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Vitamin E Isoform γ-Tocotrienol Downregulates House Dust Mite–Induced Asthma

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Inflammation and oxidative damage contribute to the pathogenesis of asthma. Although corticosteroid is the first-line treatment for asthma, a subset of patients is steroid resistant, and chronic steroid use causes side effects. Because vitamin E isoform γ-tocotrienol possesses both antioxidative and anti-inflammatory properties, we sought to determine protective effects of γ-tocotrienol in a house dust mite (HDM) experimental asthma model. BALB/c mice were sensitized and challenged with HDM. Bronchoalveolar lavage (BAL) fluid was assessed for total and differential cell counts, oxidative damage biomarkers, and cytokine levels. Lungs were examined for cell infiltration and mucus hypersecretion, as well as the expression of antioxidants and proinflammatory biomarkers. Sera were assayed for IgE and γ-tocotrienol levels. Airway hyperresponsiveness in response to methacholine was measured. γ-Tocotrienol displayed better free radical–neutralizing activity in vitro and inhibition of BAL fluid total, eosinophil, and neutrophil counts in HDM mouse asthma in vivo, as compared with other vitamin E isoforms, including α-tocopherol. Besides, γ-tocotrienol abated HDM-induced elevation of BAL fluid cytokine and chemokine levels, total reactive oxygen species and oxidative damage biomarker levels, and of serum IgE levels, but it promoted lung-endogenous antioxidant activities. Mechanistically, γ-tocotrienol was found to block nuclear NF-κB level and enhance nuclear Nrf2 levels in lung lysates to greater extents than did α-tocopherol and prednisolone. More importantly, γ-tocotrienol markedly suppressed methacholine-induced airway hyperresponsiveness in experimental asthma. To our knowledge, we have shown for the first time the protective actions of vitamin E isoform γ-tocotrienol in allergic asthma. ***The Journal of Immunology*****, 2015, 195: 000–000.**

Allergic asthma is characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (1). These pathological features are attributable to lung infiltration by eosinophils and neutrophils, augmented production of proinflammatory mediators, including IL-4, IL-5, IL-13, IL-17, and IL-33, and elevation of serum IgE level (1, 2). Aside from the inflammatory microenvironment, eosinophils and neutrophils are major sources of reactive oxygen and nitrogen species (RONS), contributing to oxidative stress and damage to the airways in asthma (3). Concomitantly, endogenous antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are weakened in asthma (3). Persistent activation of transcription factor NF-κB has been demonstrated as a pathogenic mechanism sustaining airway inflammation in asthma (4). Alternatively, downregulation of redox-sensitive nuclear erythroid 2–related factor 2 (Nrf2), essential for antioxidant gene expression, has been shown to be susceptible to allergic asthma development (5). A strategy aiming to increase nuclear Nrf2 levels may have therapeutic potential for asthma. Corticosteroid is the first-line treatment for asthma, but it does not possess direct free radical–neutralizing activity, and a subset of asthmatics is steroid resistant (6). Chronic steroid use can also lead to unwanted side effects (1). Therefore, substantial effort has been put in to identify molecules, with both anti-inflammatory and antioxidative properties offering superior protection in asthma.

Vitamin E is a lipid-soluble natural supplement with antioxidant properties, and it exists in eight distinct members comprising of α, β, γ, and δ isoforms for both tocopherols and tocotrienols (7). Tocopherol contains a chromanol ring and a saturated 15-carbon tail, whereas tocotrienol differs slightly by the presence of three *trans* double bonds within the tail (7). The different members of vitamin E vary by both the number and location of methyl groups found on the chromanol ring: α is 5,7,8-trimethyl, β is 5,8-dimethyl, γ is 7,8-dimethyl, and δ is 8-monomethyl. Tocotrienols can be found in rice bran and palm oil (8). Although α-tocopherol is regarded as the isoform that contributes to the antioxidative and anti-inflammatory effects of vitamin E, cumulative evidence has pointed to γ-tocotrienol as the superior bioactive isoform in

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Abbreviations used in this article: AHR, airway hyperresponsiveness; ALT/AST, alanine transaminase/aspartate transaminase; BAL, bronchoalveolar lavage; Cdyn, dynamic compliance; CuZnSOD, copper zinc SOD; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ECSOD, extracellular SOD; GPx, glutathione peroxidase; HDM, house dust mite; HO-1, hemeoxygenase-1; iNOS, inducible NO synthase; MnSOD, manganese SOD; NOAEL, no observed adverse effect level; NOX, NADPH oxidase; Nrf2, nuclear erythroid 2–related factor 2; 3-NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxy-2-deoxy-guanosine; PAFS, periodic acid–fluorescence Schiff; Rl, lung resistance; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase.

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vitamin E (7, 9). Not only being a more effective free radical scavenger, γ-tocotrienol has also been shown to induce production of endogenous antioxidant enzymes such as SOD and GPx and to abate proinflammatory mediators, including TNF-α, IL-1β, IL-8, VCAM-1, and NF-κB (9–11) in vitro. Tocotrienols possess a favorable therapeutic index in rodents, with the no observed adverse effect level (NOAEL) reported up to a daily dose of 130 mg/kg in rats (equivalent to 260 mg/kg in mice) for 13 wk or up to 1 y (12–14). In the present study, we hypothesized that γ-tocotrienol can protect against allergic airway inflammation via its antioxidative and anti-inflammatory properties.

Our results reveal, to our knowledge for the first time, that oral γ-tocotrienol ameliorated airway inflammation and oxidative damage in experimental mouse asthma, with efficacies similar to or even better than prednisolone. γ-Tocotrienol markedly abated house dust mite (HDM)–induced increases in airway inflammatory cell counts, cytokine and chemokine levels, mucus production, RONS, and oxidative damage biomarkers. γ-Tocotrienol reduced serum IgE levels in HDM mouse asthma and restored lung functions by suppressing AHR and improving lung dynamic compliance (Cdyn). The protective mechanisms of γ-tocotrienol are likely mediated by blocking nuclear translocation of NF-κB and augmenting Nrf2 nuclear level in HDM-challenged lungs.

