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Original research article

Anti-oxidative and anti-inflammatory properties of xanthorrhizol in airway inflammation

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ABSTRACT

Inflammation and oxidative damage contribute to the pathogenesis of chronic pulmonary diseases. Xanthorrhizol (XNT), a natural sesquiterpenoid from *Curcuma xanthorrhiza*, has been shown to have anti-microbial, anti-oxidative and anti-inflammatory properties. The present study evaluated the anti-oxidative and anti-inflammatory activities of XNT in various cell-free and cell culture models. XNT was found to possess a moderate free radical-scavenging capability using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) cell-free assay, whose potency is lower than, but efficacy is comparable to the FDA-approved antioxidant agent trolox. In aeroallergen house dust mite (HDM)- and lipopolysaccharide (LPS)-stimulated Raw 264. 7 macrophage cell line, XNT down-regulated both mRNA expression and protein production of proinflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, GM-CSF, etc. In addition, XNT was able to directly activate multiple antioxidant genes such as *Hmox1* (Hemoxygenase-1), *Nqo1* (NADPH quinone oxidoreductase-1), *Gsr* (glutathione reductase), and *Gpx1* (glutathione peroxidase 1) in Raw 264.7 cell line. Furthermore, XNT suppressed corticosteroid-resistant cytokine IL-27 and IL-6 production in mouse primary alveolar macrophages upon combined LPS/IFN-γ stimulation, which implicates a severe subtype of asthma. Particularly, by combining cell surface markers with intracellular cytokine staining strategy, it was shown, for the first time that XNT decreased Th2/Th17 cytokine IL-5-, IL-13- and IL-17-expressing CD4 T cell frequency in single cell suspension isolated from HDM- and aspergillus fumigatus (AF)-exposed mouse lungs. Taken together, our findings reveal novel anti-oxidative and anti-inflammatory properties of XNT and implicate a new potential treatment to chronic pulmonary diseases.

Keywords: xanthorrhizol, house dust mite, LPS, alveolar macrophage, Raw 264.7

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1. Introduction

Bacterial and viral infection, cigarette smoke, air pollutants and aeroallergens are widely implicated in pulmonary inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, pulmonary fibrosis, acute respiratory distress syndrome, etc. [1] The major pathological features of pulmonary inflammatory diseases are immune cell infiltration, mucus hyper-secretion, elevated pro-inflammatory cytokine and chemokine production, heightened lung oxidative damage, and deteriorated lung function.[2] Agents that possess anti-inflammatory and anti-oxidative properties have been proposed to be potential therapies for pulmonary inflammatory diseases.

As the most abundant immune cells in healthy airways and sentinel cells to external stimuli, alveolar macrophages play important roles in innate immunity including active phagocytosis, generation of danger signals and production of pro-inflammatory mediators.[3] Lipopolysaccharides (LPS), the major outer surface membrane component of Gram-negative bacteria, is a potent activator of macrophages. LPS stimulates macrophages through Toll-like receptor 4 (TLR4) activation, which activates a series of signaling cascades, resulting in increased production of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-33, etc and elevated expression of inducible nitric oxide synthase (iNOS).[4] iNOS is essential for the formation of NO and downstream free radical reactive nitrogen species generation, leading to 3-nitrotyrosine protein oxidative damage[5] Excessive acute production of pro-inflammatory cytokines by macrophages in response to LPS is regarded as the major cause of septic shock.[4]

On the other hand, CD4 T helper (Th) cells, particularly Th2 cells, are believed to play an essential role in aeroallergen-induced asthma pathogenesis and disease progression. Upon repetitive aeroallergen exposure, naïve T cells are primed and differentiated into Th2 cells, which produce typical Th2 cytokines including IL-4, IL-5 and IL-13, promoting airway eosinophilia, mucus hyper-secretion, airway remodeling and hyperresponsiveness.[6] In addition to Th2 cells, it has been shown that IL-17-expressing Th17 cells are also implicated in certain subtypes of asthma, leading to more neutrophilia-dominant than eosinophilia.[7] Furthermore, some T cells co-producing both Th2 and Th17 cytokines were also observed from asthmatic patients and mouse asthma models.[8-9]

Xanthorrhizol (XNT) is a natural sesquiterpenoid compound isolated from the rhizomes of *Curcuma xanthorrhizza* Roxb, also known as Java turmeric.[10] *C. xanthorrhizza* originates from Indonesia and has been used in the country as a folk medicine for the prevention and treatment of various ailments.[11] XNT has been shown to possess various biological activities, including anti-microbial,[12-13] anti-viral,[14] anti-hyperglycemic,[15] anti-inflammatory,[15-18] anti-oxidative[18-19] and anti-cancer activities.[20-21]

In this study, we first evaluated free radical scavenger activity of XNT, followed by anti-oxidative and anti-inflammatory effects of XNT on LPS- or LPS/ IFN-γ-stimulated mouse macrophages *in-vitro*, and as well as aeroallergens-exposed CD4 T cells *ex-vivo*. We report here for the first time that XNT not only contains efficient intrinsic free radical-neutralizing activity, but also possesses anti-oxidative and anti-inflammatory properties on type-1, type-2 and type-17 cytokines. Our findings support a novel therapeutic value for XNT to treat inflammatory lung diseases.

