, 20–1300 Da; ion mobility coefficients ($1/K_0$) scan range, 0.45–1.45 V s/cm²; and TIMS ramp time 100 ms. The precursor ions were performed with two PASEF MS/MS scans per topN acquisition cycle in PASEF mode, and the precursor ions were dynamically excluded for 0.1 min when their intensity reached a target value of 4000.

2.11.3. Data processing

Raw data from 4D-metabolomics was first processed using MetaboScape software (version 2021; Bruker). The T-ReX 4D feature finding algorithm was applied to automatically extract data from the 4D space (m/z , retention time, ion mobility and intensity) and PASEF MS/MS spectra were assigned. Feature detection was performed by using an intensity threshold of 500 counts. The minimum number of data points in the 4D TIMS space was set to 60 when using recursive features. Features were filtered if their relative standard deviations were $>30\%$ in the QC samples. The MetaboScape results were exported as a CSV file using Microsoft Excel software and uploaded to the open-access software MetaboAnalyst 4.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/>) for data analysis, including partial least squares discriminant analysis (PLS-DA), volcano plots and metabolic pathway analysis. The MS data were normalised to the total sum of all the detected ions and scaled using auto-scaling. An unpaired *t*-test was used to identify significantly differentially expressed metabolites ($P < 0.05$).

2.12. Single-cell transcriptome sequencing

The lung tissues of mice in the control, model (OVA) and PD00 (OVA + PD00 350 μ g/kg) groups were used for single-cell sequencing. The 10x Genomics platform used microfluidic technology to wrap beads and cells with Cell Barcode in droplets, collected the droplets containing cells and then lysed cells in the droplets so that the mRNA in the cells is combined with the cells on the bead to form single-cell Gel Beads in Emulsion. The Cell Ranger software pipeline (version 5.0.0), which was provided by 10 $\times$ Genomics, was used to demultiplex cellular barcodes; map reads to the genome and transcriptome using the STAR aligner, and downsample reads as required to generate normalised aggregate data across samples, producing a matrix of gene counts versus cells. We processed the unique molecular identifier count matrix using the R package Seurat [20] (version 3.1.1). Following visual inspection of the distribution of cells by the fraction of mitochondrial genes expressed, we further discarded low-quality cells, in which $>20\%$ of the counts belonged to mitochondrial genes. Additionally, we applied the DoubletFinder package [21] (version 2.0.2) to identify potential doublets. Library size normalisation was performed using the NormalizeData function of Seurat [20] to obtain the normalised count. Specifically, the global-scaling normalisation method “LogNormalize” normalised the gene expression measurements for each cell by the total expression, multiplied by a scaling factor (10,000 by default), and the results were log-transformed.

The top variable genes across single cells were identified using the method described by Macosko et al. [22]. The most variable genes were selected using the FindVariableGenes function (mean.function = FastExpMean, dispersion.function = FastLogVMR) in Seurat. Principal

component analysis was performed to reduce the dimensionality of the RunPCA function in Seurat. Graph-based clustering was performed to cluster cells according to their gene expression profiles using the FindClusters function in Seurat. Cells were visualised using a 2D t-distributed stochastic neighbour embedding algorithm with the RunTSNE function in Seurat [20]. We used the FindAllMarkers function (test use = bimod) in Seurat [20] to identify the marker genes of each cluster. For a given cluster, FindAllMarkers identified positive markers compared with all other cells. Then, we used the R package SingleR [23], a novel computational method for unbiased cell type recognition of scRNA-seq, with the reference transcriptomic datasets 'Immunological Genome Project' (<https://www.immgen.org/>) to infer the origin of each single-cell independently and identify cell types.

Differentially expressed genes (DEGs) were identified using the FindMarkers function (test use = MAST) in Seurat [20]. P value < 0.05 and $|\log_2\text{foldchange}| > 0.58$ was set as the threshold for significant differential expression.

2.13. Statistical analysis

All data are presented as mean $\pm$ standard error of mean (SEM). One-way analysis of variance was used to compare multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The peptidomimetic PD00 has good metabolic stability

Poor metabolic stability limits the druggability of peptide SP. A retro-inverse peptide (PD00) was synthesised to obtain peptides with improved metabolic stability. The ratio of the peak area of the analyte to that of tolbutamide at different incubation times of SP and PD00 in rat whole blood was compared with the zero time to obtain the residual rate (Fig. 1A). The remaining rate was plotted with the corresponding time point, and the $T_{1/2}$ of each compound was calculated using a one-

compartment metabolic model to study the in vitro metabolic stability of SP and PD00. After 60 min of incubation in rat whole blood, the residual rates of SP and PD00 were 0.67% and 103.00%, respectively, and the $T_{1/2}$ of SP was 10.4 min. However, PD00 in in vitro rat whole blood did not degrade after 60 min of incubation (Fig. 1A). Compared to SP, the metabolic stability of PD00 was markedly improved.

The average blood concentration-time curves of SP and PD00 after a single subcutaneous injection of 5 mg/kg in rats are shown in Fig. 1B and C, respectively. After subcutaneous injection of 5 mg/kg PD00 in rats, the peak plasma drug concentration $C_{\max}$ was 6474 ± 931 ng/mL (Fig. 1D), and the area under the plasma concentration-time curve (AUC_{0-t}) was 7228 ± 1372 ng min/mL (Fig. 1E). Meanwhile, the plasma elimination half-life $T_{1/2}$ was 1.05 ± 0.59 h, which was 8 times that of SP ($T_{1/2}$ was 7.78 ± 4.00 min) (Fig. 1F). Therefore, we obtained a retro-inverse peptide, PD00, with improved metabolic stability.

3.2. PD00 has a high affinity for CD81

According to Biacore analysis, the affinity constant (K_D) of PD00 to recombinant human CD81 was 2.30×10^{-7} M (Fig. 2A), which was higher than the affinity of SP ($K_D = 1.79 \times 10^{-6}$ M) (Fig. 2B). The K_D of PD00 for recombinant rat CD81 and recombinant mouse CD81 was 5.16×10^{-7} M (Figs. 2C) and 5.89×10^{-7} M (Fig. 2D), respectively. The representative binding pose of PD00 to CD81 is shown in Fig. 2E. The docked binding pose indicated that the bioactivity of PD00 possibly results from its interaction with the region near residue 188 (Glu for humans and rats, Gln for mice), which was reported to be related to the binding of CD81 and hepatitis C virus protein E2 [24]. Additionally, sequence alignment (Fig. 2F) and comparison of CD81 structures for the three involved species (Fig. 2G) showed that the binding site of PD00 is generally conserved. According to the results of molecular docking, the docked binding pose indicated that the bioactivity of PD00 possibly results from its interaction with the region near residue 188, which may be Phe186. Therefore, PD00 binding occurred in a species-independent manner in mouse, rat and human CD81.