Materials and Methods

Animals

Female BALB/c mice 6–8 wk of age (Animal Resources Center, Canning Vale, Western Australia, Australia) were anesthetized with isoflurane (Halocarbon Products, River Edge, NJ) and given 40 μl HDM (100 μg *Dermatophagoides pteronyssinus* extracts, Greer Laboratories, Lenoir, NC) or saline as a negative control on days 0, 7, and 14 via intratracheal route as described (15, 16). Because the NOAEL for tocotrienols was reported at 260 mg/kg daily in mice for 13 wk or up to 1 y (12–14), the highest oral dose of vitamin E isoforms in this study was capped at 250 mg/kg. α-Tocopherol (250 mg/kg), α-tocotrienol (250 mg/kg), δ-tocotrienol (250 mg/kg), γ-tocotrienol (30, 100, and 250 mg/kg), vehicle (oil emulsifier), or prednisolone (10 mg/kg) in 0.2 ml was given via oral gavage on days 7, 8, 9, 14, 15, and 16. Tocopherol and tocotrienols were provided by Davos Life Science (Singapore), with ≥97% purity as measured by HPLC. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the National University of Singapore.

Bronchoalveolar lavage fluid and serum analyses

Mice were anesthetized on day 17, and BAL fluid and blood were collected as previously described (17). Bronchoalveolar lavage (BAL) fluid total and differential cell counts were performed blinded. Cytokine and chemokine levels were measured using multiplex ELISA (Bio-Rad, Hercules, CA), and 8-isoprostane and 8-hydroxy-2-deoxy-guanosine (8-OHdG) were measured using enzyme immunoassays (Cayman Chemical, Ann Arbor, MI). For the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, fresh BAL fluid was incubated with 10 mM DCFH-DA (Invitrogen, Grand Island, NY) for 20 min at 37°C. DCFH-DA assay detects cellular levels of oxidative species. In the presence of RONS, DCFH is rapidly oxidized to fluorescent DCF (18). BAL fluid was then spun down, and pellets were resuspended in RPMI 1640 and measured by a spectrofluorometer with excitation at 492 nm and emission at 525 nm. Serum levels of IgE were measured using ELISA (Becton Dickinson, Franklin Lakes, NJ). Blood samples were also measured for peripheral blood counts, alanine transaminase/aspartate transaminase (ALT/AST), and creatinine levels to monitor toxicity.

Histology

Lungs were fixed in 10% neutral formalin, paraffinized, cut into 5-mm sections, and stained with H&E for examining cell infiltration and with periodic acid–fluorescence Schiff (PAFS) stain for mucus production. Scoring of cell infiltration in H&E-stained lung sections was performed blinded as previously described (19). Relative intensity analyses of PAFS stain were also performed blinded as described (19), where the ratio of red over green stains was normalized to saline control, using the ImageJ software (National Institutes of Health, Bethesda, MD).

Lung tissue analyses

Lung tissues were snap-frozen in liquid nitrogen and lyophilized using a freeze dryer (Labconco, Kansas City, MO) at −85°C. Lyophilized lung tissues were homogenized in 25 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO) using a metal bead–based Precellys homogenizer. Supernatants were assayed for 3-nitrotyrosine (3-NT) levels using an enzyme immunoassay (Cell Biolabs, San Diego, CA), and for enzymatic activities of SOD, catalase, and GPx using enzymatic assay kits (Cayman Chemical). Lung nuclear extracts (20 mg per lane) were separated by 10% SDS-PAGE. Immunoblots were probed with anti–NF-κB, anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti–TATA binding protein (Abcam, Cambridge, MA) mAb. Band intensity was quantitated using ImageJ software (National Institutes of Health). Total RNA was extracted from the lung tissues with TRIzol reagent (Invitrogen, Carlsbad, CA) and then used for first-strand cDNA synthesis. The PCR primers used are listed in Supplemental Table I. Template cDNA (100 ng) in the PCR mixture containing Fast SYBR Green master mix (Applied Biosystems, Carlsbad, CA) was amplified and quantitated using a sequence detector (ABI 7500 cycler; Applied Biosystems). The mRNA expression levels for all samples were normalized to housekeeping gene β-actin.

Measurements of AHR

Mice were anesthetized and tracheotomy was performed as previously described (19). Lung resistance (Rl) and Cdyn in response to increasing concentrations of nebulized methacholine (0.5–8.0 mg/ml) were recorded using the FinePointe data acquisition and analysis software (Buxco, Wilmington, NC). Results are expressed as a percentage of the respective basal values in response to PBS.

Pharmacokinetics analysis of γ-tocotrienol

Naive mice were given oral γ-tocotrienol (250 mg/kg) and anesthetized prior to blood collection at different time intervals during a 12-h period. Serum levels of γ-tocotrienol were measured using the Waters Alliance 2695 HPLC system (Waters, Milford, MA) as described (20). Data were pooled and analyzed using the naive pooled modeling approach (21). Data were analyzed using the average concentration at each time point. The noncompartmental analysis was performed using WinNonlin professional version 6.3 software (Pharsight, Mountain View, CA).

1,1-Diphenyl-2-picrylhydrazyl in vitro radical scavenging assay

Different concentrations of α-tocopherol, tocotrienols (Davos Life Science), prednisolone, resveratrol, or trolox (Sigma-Aldrich) were incubated with 0.1 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich) for various time intervals. Time-dependent and concentration-dependent free radical scavenging activities were measured colorimetrically at 517 nm as described (22).

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 $p < 0.05$ when compared with vehicle control.

Results

γ-Tocotrienol has superior free radical–neutralizing and anti-inflammatory activities over other vitamin E isoforms

Among the vitamin E isoforms tested, γ-tocotrienol demonstrated better free radical scavenging activity in a time-dependent (Fig. 1A) and concentration-dependent (Fig. 1B) manner, using the DPPH in vitro assay. Resveratrol and trolox were used as positive controls. Prednisolone had no free radical–neutralizing property. HDM challenge markedly increased total, eosinophil, and macrophage counts in BAL fluid, with moderate increase in lymphocyte and neutrophil counts. Additionally, BAL fluid free radical levels were strongly elevated by HDM challenge (Fig. 1C, 1D). γ-Tocotrienol effectively and consistently abated total, eosinophil, and neutrophil counts (Fig. 1C), as well as free radical levels in BAL fluid (Fig. 1D), as compared with other vitamin E members.