2. Materials and Methods

2.1 Materials

Murine macrophage cell line Raw 264.7 was purchased from ATCC (Cat# TIB-71™, Manassas, VA). Xanthorrhizol (XNT, CAS Number 30199-26-9) was obtained from Cayman Chemical (Ann Arbor, MI). House dust mite extract (HDM, dermatophagoides pteronyssinus extract, Cat# XPB82D3A2.5, Lot No. 371590) and aspergillus fumigatus

(AF, Cat# XPM3D3A4, Lot No. 359392) came from Stallergenes Greer (Lenoir, NC). Lipopolysaccharide (LPS from *E. coli*, O111:B4), Trolox, N-acetylcysteine (NAC), resveratrol (RES), dexamethasone (DEX), 2,2-Diphenyl-1- picrylhydrazyl (DPPH), 12-myristate 13-acetate (PMA), ionomycin, DMSO (99% cell culture grade), and RNAzol were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffer saline (1×PBS), DMEM, RPMI-1640, fetal bovine serum (FBS), RNAlater, Collagenase type I, DNase I, brefeldin A, Maxima First Strand cDNA Synthesis Kit, GoTaq® qPCR Master Mix, M-PER® Mammalian Protein Extraction Reagent, Pierce™ Protease and Phosphatase Inhibitor Mini Tablets, ELISA/ ELISPOT Diluent, ELISA substrate TMB (3,3',5,5'-tetramethylbenzidine) and PVDF membrane came from Thermo Fisher Scientific (Waltham, MA).

2.2 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

Various concentrations of XNT, Trolox, NAC and RES, prepared in methanol were incubated with 0.1 mM DPPH solution for 30 minutes. Concentration-dependent free radical-scavenging activities were measured colorimetrically at 517 nm on Infinite® 200 microplate reader (TECAN, Männedorf, Switzerland).

2.3 Cell culture and drug treatment *in-vitro*

Murine macrophage cell line Raw 264.7, and isolated primary mouse alveolar macrophages were cultured in DMEM medium supplemented with 10% FBS. All the cell cultures were maintained in a humidified 37°C, 5% CO_2 incubator. A 60 mM stock of XNT was prepared in DMSO. The cells were pre-treated with various concentrations of XNT (0.05% DMSO as vehicle control) or DEX, followed by stimulation with 100 ng/ml LPS or 100 μg/ml HDM for indicated time points.

2.4 MTS assay

Cell cytotoxicity was measured with CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI) according to manufacturers' instruction. Briefly, cells in a 96-well plate were incubated with MTS solution for 2 hours, and absorbance at 490 nm was recorded with TECAN Infinite® 200 microplate reader.

2.5 HDM- or AF-induced allergic asthma model

BALB/c mice of 6-8 weeks old were purchased from InVivos Pte Ltd (Singapore) and maintained in a 12-hour light-dark cycle with food and water available *ad libitum*. All experimental protocols were granted approval and performed according to the guidelines by Institutional Animal Care and Use Committee of the National University of Singapore. Mice were anesthetized with inhaled isoflurane and given 100 μg HDM extract or AF in 40 μl saline *via* intratracheal administration on days 0, 7 and 14.[22] Negative control mice were given 40 μl of sterile saline. Mice were euthanized on day 17.

2.6 Mouse lung single cell preparation and alveolar macrophage isolation

Mouse lungs were perfused with 1× PBS and minced with a surgical scissor in complete RPMI-1640 medium containing collagenase type I (2 mg/ml) and DNase I (100 U/ml), and further digested using gentleMACS Octo Dissociator Tissue Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Lung homogenate was filtered through a 70 μM MACS SmartStrainer to obtain a uniform single-cell suspension. Cell suspension was washed with MACS buffer (1× PBS/2 mM EDTA/0.5% BSA) twice and red blood cells were lysed in RBC Lysis Buffer (Biolegend, San Diego, CA). Cell suspension was then incubated with anti-mouse Siglec-F microbeads (Miltenyi Biotec) to positively label Siglec-F^+ cells. After incubation, cell suspension was transferred to a MS column for magnetic separation using OctoMACS™ Separator (Miltenyi Biotec). After washing, magnetically labeled Siglec F^+ cells were eluted from the column by a plunger. The cells were re-suspended in RPMI-1640 medium and incubated in CO_2 incubator to allow alveolar macrophage (AM) adherence. The non-adherent Siglec-F^+ eosinophil population was removed by washing.

2.7 Flow cytometry analysis

To assay IL-4-, IL-5- and IL-13-expressing CD4 T cell frequency, mouse lung single cell suspension was re-stimulated with 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (1 μg/ml), and protein transport inhibitor brefeldin A (1: 1000 dilution), in a 37°C, 5% CO_2 incubator for 5 hours. The cells were stained with surface markers CD45-FITC, CD3-APC/ eFluor780 and CD4-PerCP/Cy5.5 for 20 minutes, fixed and permeabilized with fixation buffer and 1× permeabilization buffer. Fixed cells were then stained for intracellular cytokines IL-4-PE/ Cy7, IL-5-BV421, and IL-13-PE. All samples were ran on BD LSRFortessa cell analyzer and FlowJo software (BD Biosciences) were used to analyze the results.