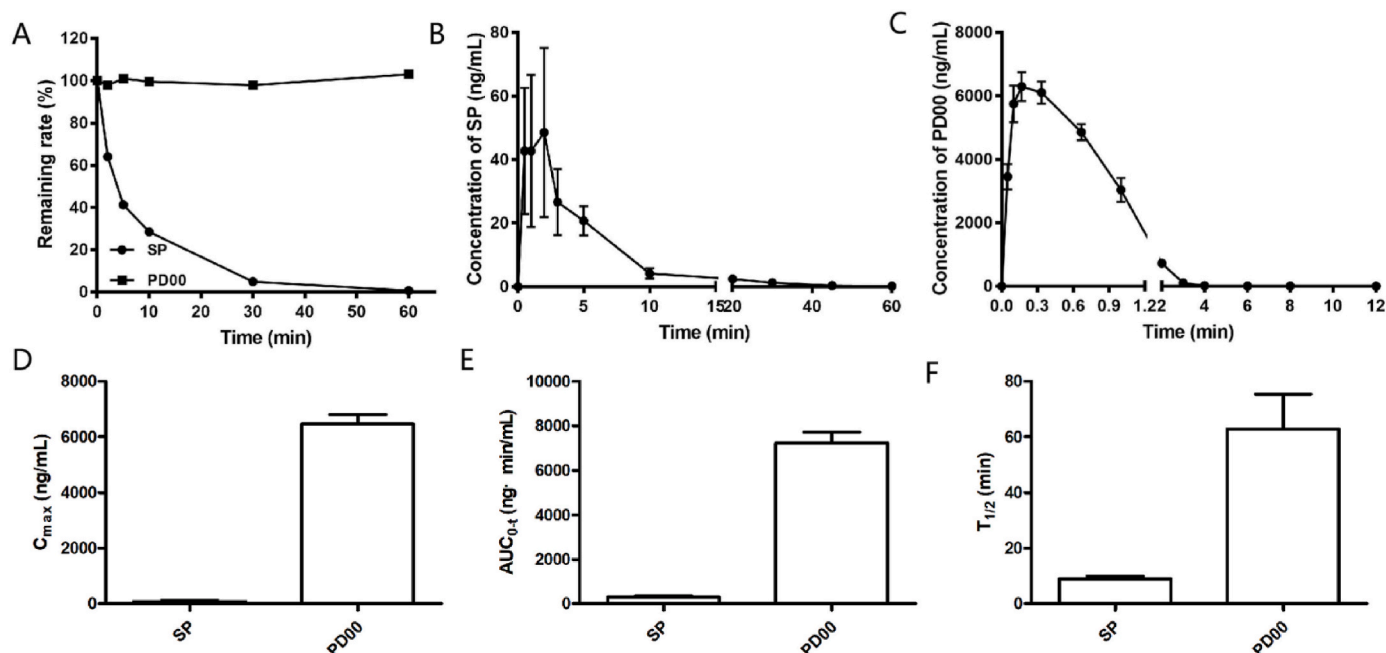


Fig. 1. Metabolic stability of PD00.

(A) The stability of PD00 in rat whole blood was improved compared with SP (duplicate tubes were set up for each sample). (B) Mean plasma concentration-time profiles of SP after a subcutaneous administration of 5 mg/kg of SP in Sprague-Dawley rats ($n = 8$). (C) Mean plasma concentration-time profiles of PD00 after a subcutaneous administration of 5 mg/kg of PD00 in Sprague-Dawley rats ($n = 8$). (D-F) Main pharmacokinetic parameters of SP and PD00 in rats after the subcutaneous administration of 5 mg/kg ($n = 8$). The $C_{\max}$, AUC_{0-t} and $T_{1/2}$ of PD00 were all increased compared with SP.