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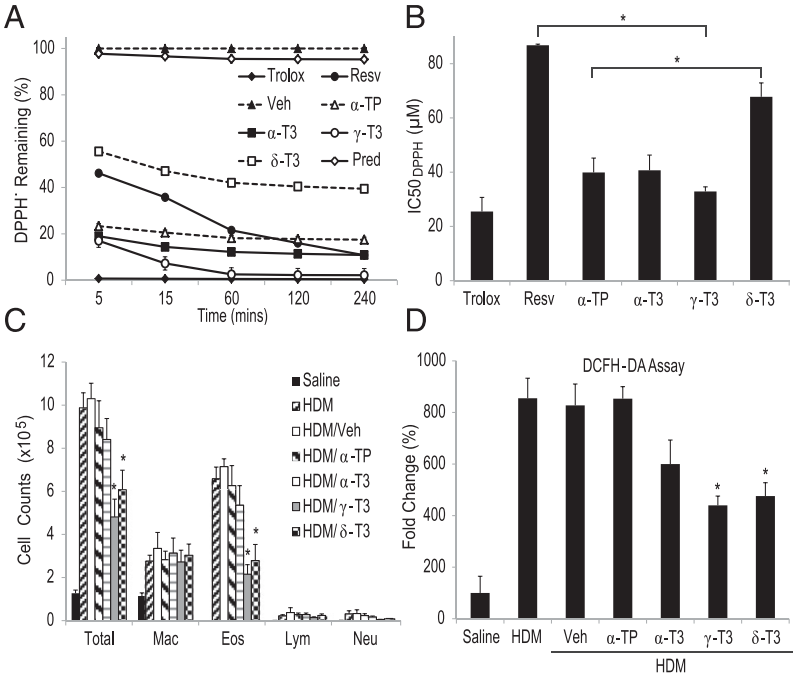


FIGURE 1. Comparison of free radical–neutralizing effects and anti-inflammatory actions of vitamin E isoforms. α-Tocopherol (α-TP), α-tocotrienol (α-T3), γ-tocotrienol (γ-T3), δ-tocotrienol (δ-T3), resveratrol (Res), prednisolone (Pred), and trolox were incubated with 0.1 mM DPPH for up to 4 h. Free radical scavenging activities were determined using the DPPH assay in a (**A**) time-dependent and (**B**) concentration (IC_{50})-dependent manner at 1 h time point. Values are expressed as means ± SEM (n = 4). *$p < 0.05$. (**C**) Inhibitory effects of vitamin E isoforms on total and differential cell counts in BAL fluid obtained from HDM-challenged mice. (**D**) Inhibitory effects of vitamin E isoforms on levels of total oxidants in the BAL fluid were determined using the DCFH-DA assay. Values are expressed as means ± SEM (n = 5). *$p <$ 0.05 compared with vehicle control.

Pharmacokinetics analysis of oral γ-tocotrienol

Pharmacokinetic parameters in plasma after a single dose of 250 mg/kg oral γ-tocotrienol were obtained using the noncompartmental analysis. Following the oral feeding, maximum plasma concentration of ~10 μg/ml was achieved within 0.5 h. Plasma half-life was estimated to be 2.8 h and total AUC (AUC0→∞) was calculated to be 38 μg/h/ml (Fig. 2A). γ-Tocotrienol levels in the lungs accumulated to ~3 ng/mg lung tissue after 12 h of a single oral dose (Fig. 2B), and they remained the same at 3.8 ng/mg lung tissue after a daily oral dose for 3 consecutive days.

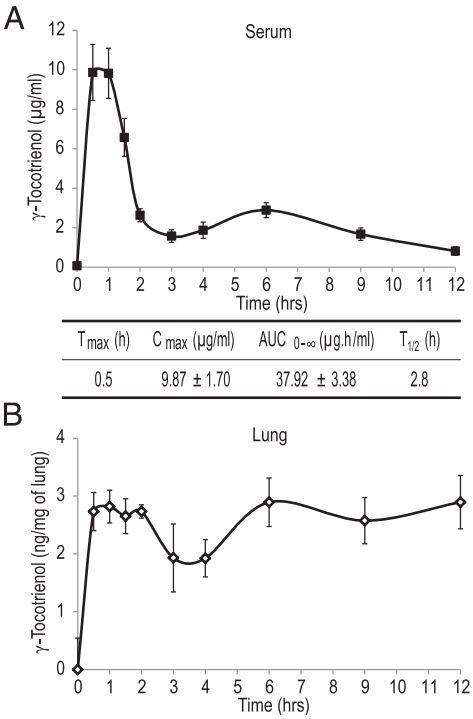
γ-Tocotrienol attenuates HDM-induced airway inflammation

γ-Tocotrienol (30, 100, and 250 mg/kg) significantly decreased total, eosinophil, and neutrophil counts in a dose-dependent manner (Fig. 3A). Maximum inhibitory effects of γ-tocotrienol on total and eosinophil counts were comparable to those of prednisolone (10 mg/kg). More importantly, only γ-tocotrienol markedly reduced neutrophil count in BAL fluid. γ-Tocotrienol had no effect on BAL fluid cell profile in naive mice, or serum levels of leukocytes, AST/ALT, and creatinine in mice (Supplemental Table II).

Besides, HDM challenge induced bronchial epithelial hypertrophy as well as marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues (Fig. 3B). γ-Tocotrienol (250 mg/kg) significantly diminished epithelial hypertrophy and decreased inflammatory cell infiltrations into the airways, with effects comparable to prednisolone (10 mg/kg) (Fig. 2B). HDM challenge also developed distinct goblet cell hyperplasia and mucus hypersecretion in the bronchial epithelium. γ-Tocotrienol significantly suppressed mucus production to a similar extent as prednisolone (Fig. 3C).

Serum total and HDM-specific IgE levels were heightened upon HDM challenge, and γ-tocotrienol dose-dependently abrogated total and HDM-specific IgE levels substantially (Fig. 3D). Notably, prednisolone (10 mg/kg) failed to significantly lower both total and HDM-specific IgE levels.

HDM challenge upregulated BAL fluid levels of Th2 and Th17 cytokines (IL-4, IL-5, and IL-17), the chemokine RANTES, and G-CSF, but it repressed Th1 and Th10 cytokines (IL-1β, IL-12p70, IFN-γ and IL-10). γ-Tocotrienol dose-dependently reduced IL-4, IL-5, IL-17, RANTES, and G-CSF, and it restored Th1 and Th10 cytokine levels (Fig. 4A). Overall, the inhibitory efficacies of γ-tocotrienol were comparable to those by prednisolone, with the exception that prednisolone failed to decrease G-CSF level (Fig. 4A), further distinguishing the ability of γ-tocotrienol in modulating neutrophilic inflammation.