2.8 ELISA

Levels of TNF-α, IL-1β, IL-6, IL-27p28 and IL-33 were measured with sandwich ELISA according to the manufacturer' s instructions. IL-1β and IL-33 were measured in total cell lysates which were prepared in M-PER® Mammalian Protein Extraction Reagent containing protease and phosphatase inhibitors. Briefly, 96-well BD Falcon ELISA plates were coated with capture antibodies at 4°C overnight. After blocking with ELISA diluent, culture medium or cell lysate samples were added and incubated for 2 hours at room temperature. After washing, biotinylated detection antibodies, mixed with enzyme reagent streptavidin-HRP conjugate were added and incubated for 1 h at room temperature. The plates were washed and developed with TMB substrate. The reaction was stopped with stopping solution (1 M H_2SO4) and the OD was read at 450 nm on Infinite® 200 microplate reader (TECAN).

2.9 RNA extraction and qPCR

Total RNA was extracted from lung tissues or cells using RNAzol according to the manufacturer' s instructions. cDNAs were synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with Biometra gradient thermal cycler (Goettingen, Germany). qPCR was performed with GoTaq® qPCR Master Mix (Promega, Madison, WI) as a detection dye in the ABI 7900 Real-Time PCR machine (Thermo Fisher Scientific) and presented as fold differences over the controls by the $2^{-\Delta\Delta Ct}$ method. The efficiency of all used primer pairs was pre-tested. Mouse *Actb* (β-actin) gene was used as an endogenous control. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The primer pairs are listed in Table 1.

2.10 Statistical analysis

Data are presented as means ± SEM. One-way ANOVA followed by a Dunnett test was used to determine significant differences between treatment groups. Significant levels were set at $p < 0.05$.

3. Results

3.1 XNT possesses free radical- neutralizing activity

The chemical structure of XNT is shown in Fig. 1A. XNT contains a phenolic hydroxyl group, indicating its potential free radical-neutralizing activity. DPPH assay was used to evaluate the free radical scavenging capability of XNT. XNT exhibited a milder free radical-scavenging activity as compared to the well-established antioxidant reagents trolox, resveratrol (RES), and N-acetylcysteine (NAC) (Fig. 1B). XNT had an EC_{50} value of 787 μM, approximately 47-fold, 30-fold and 9-fold weaker than trolox, RES and NAC, respectively (Fig. 1C) . Although XNT displayed a relatively weaker potency, it had a comparable and high efficacy (E_{max} = 80%) among the compounds tested (Fig. 1C).

3.2 Cytotoxicity of XNT

MTS assay was performed to measure the impact of various concentrations of XNT on Raw 264.7 macrophage cell viability. XNT, up to 30 μM did not show any effect on Raw 264.7 cell viability after incubation for 24 hours (Fig. 2). Stimulation of Raw cells with LPS for 24 hours slightly promoted the cell proliferation, but the presence of XNT in combined with LPS stimulation did not have any additional effect on Raw cell viability (Fig. 2).

Table 1. Sequence of primer pairs used in this study.

Gene	Full name	Gene ID	Primer	Sequence (5'-3')
Actb	Actin, beta	11461	Forward	TCATGAAGTGTGACGTTGACATCCG
			Reverse	CCTAGAAGCATTTGCGGTGCACGATG
Csf2	Colony-stimulating factor-2	12981	Forward	GGCCTTGGAAGCATGTAGAGG
			Reverse	GGAGAACTCGTTAGAGACGACTT
Gpx1	Glutathione peroxidase-1	14775	Forward	GGTTCGAGCCCAATTTTACA
			Reverse	TCGATGTCGATGGTACGAAA
Gsr	Glutathione reductase	14782	Forward	GCGTGAATGTTGGATGTGTACC
			Reverse	GTTGCATAGCCGTGGATAATTTC
Hmox1	Heme oxygenase-1	15368	Forward	CCTCACTGGCAGGAAATCATC
			Reverse	CCTCGTGGAGACGCTTTACATA
Il1b	Interleukin-1 beta	16176	Forward	AGCTTCAGGCAGGCAGTATC
			Reverse	AAGGTCCACGGGAAAGACAC
Il33	Interleukin-33	77125	Forward	CCTCCCTGAGTACATACAATGACC
			Reverse	GTAGTAGCACCTGGTCTTGCTCTT
Il6	Interleukin-6	16193	Forward	TAGTCCTTCCTACCCCAATTTCC
			Reverse	TTGGTCCTTAGCCACTCCTTC
Nos2	Nitric oxide synthase-2, inducible	18126	Forward	CGGGCAAACATCACATTCAGATCCCG
			Reverse	TATATTGCTGTGGCTCCCATGTT
Nqo1	NAD(P)H dehydrogenase, quinone-1	18104	Forward	TCGCCATTTTCTTCGGGCTA
			Reverse	GAGCAATTCCCTTCTGCCCT
Tnf	Tumor necrosis factor	21926	Forward	GATCGGTCCCCAAAGGGATG
			Reverse	GTGGTTTGTGAGTGTGAGGGT
Tslp	Thymic stromal lymphopoietin	53603	Forward	AGTCTTTCTCACCTCCCCTG
			Reverse	GGGTTTAGATGCTGTCATTGGT