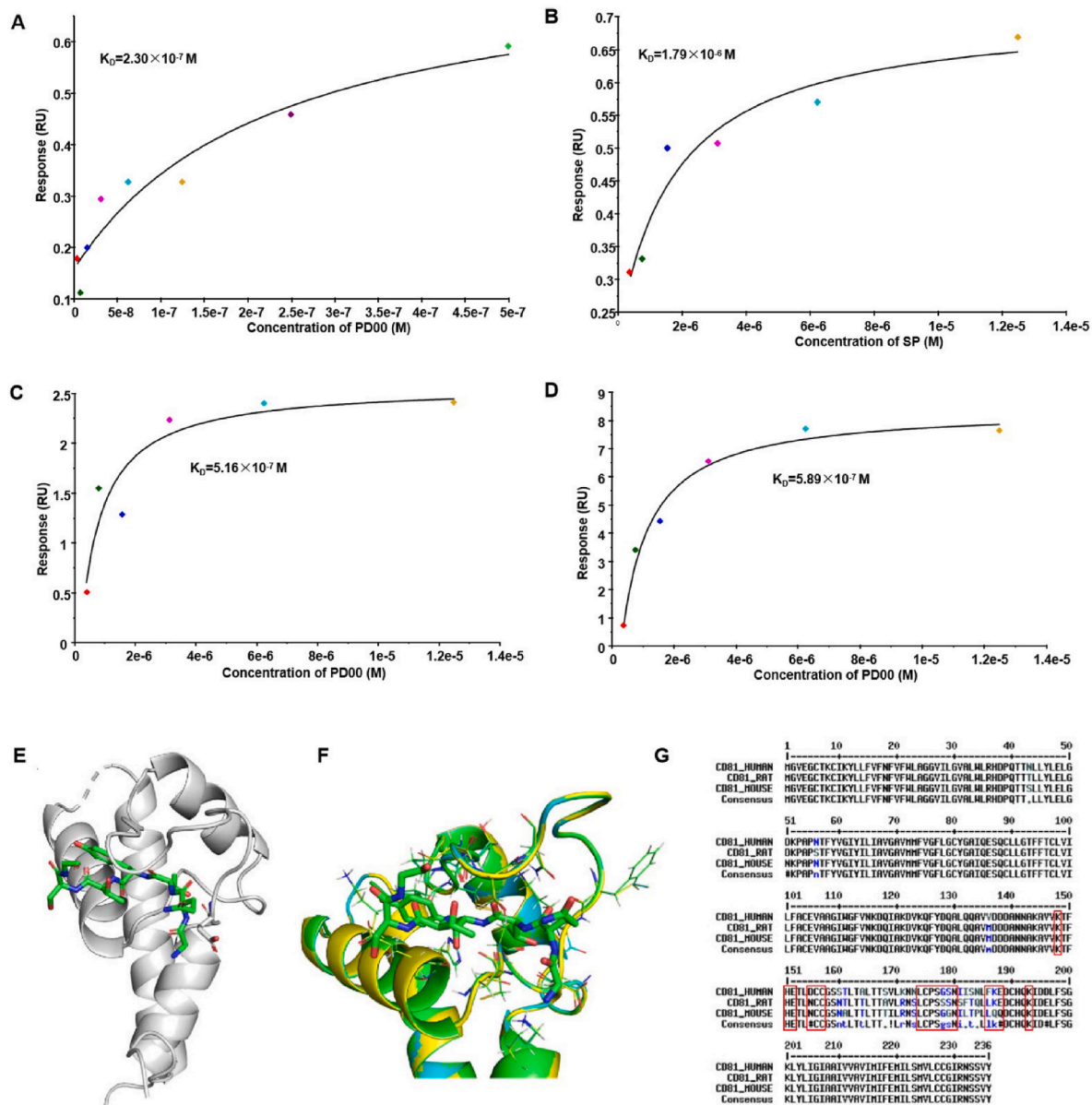


Fig. 2. PD00 has high affinity with recombinant CD81 of different species.

(A–B) The affinity of PD00 and SP to human CD81 was compared using Biacore, and it was found that the affinity of PD00 and CD81 was higher than that of SP. The experiments were repeated twice. (C) The affinity of PD00 to rat CD81. (D) The affinity of PD00 to mouse CD81. The experiment was repeated twice. (E) A representative binding pose of PD00 (green sticks) with human CD81 (Glu 188 was shown as white sticks). (F) Superposition of the CD81 structures for human (green), mouse (cyan) and rat (yellow), the structures of CD81 for mouse and rat were modelled using the human CD81 structure as the template to exclude the influence of the conformational changes of the flexible loop in the structure. (G) Sequence alignment results of CD81 for mouse, rat and human residues constituting the peptide-binding site (defined as residues within 4 Å of PD00) are highlighted with red frames.

3.3. PD00 reduced AHR in OVA-induced murine asthma model

To investigate the effect of PD00 on anti-allergic reactions, an OVA-induced asthma mouse model was established (Fig. 3A). Effective modulation of AHR, including the airway resistance (R_L) and respiratory dynamic compliance (C_{dyn}), was detected. R_L and C_{dyn} were significantly enhanced in the OVA group compared with the control group ($P \leq 0.01$). Mice in the Dex-treated (2 mg/kg) and PD00 (175 and 350 µg/kg) groups showed a marked decrease in R_L and a significant increase in C_{dyn} compared with the OVA group ($P < 0.05$ or $P < 0.01$). There was no significant difference among SP (175 and 350 µg/kg) treatment groups compared with the OVA group in AHR ($P > 0.05$). Treatment with PD00 (175 and 350 µg/kg) also resulted in a marked decrease in R_L and a significant increase in C_{dyn} compared to the high dose (350 µg/kg) of the

SP group ($P < 0.05$ or $P < 0.01$). The results demonstrated that the suppressive effect of PD00 on AHR was greater than that of SP (Fig. 3B and C).

3.4. PD00 effectively inhibited airway inflammatory cell accumulation

The accumulation of total inflammatory cells and eosinophils in BALF was significantly elevated in the OVA group compared with the control group ($P < 0.01$). Treatment with PD00 (175 and 350 µg/kg) and Dex (2 mg/kg) significantly reduced the counts of eosinophils in BALF when compared to the OVA group ($P < 0.05$ or $P < 0.01$). There was no significant effect of SP treatment in inhibiting airway inflammatory cell accumulation compared to the OVA group ($P > 0.05$) (Fig. 3D–G).