T_{max} (h)	C_{max} (μg/ml)	AUC $_{0-\infty}$ (μg.h/ml)	$T_{1/2}$ (h)
0.5	9.87 ± 1.70	37.92 ± 3.38	2.8

FIGURE 2. Pharmacokinetics analysis of oral γ-tocotrienol in mice. (**A**) Serum levels and (**B**) lung tissue levels of γ-tocotrienol were measured using HPLC system. Pharmacokinetics parameters were calculated using WinNonlin software program. Values are expressed as means ± SEM (n = 6/time point).

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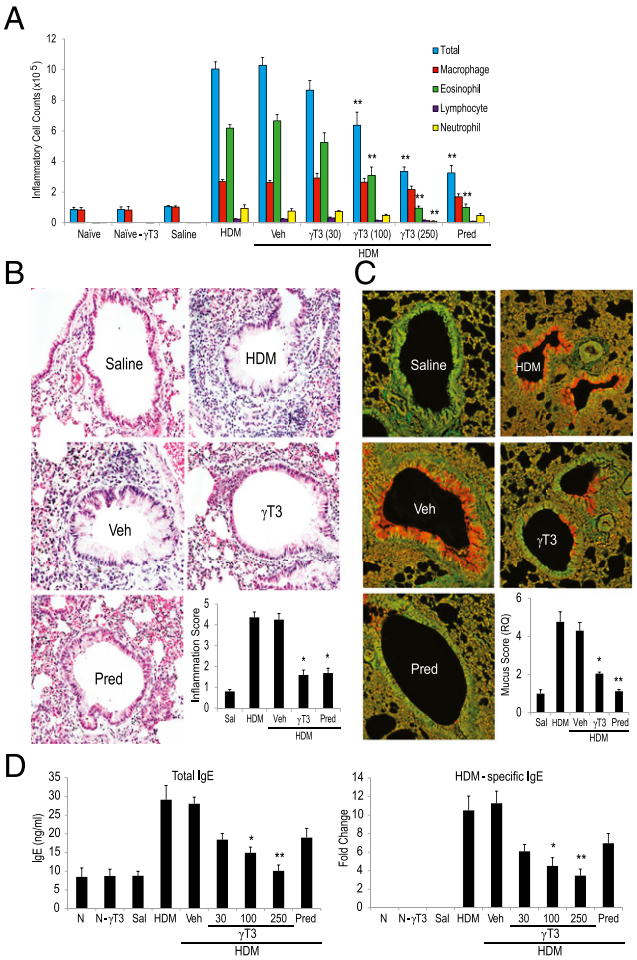

FIGURE 3. Protective effects of γ-tocotrienol on HDM-induced allergic airway inflammation. (**A**) γ-Tocotrienol dose-dependently suppressed total and differential inflammatory cell counts in BAL fluid obtained from HDM-challenged mice. Values are expressed as means ± SEM (n = 8). (**B**) Representative photomicrographs of H&E-stained lung sections at original magnification ×200. Scoring of cell density and bronchial epithelium thickness was performed. (**C**) Representative photomicrographs of PAFS-stained lung sections at original magnification ×200, where red color stain demarcates mucus. Relative intensity scoring of PAFS-stained mucus was quantitated using ImageJ. Values are expressed as means ± SEM (n = 4). (**D**) Serum levels of total IgE and HDM-specific IgE were measured using ELISA. Values are expressed as means ± SEM (n = 8). *$p < 0.05$, **$p < 0.01$ compared with vehicle control.

Lung mRNA expression of proinflammatory cytokines IL-13, IL-17, IL-33, periostin, and TNF-α, adhesion molecules E-selectin and VCAM-1, and mucin MUC5AC were markedly elevated by HDM challenge (Fig. 4B). Both γ-tocotrienol (250 mg/kg) and prednisolone (10 mg/kg) were equally effective in abating gene expression of these proinflammatory biomarkers in the allergic airways.

γ-Tocotrienol ameliorates HDM-induced oxidative stress in the airways

HDM challenge strongly increased RONS levels in BAL fluid cells. γ-Tocotrienol significantly lowered cellular RONS levels in a dose-dependent manner (Fig. 5A). Although prednisolone did not have any free radical scavenging property (Fig. 1A), it suppressed BAL fluid cellular RONS level to the same extent as γ-tocotrienol, suggesting a consequence of its potent anti-inflammatory action. Similarly, oxidative damage biomarkers 8-isoprostane, 8-OHdG, and 3-NT were heightened with HDM challenge (Fig. 5B–D), and both γ-tocotrienol and prednisolone abrogated all three oxidative damage markers in the allergic airways.

Besides, HDM challenge promoted gene expression of prooxidants inducible NO synthase (iNOS) and NADPH oxidase (NOX) isoforms (NOX1–4) and their related regulatory subunits $p22^{phox}$ and $p67^{phox}$ (Fig. 5E), which generate NO and superoxide anion ($O_2^{\cdot -}$), respectively (23). Both γ-tocotrienol and prednisolone significantly diminished iNOS and NOX levels in allergic airways.

Alternatively, antioxidant activities of SOD, catalase, and GPx declined substantially in HDM-challenged mice (Fig. 6A–C). γ-Tocotrienol restored antioxidant enzymatic activities of SOD, catalase, and GPx to normal levels in a dose-dependent manner. Additionally, mRNA levels of all three isoforms of SOD, that is, copper zinc SOD (CuZnSOD; SOD1), manganese MnSOD (MnSOD; SOD2), and extracellular SOD (ECSOD; SOD3), dropped by HDM challenge, and γ-tocotrienol significantly restored SOD2 and SOD3 but not SOD1 gene expression (Fig. 6D). In contrast, prednisolone was only able to restore SOD3 gene expression and GPx antioxidant activity in allergic airways (Fig. 6C, 6D). Hemeoxygenase-1 (HO-1) is another antioxidant that is inducible by inflammation to combat oxidative stress (24), and it was found upregulated in HDM-challenged airways (Fig. 6D). γ-Tocotrienol, but not prednisolone, markedly suppressed HO-1 mRNA level in the inflamed lungs.