3.3 Effect of XNT on pro-inflammatory cytokine and chemokine gene induction

As sentinel cells to external stimuli, macrophages play an important role in innate immunity by executing phagocytosis, producing pro-inflammatory mediators, and facilitating activation of adaptive immunity. Upon aeroallergen HDM stimulation, genes of pro-inflammatory cytokines TNF-α (*Tnfa*) and IL-1β (*Il1b*), chemokine GM-CSF (*Csf2*) and enzyme iNOS (*Nos2*) were significantly induced in Raw cells, and pre-treatment with XNT inhibited the transcription of these genes in a concentration-dependent manner (Fig. 3A). Besides, bacterial component LPS not only drastically stimulated gene *Tnfa, Il1b,* and *Csf2,* but also activated genes of acute phase protein *Il6,* alarmin *Il33* and pleiotropic cytokine *Tslp* in Raw 264.7 cells. Consistently, XNT suppressed the above gene induction in a concentration-dependent manner, indicating potential anti-inflammatory effects (Fig. 3B).

3.4 Regulation of antioxidant genes by XNT in LPS-stimulated Raw 264.7 cells.

Having demonstrated a mild free radical-scavenging capability of XNT in the cell-free DPPH assay, we further examined if XNT was able to modulate antioxidant genes. Stimulation of Raw 264.7 cells with LPS increased the mRNA expression of antioxidant genes *Hmox1 (Hemoxygenase-1, HO-1), Nqo1 (NADPH quinone oxidoreductase-1), Gsr (glutathione reductase)*, but decreased that of *Gpx1* (*glutathione peroxidase 1)*. Treatment of XNT of 30 μM was able to significantly augment the expression of these antioxidant genes, indicating anti-oxidative effects of XNT *in-vitro* (Fig. 4)

3.5 Effects of XNT on pro-inflammatory cytokine production in mouse macrophages.

In Raw 264.7 macrophage cell line, LPS not only stimulated the mRNA level of pro-inflammatory mediators, but also induced pro-inflammatory cytokines TNF-α and IL-6 protein secretion into culture media, and as well as IL-1 family members IL-1β and IL-33 in the total cell lysates. Pre-treatment of XNT reduced LPS-triggered protein production of TNF-α, IL-6, IL-1β and IL-33 in Raw 264.7 cells (Fig. 5A). It has been shown that combined LPS and IFN-γ was able to induce steroid-resistant airway hyperresponsiveness in mice by producing steroid-resistance cytokine IL-27 in macrophages.[23] In primary alveolar macrophages isolated from naïve mouse, combined LPS and IFN-γ treatment promoted a substantial increase in IL-27 and IL-6 protein production, and XNT was able to decrease their levels in the culture media concentration-dependently (Fig. 5B).

3.6 Effects of XNT on Th2/Th17 cytokine expression from pulmonary $CD4^+$ T cells *ex-vivo*.

Having demonstrated the anti-inflammatory effects of XNT on LPS or LPS/IFN-γ-induced Th1 cytokines including TNF-α, IL-1β, IL-6, IL-27 and IL-33 in macrophages, we further sought to investigate if XNT was able to modulate Th2 and Th17 cytokines in $CD4^+$ T cells. Mouse allergic asthma models were developed by repetitive exposure to aeroallergen HDM or AF. Lung single cell suspension was isolated and pre-treated with various concentrations of XNT or dexamethasone (DEX, positive control) before re-stimulation with PMA/ionomycin and treatment with golgi blocker brefeldin A. Th2/Th17 cytokines IL-5, IL-13 and IL-17 were analyzed by intracellular cytokine staining with flow cytometry. The gating strategy of pulmonary $CD4^+$ T cells for intracellular cytokine staining is shown in Fig. 6A. We detected significant increases in the frequency of IL-5-, IL-13- and IL-17-expressing $CD4^+$ T cells from HDM- or AF-exposed mouse lung (Fig. 6B and 6C). Treatment of XNT was able to reduce IL-5-, IL-13- and IL-17-expressing $CD4^+$ T cell frequency in a concentration-dependent manner. Besides, DEX, as a positive control, exhibited potent inhibitory effects on both Th2 and Th17 cytokines (Fig. 6B and 6C).