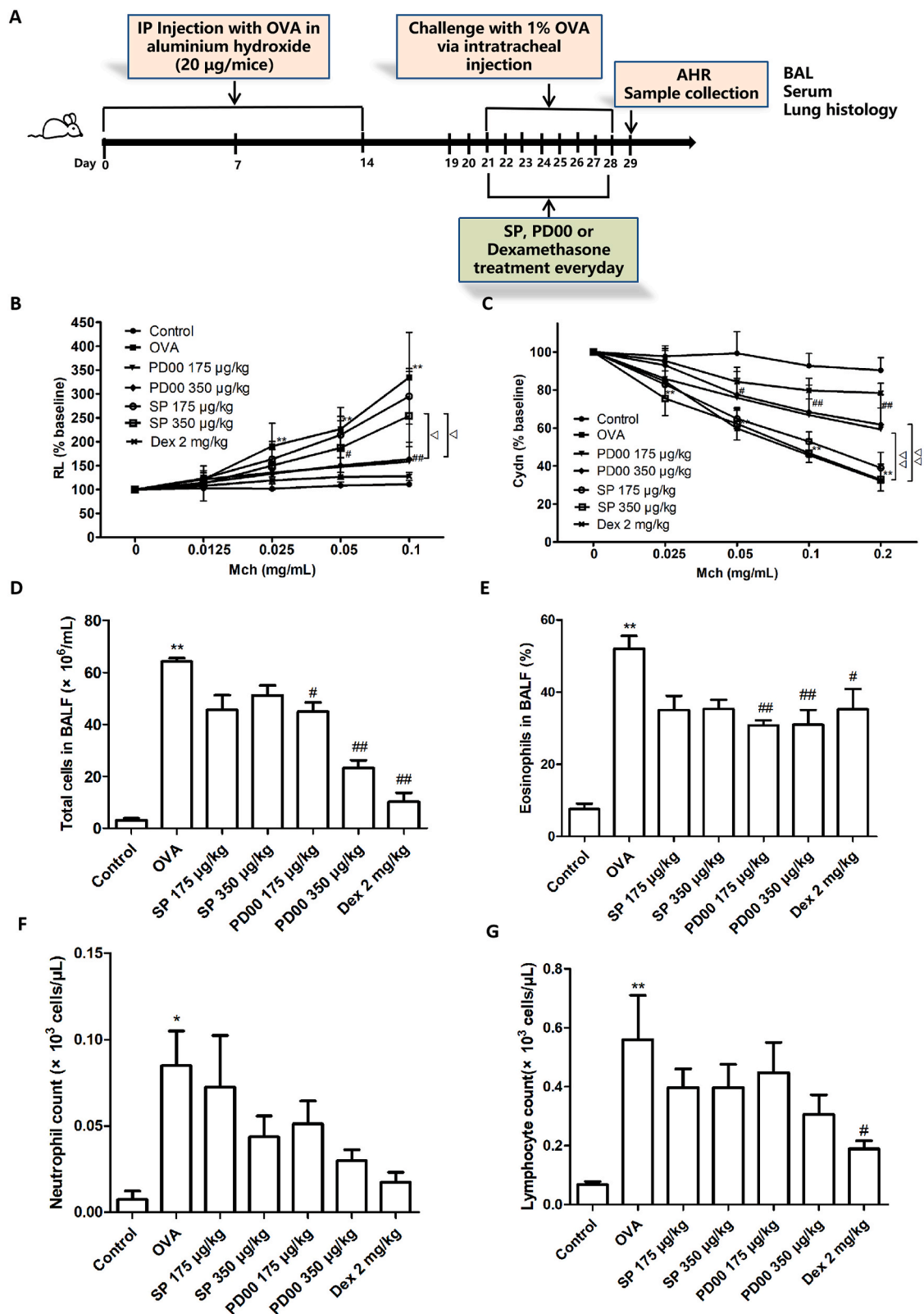


Fig. 3. Effects of PD00 on AHR and BALF inflammatory cell accumulation in OVA-induced asthmatic mice.

(A) Mice sensitised with OVA on day 0, 7 and 14 before subcutaneous injection of vehicle, SP, PD00 or dexamethasone. Mice were challenged with OVA from day 21–28. (B–C) Mice anaesthetised and forced to breathe with increasing concentrations of Mch 24 h after last OVA challenge; changes in AHR, including airway resistance (R_L) and respiratory dynamic compliance (C_{dyn}) were detected. PD00 treatment improved the AHR in asthmatic mice, exhibiting favourable effects compared with SP ($n = 8$). (D–G) The BALF was collected, and inflammatory cells in BALF were classified and counted; PD00 treatment decreased total and eosinophil counts in asthmatic mice; its effect was superior to SP ($n = 10$). The values indicate the mean $\pm$ SEM. Significance is represented by $**P < 0.01$ vs. control; $\#P < 0.05$, $##P < 0.01$ vs. OVA; $\triangle P < 0.05$, $\triangle\triangle P < 0.01$ vs. SP.

3.5. PD00 reduced airway inflammation and collagen deposition in lung tissue

H&E staining was performed to determine the histological features of the lung tissue. H&E staining showed a significant increase in perivascular and peribronchial infiltration in the OVA group compared to that in the control group ($P < 0.01$). Treatment with PD00 (175 and 350 $\mu\text{g}/\text{kg}$) and Dex (2 mg/kg) reduced the infiltration of inflammatory cells into the airways ($p < 0.01$). There was no significant difference between the SP and OVA groups ($P > 0.05$) (Fig. 4A and B). In addition, PD00 could inhibit collagen deposition in the lung tissue of asthmatic mice. Treatment with PD00 (175 and 350 $\mu\text{g}/\text{kg}$) reduced the collagen deposition score of the lung tissues ($P < 0.01$) (Fig. 5A and B).

3.6. PD00 reduced the level of OVA-induced serum IgE

Compared with the control group, sensitisation and challenge with OVA promoted a significant increase in serum OVA-specific IgE levels in mice ($P < 0.01$). Treatment with PD00 (175 and 350 $\mu\text{g}/\text{kg}$) and Dex (2 mg/kg) significantly reduced OVA-specific IgE levels ($P < 0.05$ or $P < 0.01$). A notable reduction in serum OVA-specific IgE level was also detected in the low-dose SP-treated group; however, there was no

significant difference between the high-dose SP and OVA groups, suggesting that SP did not have a good dose-response relationship due to the instability of SP metabolism in mice, whereas PD00 had a better dose-response relationship.

3.7. PD00 treatment affected glycerophospholipid and purine metabolism

The lung metabolites were significantly altered in the OVA group compared with the control group. Fold-changes are depicted in the heatmap for each treatment group (Fig. 6A). Altered lipid metabolism was noted in allergic airway inflammation, with significant increases in the pulmonary levels of phosphatidylcholine (PC), phosphatidylethanolamine (PE), LysoPC (P-18:0/0:0), LysoPC (O-20:2), glycerophosphocholine, glycerylphosphorylethanolamine and glycerol 3-phosphate. In contrast, the concentrations of three phosphatidic acids (PA) containing PA (20:3(8Z,11Z,14Z)/12:0), PA (18:3(9Z,12Z,15Z)/14:0) and PA (14:0/18:3(9Z,12Z,15Z)) were significantly reduced (Fig. 7A). Alterations in purine metabolism were also observed, with obvious increases in D-ribose 5-phosphate (Ribose 5P), 5-aminoimidazole ribonucleotide (AIR), guanosine monophosphate (GMP), guanosine, deoxyguanosine monophosphate (dGMP), adenosine monophosphate (AMP) and deoxyadenosine monophosphate (dAMP);