γ-Tocotrienol abrogates HDM-induced AHR

Rl is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. HDM-challenged mice developed AHR, which is typically manifested with high Rl (Fig. 7A) and low Cdyn (Fig. 7B). Both γ-tocotrienol (250 mg/kg) and prednisolone (10 mg/kg) effectively blocked methacholine-induced AHR by restoring Rl and Cdyn to their basal levels.

γ-Tocotrienol reduces nuclear NF-κB and promotes nuclear Nrf2

HDM challenge promoted NF-κB nuclear translocation (Fig. 8A) and decreased nuclear Nrf2 levels (Fig. 8B) in the inflamed airways. NF-κB is a master switch for proinflammatory gene production, whereas the redox-sensitive Nrf2 facilitates antioxidant expression (4, 5). Both γ-tocotrienol and prednisolone drastically blocked NF-κB nuclear translocation. In contrast, α-tocopherol failed to prevent HDM-induced NF-κB nuclear translocation in lung tissues (Fig. 8A). Alternatively, γ-tocotrienol markedly augmented Nrf2 nuclear accumulation. However, both prednisolone and α-tocopherol failed to elevate nuclear Nrf2 to a significant extent in HDM-challenged lung tissues (Fig. 8B).

Discussion

In this study, we have shown, to our knowledge for the first time, that vitamin E isoform γ-tocotrienol not only can directly neutralize free radicals, but also elicit anti-inflammatory and antioxidative actions in HDM-mediated experimental asthma by inhibiting NF-κB activity and promoting Nrf2 activation, leading to amelioration of AHR.

The highest γ-tocotrienol dose exposure in the current study was capped at 250 mg/kg in mice, which is below the reported NOAEL of 260 mg/kg given daily to rodents for 13 wk or up to 1 y (12–14). γ-Tocotrienol showed no effect on BAL fluid cell profile in naive mice, or serum levels of leukocytes, hematocrit, AST/ALT, and creatinine in mice (Supplemental Table II). Nevertheless,

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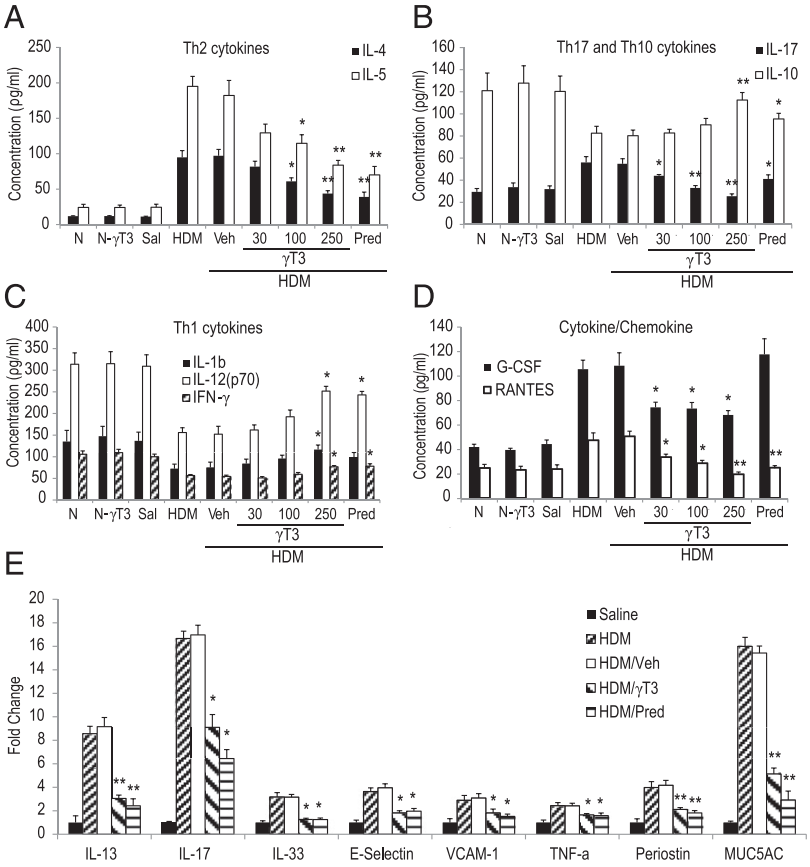

FIGURE 4. Inhibitory effects γ-tocotrienol on HDM-induced lung cytokines and chemokines. (**A**–**D**) BAL fluid levels of cytokines and chemokines were measured using ELISA. Values are expressed as means ± SEM (n = 8). (**E**) Lung mRNA expression of proinflammatory mediators was assayed by real-time PCR. Values are expressed as means ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$ compared with vehicle control.

subchronic and chronic toxicity studies of oral γ-tocotrienol should be conducted to properly address the issue of possible systemic toxic effects on prolonged use of γ-tocotrienol.

α-Tocopherol has been regarded as the major isoform of vitamin E with the most potent antioxidant property (7). In this study, we observed that the basal serum level of α-tocopherol in mice was 8.76 ± 0.46 μM and of γ-tocotrienol was 0.18 ± 0.03 μM. Upon a single oral dose of 250 mg/kg, the highest serum γ-tocotrienol level reached 24.04 ± 4.14 μM in a 12-h time course study. Other laboratories have recently revealed superior antioxidant activities of tocotrienols over tocopherols (9, 25). In line with those observations, we found that γ-tocotrienol produced significantly more reduction of free radicals in BAL fluid from HDM-challenged mice using DCFH-DA assay, as compared with α-tocopherol. γ-Tocotrienol was also more effective than α-tocopherol in ameliorating inflammatory leukocytes into the airways and produced greater beneficial modulation of NF-κB and Nrf2 in the lungs.