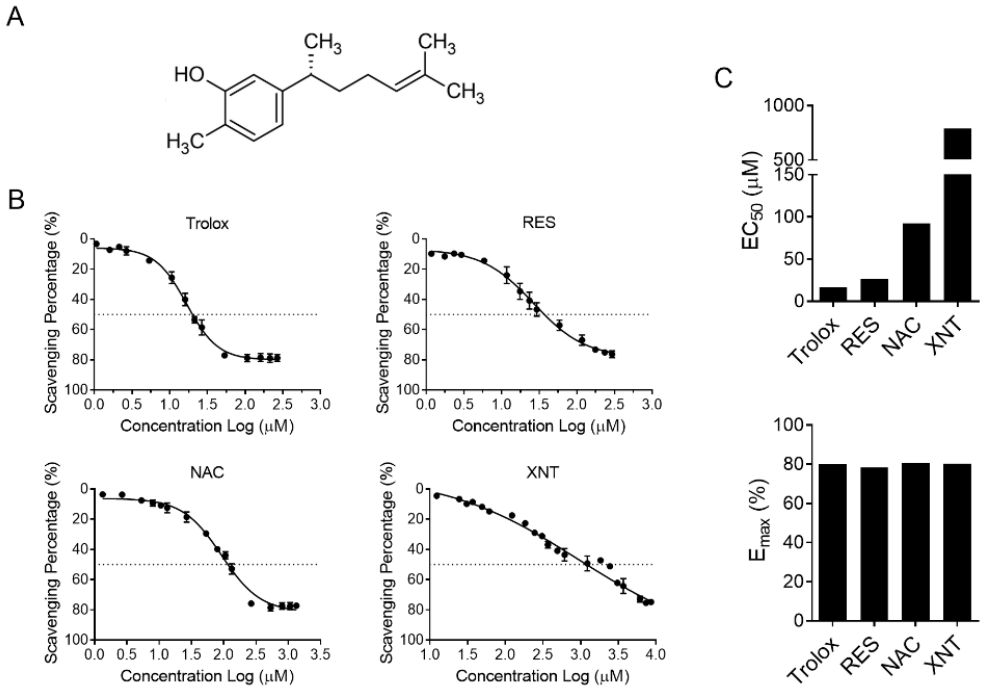

Fig.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) cell-free radical-scavenging activity assay. (A) Chemical structure of xanthorrhizol (XNT). (B) DPPH scavenging activity (%) is plotted against concentrations of antioxidant agents trolox, resveratrol (RES), N-acetylcysteine (NAC) and XNT. Values are expressed as mean ± SEM (n=3-5 for each concentration). (C) EC_{50} (μM) and maximal efficacy E_{max} (%) of free radical-scavenging activity of trolox, RES, NAC and XNT.

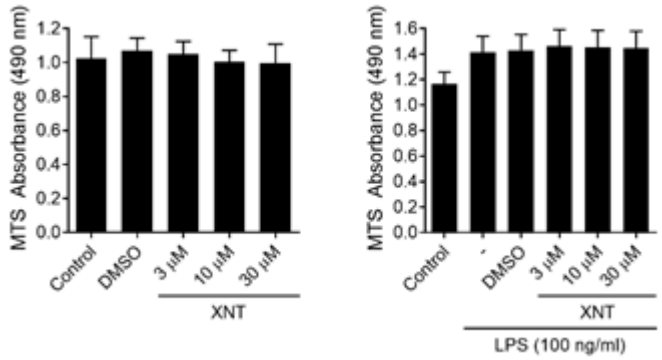

Fig. 2. MTS cytotoxicity assay of XNT in mouse macrophage cell line Raw 264.7. Raw cells were incubated with various concentrations of XNT for 24 hours in the presence or absence of LPS treatment. Values are expressed as mean ± SEM (n=6).