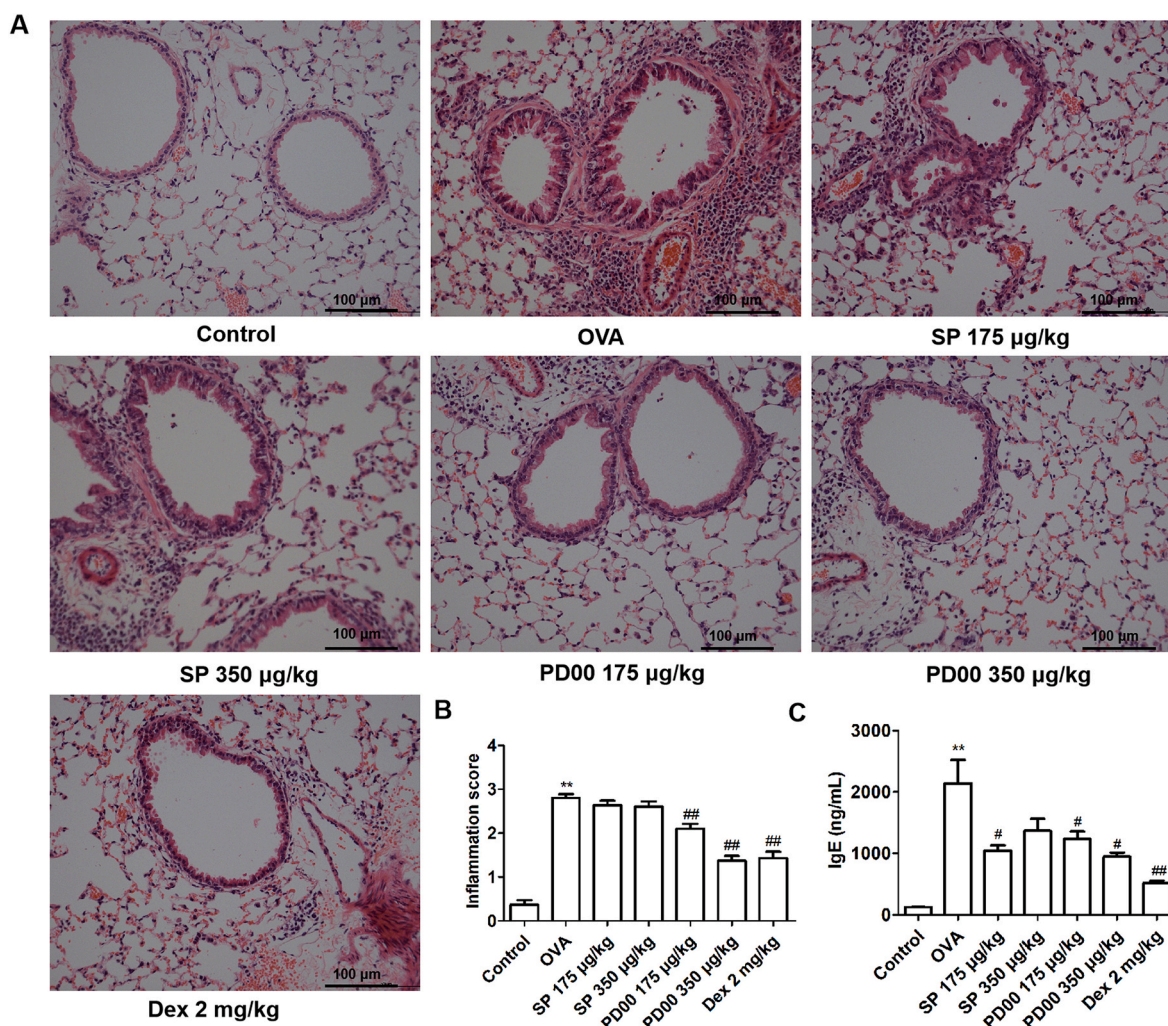


Fig. 4. Effect of PD00 treatment on airway inflammation and IgE concentrations in blood serum.

(A) H&E staining showed PD00 suppressed airway inflammation. Scale bar: 100 μm . (B) Semiquantitative scoring of the severity of inflammatory infiltrates in the airways. PD00 significantly inhibited the infiltration of inflammatory cells into lung tissues in asthmatic mice. (C) Blood samples were collected by cardiac puncture and level of serum IgE was determined. PD00 reduces OVA-induced serum IgE levels in mice. The data are presented as the means $\pm$ SEM of $n = 10$. ** $P < 0.01$ compared to control group; # $P < 0.05$, ### $P < 0.01$ compared to OVA group.