Persistent NF-κB activation and excessive oxidative stress in association with dampened Nrf2 activity have been observed in allergic airway inflammation (4, 5). Nrf2 is a redox-sensitive transcription factor involved in binding and activating the antioxidant response element located in the promoter region of many antioxidant genes (5). Disruption of Nrf2 in mice was found to be more susceptible to allergen-mediated airway inflammation (5). Strategies targeting the NF-κB signaling pathway using NF-κB–specific decoy oligonucleotide, IKKβ-selective small molecule inhibitor, and RIP-2 small interfering RNA have demonstrated beneficial effects in experimental asthma models (19, 26). Compounds capable of promoting Nrf2 transcriptional activity have demonstrated protection against experimental asthma (5, 17). In this study, γ-tocotrienol not only functioned as a free radical scavenger, but it also acted by blocking NF-κB nuclear translocation and upregulating nuclear Nrf2 levels in lung tissues from HDM-challenged mice. In contrast, α-tocopherol failed to block NF-κB nuclear translocation and to promote nuclear Nrf2 levels. As a result, γ-tocotrienol markedly reduced HDM-induced eosinophil lung infiltration, mucus hypersecretion, and AHR to the same extent as prednisolone. More importantly, γ-tocotrienol elicited much stronger protection against neutrophil lung infiltration, serum IgE elevation, and oxidative stress than did prednisolone. These findings implicate a potential role of γ-tocotrienol in preventing neutrophil-induced chronic persistent asthma and airway damage (2).

Asthma is primarily driven by Th2 immune response, where Th1 immunity is negatively regulated (2). γ-Tocotrienol markedly abated pulmonary Th2 cytokines (IL-4, IL-5, IL-13), IL-17, IL-33, TNF-α, the chemokine RANTES, periostin, and adhesion molecules (E-selectin and VCAM-1), but it restored Th1 cytokines (IL-1β, IL-12p70, and IFN-γ) and IL-10. Pulmonary eosinophil trafficking is orchestrated by IL-5, IL-13, TNF-α, and RANTES in combination with VCAM-1 and E-selectin (27). It has been shown that IL-13 induces periostin production from the airway epithelial cells to augment epithelial–mesenchymal interactions and extracellular matrix organization, leading to airway remodeling (28). Besides, periostin is increasingly used as a clinical biomarker for eosinophilic asthma (28). IL-4, IL-5, IL-13, IL-17, IL-33, and TNF-α have all been shown to promote MUC5AC expression and goblet cell hyperplasia, and MUC5AC gene expression hinges on the transcriptional activity of NF-κB (29). IL-4 and IL-13 induce B cells to undergo isotype class switching to IgE production (30), and IgE cross-linking with FcεRI on mast cells leads to mast cell degranulation and bronchoconstriction (31). IL-10 plays a central role in potentiating regulatory T cell function and acts as an anti-inflammatory cytokine in asthma (32). IL-17 and G-CSF can contribute to the neutrophilic lung inflammation (33, 34), and IL-17 has also been linked to the development of steroid-resistant asthma. The observed anti-inflammatory effects of γ-tocotrienol

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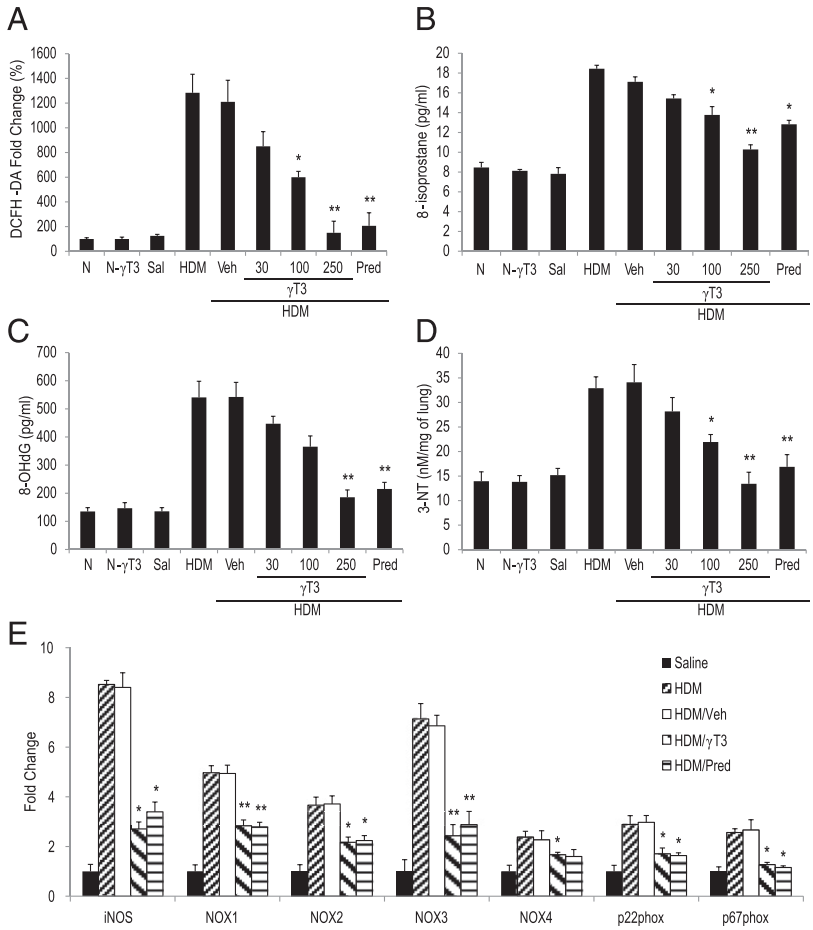

FIGURE 5. Protective effects of γ-tocotrienol on HDM-induced oxidative stress in the lungs. (**A**) Levels of total oxidants in the BAL fluid were measured using the DCFH-DA assay. Levels of (**B**) 8-isoprostane and (**C**) 8-OHdG in BAL fluid and (**D**) 3-NT in lung homogenate were measured by ELISA. Values are expressed as means ± SEM (n = 8). (**E**) Lung mRNA expression of iNOS, NOX isoforms, and regulatory subunits of NOX $p22^{phox}$ and $p67^{phox}$ were assayed by real-time PCR. Values are expressed as means ± SEM (n = 5). $*p < 0.05$, $**p < 0.01$ compared with vehicle control.

in HDM-induced experimental asthma are likely due to its inhibition on NF-κB activation, as many of those proinflammatory genes encoding for TNF-α, IL-17, MUC5AC, VCAM-1, RANTES, and E-selectin contain the κB site for NF-κB binding within their promoter regions and/or act through NF-κB signaling pathway (4, 29).