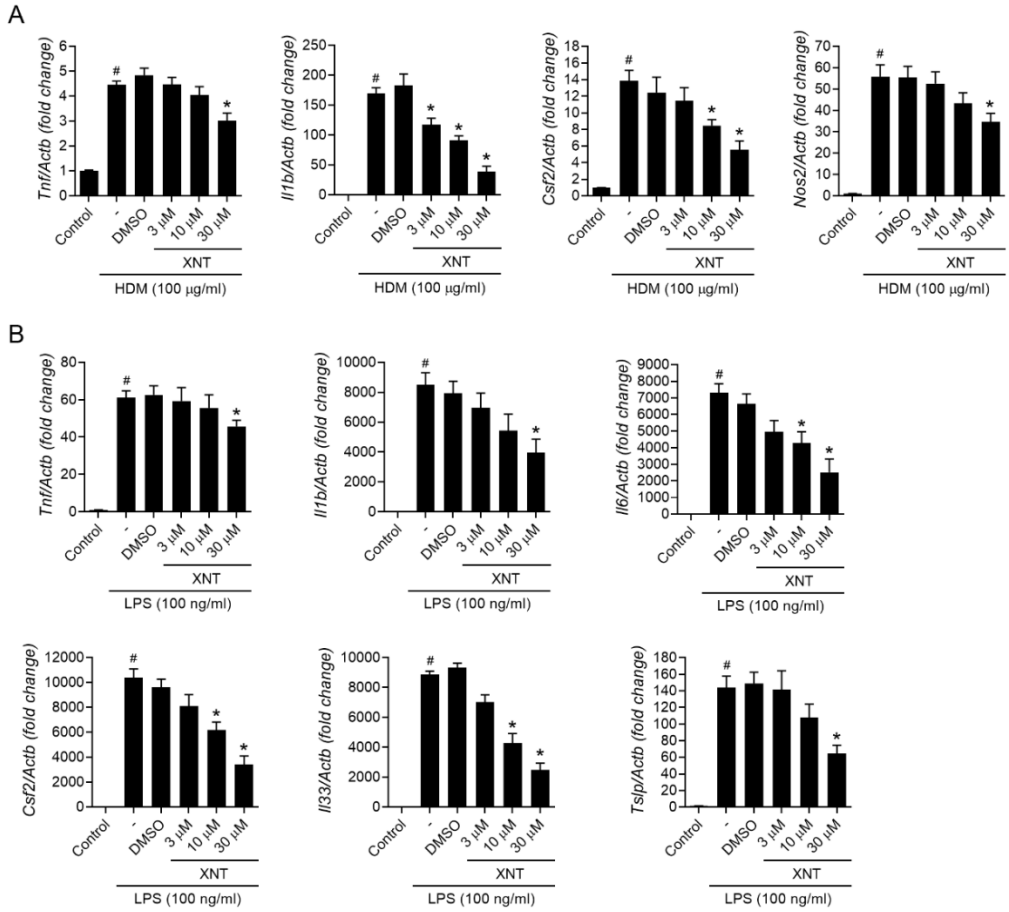

Fig.3. Effects of XNT on HDM or LPS-stimulated cytokine and chemokine gene induction in mouse macrophage cell line Raw 264.7. mRNA expression of cytokine and chemokine genes upon (A) HDM- or (B) LPS-stimulation for 4 hours after pre-treatment with various concentrations of XNT (3 μM, 10 μM and 30 μM) for one hour in Raw 264.7 cells. Values are expressed as mean ± SEM (n=6-8). $^{\#}p < 0.05$, compared with control; $^{*}p < 0.05$, compared with vehicle control 0.5% DMSO.

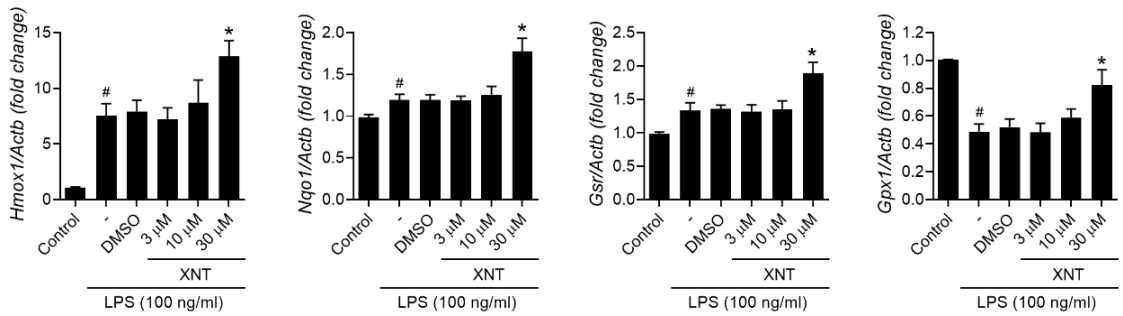

Fig.4. Regulation of antioxidant genes by XNT in LPS-stimulated Raw 264.7 cells. mRNA expression of antioxidant genes upon LPS-stimulation for 4 hours after pre-treated with various concentrations of XNT (3 μM, 10 μM and 30 μM) for one hour. Values are expressed as mean ± SEM (n=5-8). $^{\#}p < 0.05$, compared with control; $^{*}p < 0.05$, compared with vehicle control 0.05% DMSO.

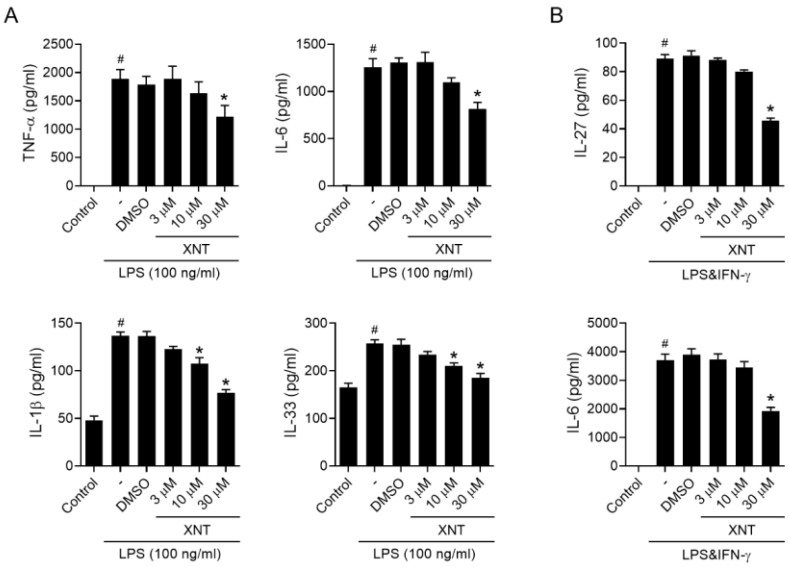

Fig. 5. Effects of XNT on pro-inflammatory cytokine production in mouse macrophages. Increasing concentrations of XNT were pre-treated with (A) Raw 264. 7 cell line for one hour before LPS stimulation for overnight (n=6), or (B) mouse primary AMs for one hour before combined LPS/IFN-γ stimulation for overnight (n=4). Cytokine levels are expressed as mean ± SEM. $^{\#}p < 0.05$, compared with control; $^{*}p < 0.05$, compared with vehicle control 0.05% DMSO.