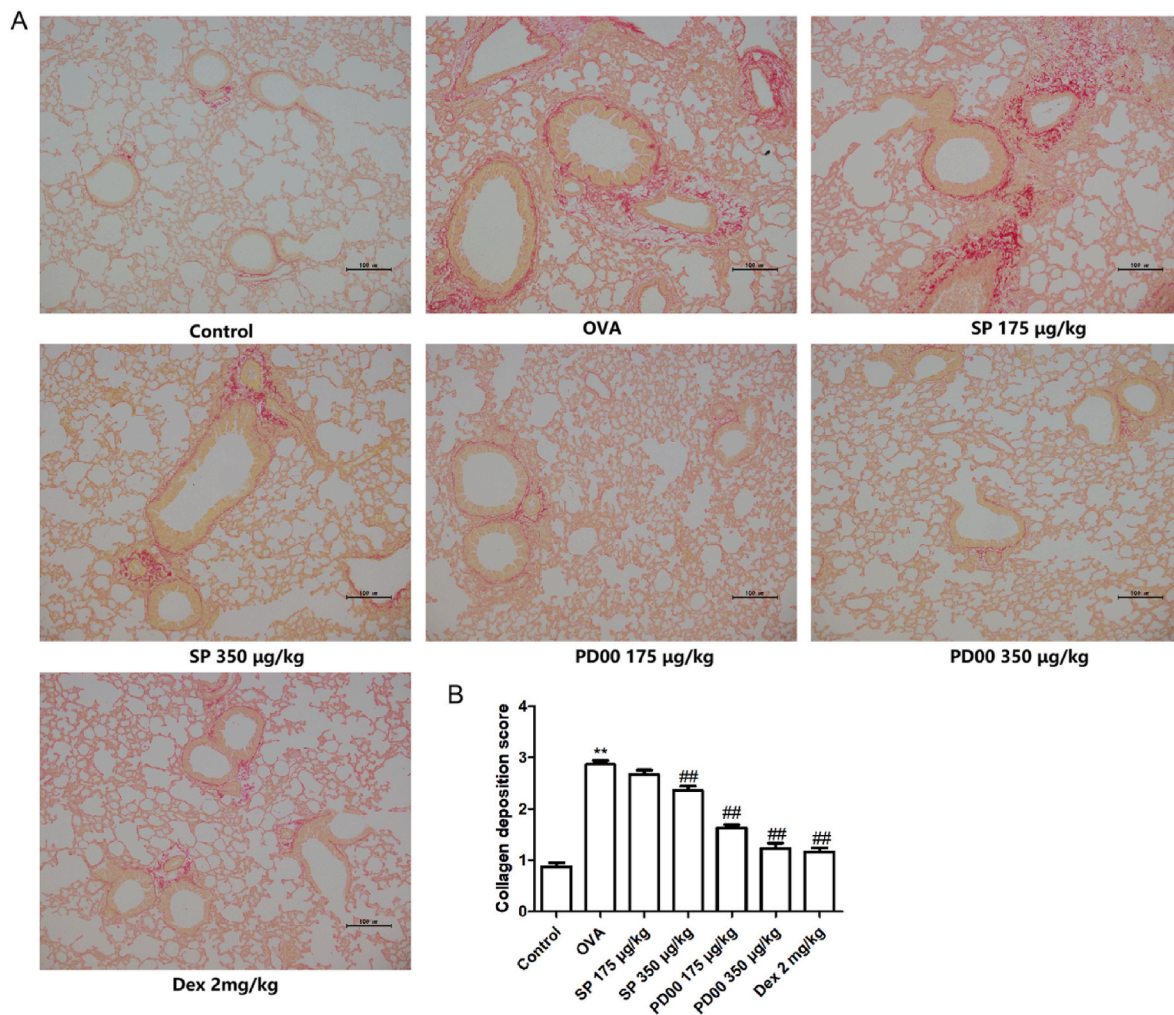


Fig. 5. Effect of PD00 treatment on collagen deposition in tissues.

(A) Sirius red staining showed PD00 suppressed collagen deposition in the lungs. Scale bar: 100 µm. (B) Semiquantitative scoring of the severity of collagen deposition in the lung tissues. PD00 considerably inhibited collagen deposition in the lung tissues in asthmatic mice.

detailed data on fold-changes in various choline glycerophosphates (PCs), diacylglycerols (DGs), triacylglycerols (TGs) and other metabolites are available in the online Supplementary Materials (see [Supplementary Table 1](#) in the online Supplementary Materials). PD00 showed reversed changes in most of the altered metabolites ([Fig. 7B](#)).

Moreover, a complete metabolic pathway analysis using discriminating metabolites was performed to explore the pathway-based metabolomic features. Four differential metabolic pathways ([Fig. 6B](#)) were enriched after pathway analysis: glycerophospholipid metabolism (impact value = 0.337; $p = 0.000058$), purine metabolism (impact value = 0.188; $p = 0.00026$), glycerolipid metabolism (impact value = 0.056; $p = 0.041$) and pyrimidine metabolism (impact value = 0.100; $p = 0.044$). In addition, based on the transcriptome data, the glycerophospholipid and purine metabolism pathway scores ([Fig. 6C](#)) were significantly different in the control, OVA and PD00 groups, further confirming that PD00 inhibited OVA-induced asthma symptoms by regulating glycerophospholipid and purine metabolism.

3.8. PD00 affected the expression of genes related to glycerophospholipid metabolism and purine metabolism pathways in immune cells

After applying the QC criteria, 9109–10,956 single cells were analysed. Based on the gene reference dataset combined with specific marker genes, 13 cell types were identified, and one cell cluster could not be identified based on existing markers ([Fig. 8A](#)). After PD00

treatment, the expression of glycerophospholipid and purine metabolism pathway genes in various immune cells changed significantly ([Fig. 8B](#) and [C](#)). Analysis of DEGs involved in glycerophospholipid and purine metabolism pathways revealed that the mRNA expression of glycerophospholipid metabolism pathway-related genes *Pparg*, *Gpcpd1*, *Lpcat3*, *Pnpla7*, *pla2g1b*, *Adss* and *Lcat* significantly increased after PD00 treatment, and the mRNA levels of *pld4* and *lypla1* were significantly decreased; the mRNA expression of purine metabolism pathway-related genes *Gmpr*, *Ampd2*, *Adssl1* and *Adss* significantly increased after PD00 treatment, while the mRNA expression of *Gmps*, *Nt5c*, *Adsl* and *lmpdh2* significantly decreased, as compared with the OVA group ([Fig. 8D](#)). This is consistent with the results of metabolomics. These results suggest that PD00 affects the state of immune cells by affecting the glycerophospholipid and purine metabolism pathways of immune cells. Therefore, it plays a role in regulating the immune microenvironment and improving inflammation.

4. Discussion

Asthma is a heterogeneous disease characterised by chronic airway inflammation, mucus overproduction, bronchial hyperreactivity and airway remodelling. The onset of asthma is a result of the complex interplay between genetic and environmental factors [25]. GWASs have already identified multiple chromosomal loci associated with higher asthma susceptibility [26], but none of these genes affect all features of