Oxidative and antioxidative imbalance is critically associated with the pathogenesis of asthma (3). Infiltrated cells such as eosinophils and neutrophils can produce RONS, including superoxide anion ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), NO, and H_2O_2 (3, 35). NOX is the major producer of $O_2^{\cdot -}$ and its expression levels are elevated in asthma (23). NOX has been shown to regulate the trafficking of both eosinophils and neutrophils into the lungs (23). Increases in airway oxidative damage markers 8-isoprostane (for lipid), 8-OHdG (for DNA), and 3-NT (for protein) are linked to

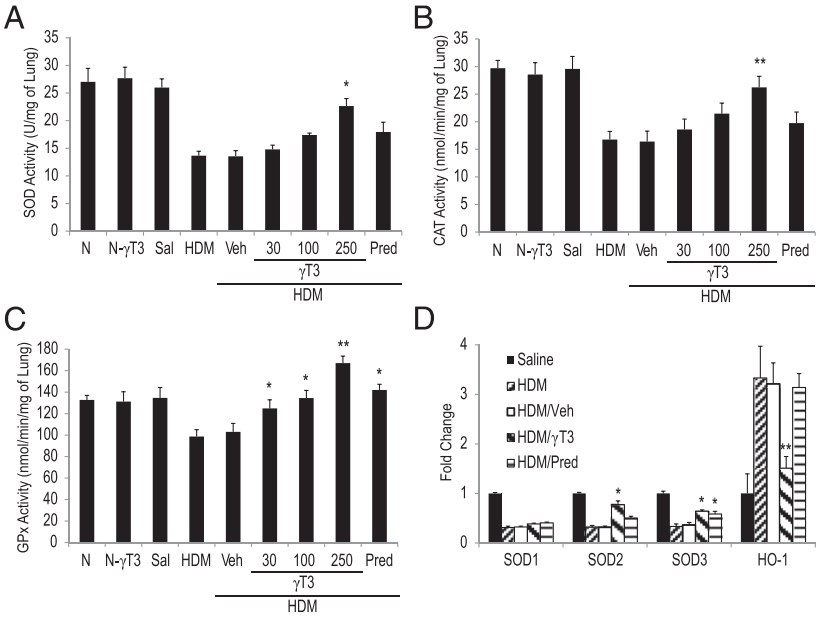

FIGURE 6. γ-Tocotrienol strengthens antioxidant defense in HDM-challenged lungs. Enzymatic activities of (**A**) SOD, (**B**) catalase, and (**C**) GPx in lung tissues were measured using activity-based ELISA. Values are expressed as means ± SEM (n = 8). (**D**) Lung mRNA expression of three isoforms of SOD (CuZnSOD, MnSOD, and ECSOD) and HO-1 was analyzed using real-time PCR. Values are expressed as means ± SEM (n = 5). $*p < 0.05$, $**p < 0.01$ compared with vehicle control.

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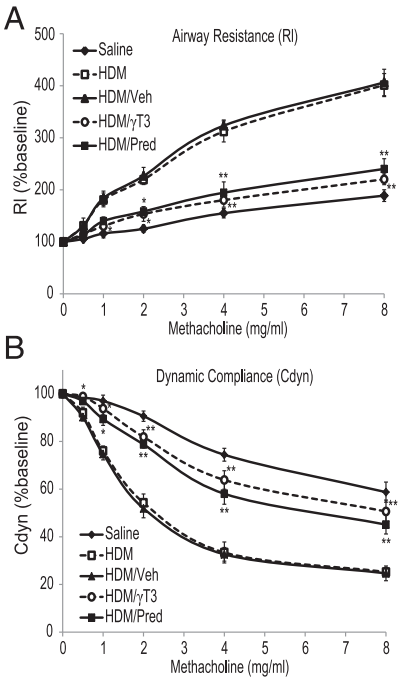

FIGURE 7. γ-Tocotrienol prevents HDM-induced AHR to increasing concentrations of methacholine. AHR is expressed as percentage change from the baseline level of (**A**) RI and (**B**) Cdyn. Values are expressed as means ± SEM (n = 5). *$p < 0.05$, **$p < 0.01$ compared with vehicle control.

asthma severity (3, 36, 37). iNOS level has also been found to be heightened in asthma and is responsible for the formation of NO and the downstream free radical peroxynitrite ($ONOO^-$), leading to 3-NT protein oxidative damage (38). Besides, peroxynitrite is involved in the activation of the PI3K/Akt signaling pathway, supporting the notion that oxidative stress can positively regulate

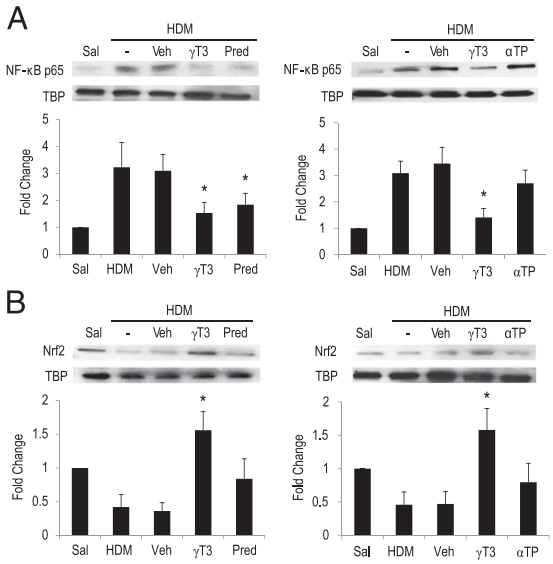

FIGURE 8. Protective effects of γ-tocotrienol are mediated through regulation of nuclear levels of NF-κB and Nrf2 in HDM-challenged lungs. Immunoblots were probed with (**A**) anti–NF-κB or (**B**) anti-Nrf2 mAb. Nuclear protein TATA-binding protein (TBP) was used as an internal control. The experiments were repeated three times with similar patterns of results. Protein band intensities were analyzed using ImageJ software and normalized against TBP controls. Values are expressed as mean ± SEM (n = 3). *$p < 0.05$ compared with vehicle control.

inflammation in asthma (38). We observed that NOX1–4, NOX essential regulatory subunits $p22^{phox}$ and $p67^{phox}$, iNOS, and oxidative damage markers were upregulated in the HDM-challenged lungs. γ-Tocotrienol was found to decrease free radical levels and oxidative damage markers in the BAL fluid and also suppress NOX and iNOS gene expression, which have been shown to be transcriptionally regulated by NF-κB (39). The reduced expression of NOX and iNOS may also be contributed by the drop in lung infiltration of granulocytes such as eosinophils and neutrophils.