4. Discussion

It has been shown that the methanol extracts and essential oils of herbal plant *C. xanthorrhizza* contain antioxidant activity against human low-density lipoprotein, which is positively correlated with the percentage of XNT in the isolated fractions.[24] In a separate study, the influence of side chain functional groups of XNT and its analogues on DPPH radical-scavenging activity revealed that XNT had an EC_{50} of 817 μM, which is consistent with our current study reporting an EC_{50} of 787 μM (Fig. 1). It could be inferred that XNT exhibited the smallest ionization potential among the tested analogues because of stabilization of the radical cation through the double-bond moiety on the side chain, and as a result, had a higher radical-scavenging ability.[25]

In addition to its direct radical-scavenging capability, XNT also exhibited neuroprotective effects through its antioxidant activity on inhibiting glutamate-induced reactive oxygen species (ROS) production in murine hippocampal HT22 cell line, and H_2O_2-induced lipid peroxidation in rat brain homogenates. [26] Oxidative damage causes declined activity of neprilysin, the major protease which cleaves amyloid-β peptide in the brains of patients with Alzheimer' s disease. XNT was able to reduce the level of 4-hydroxynonenal, a lipid peroxidation marker, on neprilysin, and restore its enzymatic activity in oligomeric amyloid-β42-treated human neuroblastoma cells. [27] Although the above two studies supported the anti-oxidative effect of XNT for its role in neuroprotection; however, neither study identified the underlying mechanism of

action of XNT as an antioxidant in tissue culture systems. Our data have revealed, for the first time that XNT could directly regulate antioxidant gene transcription, in addition to its free radical-neutralizing capability. Glutathione peroxidase (Gpx1) functions in the detoxification of dangerous intracellular hydrogen peroxide and, as well as lipid peroxides, by catalyzing their reduction into water and corresponding alcohols, respectively.[28] On the other hand, glutathione reductase (Gsr) catalyzes the oxidized glutathione disulfide to the reduced form glutathione, which is essential for the cell to maintain the reducing environment and to prevent oxidative stress.[29] XNT was found to up-regulate both Gpx1 and Gsr gene expression, indicating glutathione might play an important role in executing the antioxidant effect of XNT. In addition, XNT also enhanced gene expression of phase II detoxification and anti-oxidative enzymes HO-1 and NQO-1, which are predominantly regulated by transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), to eliminate ROS and inhibit inflammatory responses.[30] Our data suggest that XNT might be able to activate Nrf-2-mediated antioxidant signaling pathway and Nrf-2-regulated antioxidant gene expression, which warrants further investigation.

It has been shown that XNT reduced the production of pro-inflammatory cytokines IL-6 and TNF-α, down-regulated the expression of pro-inflammatory enzymes cyclooxygenase-2 and iNOS in LPS-stimulated microglial cells, and as well as inhibited the levels of IL-1β and matrix metalloproteinases (MMPs) in LPS-induced HGF-1 cells.[18,31] It has also been demonstrated that XNT suppressed the inflammatory responses in various murine models including 12-O-tetradecanoylphorbol-13-acetate (TPA) -induced papillomas, dextran sulfate sodium (DSS) -induced colitis, high-fat diet-induced obesity, and LPS-induced periodontitis models.[15-17, 32] The anti-inflammatory effects of XNT could be attributed to inhibition of mitogen-activated protein kinase (MAPK) pathway and transcription factor NF-κB.[31-32] In line with the reports mentioned, we confirmed that XNT down-regulated HDM- or LPS-stimulated pro-inflammatory cytokines, chemokines, and other mediators at both mRNA and protein level in mouse macrophage cell line Raw 264. 7 cells. Particularly, high concentration of XNT (30 μM) was able to significantly decrease the production of combined LPS/ IFN-γ-stimulated IL-27, a cytokine that was shown to mediate steroid-resistant airway hyperresponsiveness,[23] suggesting XNT has a potent anti-inflammatory activity and could be a potential steroid re-sensitizer.

In contrast to the well-studied anti-inflammatory effects of XNT on type-1 inflammatory responses characterized by elevation of macrophage-derived TNF-α, IL-1β, IL-27, iNOS, etc., little is known about the effects of XNT on type-2 and type-17 inflammatory responses such as Th2/ Th17 cytokines IL-4, IL-5, IL-13 and IL-17. In assessing the percentage of isolated $CD4^+$ T cells expressing IL-5, IL-13 or IL-17 from aeroallergen-exposed allergic airways, pre-treatment of XNT was able to decrease the percentage of Th2/ Th17 cytokine expressing-$CD4^+$ T cells, implicating that XNT is also effective in suppressing type 2 and type 17 immune responses such as those occur in allergic asthma. It is of note that in our study, $CD4^+$ T cells were assayed from mixed lung cell suspension, so a secondary

effect on the $CD4^+$ T cells from other cells in the mixture should be of consideration. It is desirable that purified CD4 T cells be used, and as well as cytokine levels be quantified in future studies.