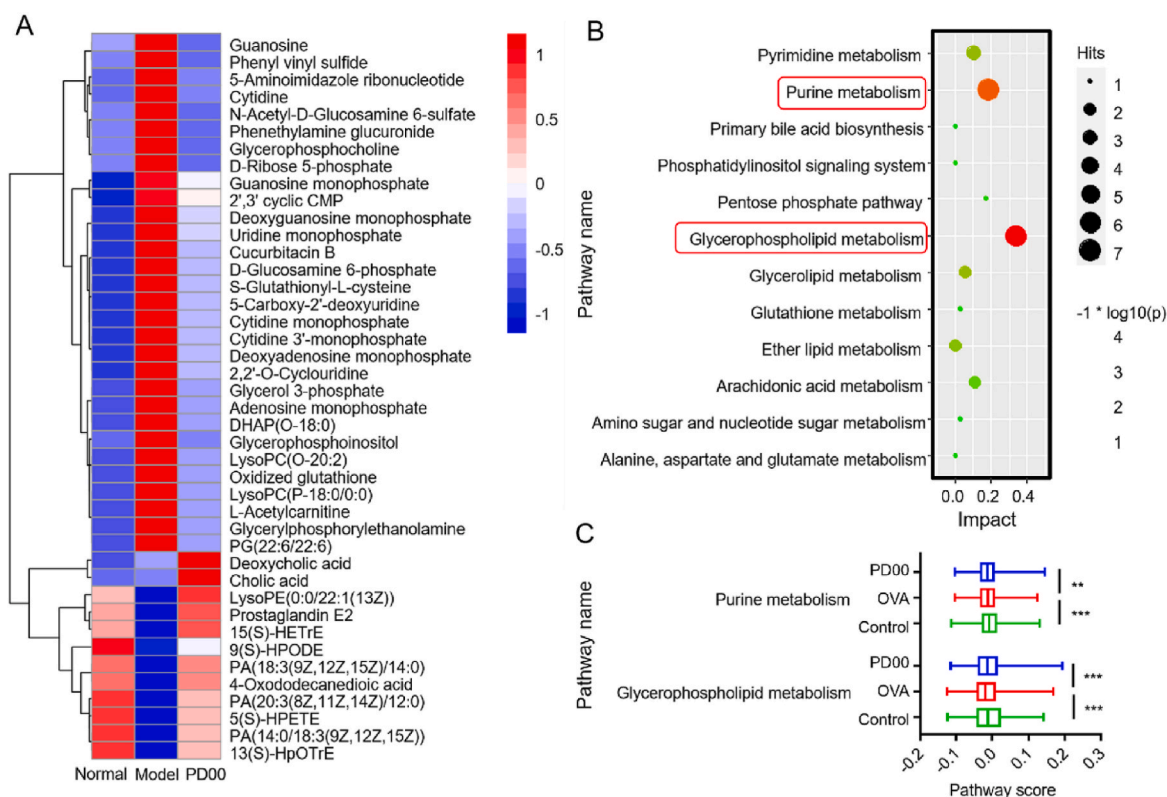


Fig. 6. Metabonomics and transcriptomics revealed the effect of PD00 on purine and glycerophospholipid metabolism in OVA-induced asthmatic mice. (A) Heatmap overview of the 42 differential metabolites in the control, OVA and PD00 groups. (B) Dot plot resulting from metabolic pathway enrichment analysis of 42 differential metabolites confirmed that PD00 significantly changed purine and glycerophospholipid metabolism. (C) The transcriptome pathway score illustrated that PD00 also regulated purine and glycerophospholipid metabolism. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

this heterogeneous disease [27]. Despite compounds or antibodies that act on different targets with certain pharmacodynamic effects in the treatment of asthma have been employed recently, some patients still show uncontrollable asthma symptoms. Therefore, the development of anti-asthma drugs with new mechanisms of action is still an important issue.

CD81 is an aglycosylated membrane protein expressed on immune cells such as T cells, B cells and macrophages [28–30]. Owing to its special transmembrane structure, it can be used as a platform for receptor clustering and signal transmission, which is involved in regulating cell proliferation, differentiation, adhesion and metabolic homeostasis. Polypeptides are a class of single-chain compounds formed by the dehydration condensation of 3–50 amino acids. Compared to general small-molecule drugs, they exhibit higher activity and stronger selectivity [31]. However, their use is limited by intrinsically low bioavailability and poor stability [32]. To overcome these issues, retro-inverse analogues with reversed sequences of mirror-image amino acid residues have been investigated as more stable surrogates [33]. Here, we synthesised the retro-inverse peptide PD00 based on the amino acid sequence of the SP targeting CD81. We found that the metabolic stability of PD00 was considerably increased, and that it had a higher affinity for CD81. We also found that the metabolic half-life of the retro-inverse peptide PD00 was substantially higher in rats than that of the prototype peptide SP, and the affinity with the target protein CD81 was not lower than that of SP. Moreover, when administered subcutaneously without the aid of a minipump, its therapeutic effect on OVA-induced asthma in mice was better than that of SP.

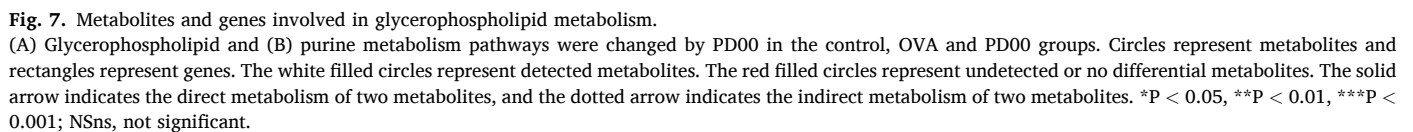
A large number of inflammatory cells and a variety of inflammatory mediators are involved in the regulation of airway allergic inflammation in bronchial asthma. Lymphocytes, eosinophils, macrophages, neutrophils, airway epithelial cells, vascular endothelial cells and platelets all

play a certain role in the pathogenesis of bronchial asthma. Treatment with PD00 significantly reduced the counts of eosinophils in BALF but there was no significant effect of PD00 treatment in inhibiting airway neutrophils or lymphocytes accumulation compared to the OVA group ($P > 0.05$) (Fig. 3F and G). This suggests that it may have a better effect on asthma patients with eosinophilia.

As asthma is a heterogeneous disease, non-targeted metabolome analysis and transcriptome sequencing are often used to study its pathogenesis and the efficacy of drug intervention. In order to find the signal pathway regulated by PD00 and point out the research direction for further exploring its molecular mechanism, single-cell transcriptome sequencing technology was used to establish the differential gene expression profile of cells in the lung tissue of OVA-induced asthma model mice after PD00 treatment. We also observed that the anti-asthma mechanism may be related to the regulation of glycerophospholipid and purine metabolism.

Based on the non-targeted metabolomics analysis of mouse lung tissue, LysoPCs were dramatically increased in asthmatic lung tissues. Previous results showed that lysophospholipids have a wide variety of functions, as lipid mediators, that are exerted through GPCRs specific to each lysophospholipid [13]. CD81 plays a central role in GPR56-CD81-G q/11-G complexes [34]. In particular, full-length CD81 tetraspanin has interesting cholesterol-related properties that modulate its activity in cells [35,36]. PD00 considerably suppressed LysoPC, glycerophosphocholine and glycerylphosphorylethanolamine production, suggesting that PD00 may ameliorate lung inflammation by regulating glycerophospholipid metabolism.