C. xanthorrhizza is traditionally used as folk medicine for the prevention and treatment of various ailments. XNT, as the major bioactive natural compound isolated from *C. xanthorrhizza*, has been shown to be effective in various disease models including infection, inflammation, metabolic syndromes, neurological disorders, liver diseases and cancer.[10] The present study supports further investigations of potential therapeutic values of XNT in various models of airway inflammation including asthma, COPD and idiopathic pulmonary fibrosis.

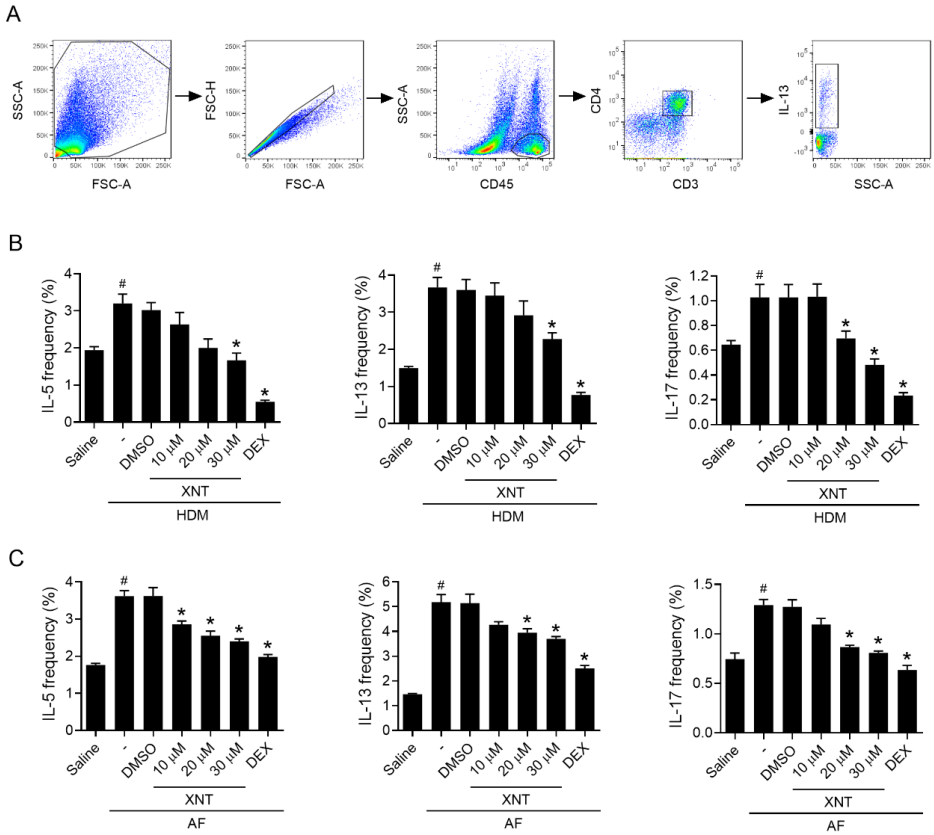

Fig.6. Effects of XNT on Th2/Th17 cytokine expression from pulmonary $CD4^+$ T cells. Murine allergic asthma models were developed using aeroallergen HDM or AF. Lung single cell suspensions were obtained from aeroallergen-challenged mice, and then stimulated *ex vivo* with PMA/ ionomycin/ brefeldin A for 5 hours after pre-treatment with increasing concentrations of XNT or dexamethasone (DEX) for one hour. (A) Gating strategy of airway $CD4^+$ T cells for intracellular cytokine staining. IL-5, IL-13, and IL-17-expressing $CD4^+$ T cell frequency upon above treatment and stimulation in lung single cell suspension from (B) HDM- or (C) AF-exposed allergic airways was determined by intracellular staining and flow cytometry analysis. Values are expressed as mean ± SEM (n=4). $^{\#}p < 0.05$, compared with control; $^{*}p < 0.05$, compared with vehicle control 0.05% DMSO.

5. Conclusion

In this study, we have confirmed free radical-scavenging activity of XNT and also inhibitory effect of XNT on pro-inflammatory cytokines and chemokines in mouse macrophages. More importantly, for the first time, we have demonstrated that XNT is able to up-regulate antioxidant genes and suppress type-2 and type-17 cytokines from aeroallergen-exposed CD4 T cells as well. However, other than the MAPK pathway and NF-κB inhibition reported in the literature, the mechanisms of action by XNT are still far from established. In a recent study, the potential binding targets of XNT have been investigated by computational target fishing strategy. It was found that multiple proteins and pathways may be exploited by XNT to form a network for it to execute systematic pharmacological effects.[33] These proteins and pathways should be further validated experimentally. Besides, comprehensive animal work should be performed to evaluate the anti-inflammatory and anti-oxidative efficacy of XNT *in-vivo*, especially in type-2 and type-17 immunity-dominated disease models such as allergic asthma model and autoimmune disease multiple sclerosis model. Our findings reveal novel anti-oxidative and anti-inflammatory properties of XNT, and implicate a new potential treatment to inflammation and oxidative damage in pulmonary diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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