Additionally, Ribose 5P, AIR, GMP, guanosine, dGMP, AMP and dAMP involved in the purine metabolism pathways were upregulated in the OVA group. AMP can induce eosinophilia and is an indirect stimulus to induce bronchial hyperresponsiveness because it works by releasing



regulates lipid [11] and dNTP metabolism [12]. Our results showed that both lipid and nucleotide metabolism in immune cells were altered in the OVA-induced mouse asthma model. Moreover, immune cell activation is intimately linked to metabolic reprogramming [39]. This suggests

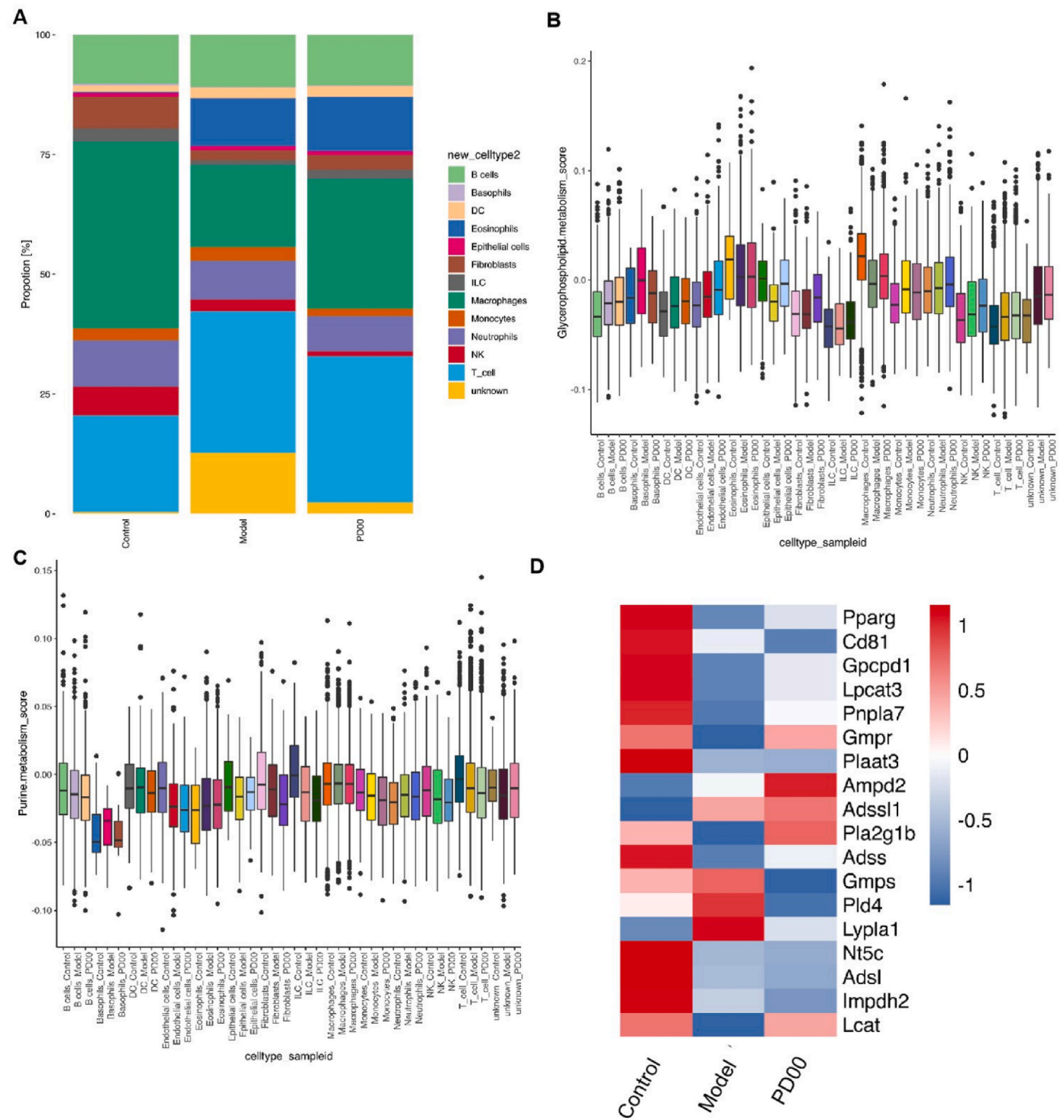


Fig. 8. PD00 affects the mRNA expression of genes related to glycerophospholipid and purine metabolism. (A) Histogram of the proportion of cell populations between samples. (B–C) Expression of differential genes in glycerophospholipid metabolism pathway and purine metabolism pathway in different cells. (D) Heatmap of significantly differentially expressed genes between groups. The Model group is the OVA treatment group.

that metabolic reprogramming of immune cells plays a role in the occurrence and development of asthma. This may provide a new direction for asthma treatment. Although we have shown the utility of PD00 in the treatment of allergic asthma through various studies, its specific molecular mechanisms, such as how PD00 and CD81 combine to mediate the conformational change of CD81 and how the downstream immune signaling cascade is regulated, need to be further studied. We are currently conducting protein crystal structure analysis to determine

the amino acid binding site of PD00 on CD81 protein, and aim to explore how the conformation change of CD81 caused by PD00 mediates the activation of downstream signal proteins, especially the expression changes of glycerophospholipid and purine metabolism signal pathway proteins.

5. Conclusions

Our study demonstrated that a retro-inverse-modified peptide PD00 with favourable metabolic stability, had a high affinity for CD81. PD00 reduced the inflammatory response and AHR in OVA-induced allergic asthma by ameliorating glycerophospholipid and purine metabolism disorders of immune cells in mouse lungs. Our results demonstrate that PD00 has a similar affinity to rat, mouse and human recombinant CD81 proteins. This suggests that the pharmacological effects of PD00 in rodent models may have similar effects in humans.

Funding

This study was funded by Science and Technology Commission of Shanghai (20S11903500).

Author contributions

Shumei Ma, data curation and original draft; Kuan Yang, formal analysis; Zhihong Li and Liang Li, methodology; Yue Feng, project administration; Xiaowei Wang, resources; Zhengdan Zhu, software and visualisation; Zhiyong Wang and Juan Wang, supervision; Yizhun Zhu, investigation; Li Liu, conceptualisation, funding acquisition and review and editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

We thank P.D. Zhonglian Cao for assistance with the Biacore experiments, OE Biotech Co. Ltd. (Shanghai, China) for providing single-cell RNA-seq, and Suling Zhang and Muladili Maimaituoheti for assistance with bioinformatics analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pupt.2022.102185>.

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