SODs are metalloenzymes that can dismutate $O_2^{\cdot -}$ into relatively less toxic H_2O_2. Catalase and GPx are responsible for neutralizing H_2O_2 into water and oxygen (3). In asthma, SOD, catalase, and GPx antioxidant activities are reduced, resulting in lung function impairment (40, 41). In this study, prednisolone failed to restore SOD, catalase, or GPx antioxidant activity to a significant extent in HDM-challenged mice. In contrast, γ-tocotrienol elevated the expression of MnSOD and ECSOD, as well as restored SOD, catalase, and GPx activities back to normal levels. Our findings revealed, to our knowledge for the first time, that γ-tocotrienol could confer protection against oxidative damage in the allergic airway by enhancing nuclear Nrf2.

IL-5, IL-13, IL-17, and IL-33 have been shown to contribute to the development of AHR in asthma (33, 42, 43). IgE-mediated mast cell degranulation is necessary for AHR by producing mediators, including IL-13, IL-17, IL-33, and histamine (31, 44). Overproduction of $ONOO^-$ or accumulation of $O_2^{\cdot -}$ can induce AHR in experimental asthma (3, 45). γ-Tocotrienol was able to impede HDM-induced AHR by abrogating proinflammatory cytokines and serum IgE levels, as well as neutralizing oxidative stress and restoring antioxidant defenses, probably via inhibition of NF-κB activity and promotion of nuclear Nrf2 level. Indeed, recent findings reveal a direct role of epithelial NF-κB in orchestrating HDM-induced AHR (46).

We report, to our knowledge for the first time, that vitamin E isoform γ-tocotrienol effectively prevented HDM-induced airway inflammation and oxidative stress, as well as AHR in experimental asthma. γ-Tocotrienol acts not only as a direct free radical scavenger, but also as an anti-inflammatory and antioxidative agent by inhibiting NF-κB nuclear translocation and promoting nuclear Nrf2 level. Overall, γ-tocotrienol has similar anti-inflammatory efficacies to prednisolone in asthma, and it demonstrates greater antioxidative actions than prednisolone. Therefore, γ-tocotrienol may have therapeutic potential for the treatment of asthma, and it may be considered as an add-on therapy to glucocorticoid for optimal asthma control. It would be essential to confirm these beneficial effects of γ-tocotrienol in human asthma in future clinical studies.

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Disclosures

The authors have no financial conflicts of interest.

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ONLINE SUPPLEMENTARY MATERIAL

Vitamin E Isoform γ-Tocotrienol Down Regulates House Dust Mite-Induced Asthma

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Supplementary Table I Primers sequences used in this study

Gene	Primer	Primer Sequences (5' → 3')
Cu,Zn-SOD	Forward	5’-ACCAGTGCAGGACCTCATTTTAA-3’
	Reverse	5’-TCTCCAACATGCCTCTCTTCATC-3’
E-Selectin	Forward	5’-AACGCCAGAACAACAATTCC-3’
	Reverse	5’-TGAATTGCCACCAGATGTGT-3’
Ec-SOD	Forward	5’- GTGTCCCAAGACAATC-3’
	Reverse	5’- GTGCTATGGGGACAGG-3’
HO-1	Forward	5’-CCTCACTGGCAGGAAATCATC-3’
	Reverse	5’-CCTCGTGGAGACGCTTTACATA-3’
iNOS	Forward	5'-GTTCTCAGCCCAACAATACAAGA-3'
	Reverse	5'-GTGGACGGGTCGATGTCAC-3'
IL-13	Forward	5'-CAGAGGCCATGCAATATCCTC-3'
	Reverse	5'-CAGCATGGTATGGAGTGTGGA-3'
IL-17	Forward	5'-GACCAGGATCTCTTGCTGGA-3'
	Reverse	5'-GAACTCTCCACCGCAATGA-3'
33	Forward	5'-CGGATCCACTTCACTTTTAACACAGTC-3'
	Reverse	5'-GAGATCTTTAGATTTTCGAGAGCTTA-3'
Mn-SOD	Forward	5’-CACATTAACGCGCAGATCATG-3’
	Reverse	5’-CCAGAGCCTCGTGGTACTTCTC-3’
MUC5AC	Forward	5'-GGACTTCAATATCCAGCTACGC-3'
	Reverse	5'-CAGCTCAACAACTAGGCCATC-3'
NOX1	Forward	5’-AGGTCGTGATTACCAAGGTTGTC-3’
	Reverse	5’-AAGCCTCGCTTCCTCATCTG-3’
NOX2	Forward	5’-AGCTATGAGGTGGTGATGTTAGTGG-3’
	Reverse	5’-CACAATATTTGTACCAGACAGACTTGAG-3’
NOX3	Forward	5’-GCTGGCTGCACTTTCCAAAC-3’
	Reverse	5’-AAGGTGCGGACTGGATTGAG-3’
NOX4	Forward	5’-CCCAAGTTCCAAGCTCATTTCC-3’
	Reverse	5’-TGGTGACAGGTTTGTTGCTCCT-3’
p22phox	Forward	5’-CGTGGCTACTGCTGGACGTT-3’
	Reverse	5’-GCACACCTGCAGCGATAGAG-3’
p67phox	Forward	5’-CTTCGGATTCACCCTCAGTC-3’
	Reverse	5’-CACCTTGAGCATGTAAGGCATAGG-3’
Periostin	Forward	5'-TAGCCCAATTAGGCTTGGCATCC-3'
	Reverse	5'-TAAGAAGGCGTTGGTCCATGCT-3'
RANTES	Forward	5'-TTTGCCTACCTCTCCCTCG-3'
	Reverse	5'-CGACTGCAAGATTGGAGCACT-3'
TNF-a	Forward	5'-CCAGTGTGGGAAGCTGTCTT-3'
	Reverse	5'-AAGCAAAAGAGGAGGCAACA-3'
VCAM-1	Forward	5'-CCAGCACTCCGTGAAGATCC-3'
	Reverse	5'-CTGGTCTGGGAAGGCATACA-3'

Supplementary Table II Complete blood count and chemical analysis of serum in mice

Peripheral Blood Count	Units	Saline	HDM	HDM / γ-T3
White blood cell	K/uL	1.94 ± 0.53	2.11 ± 0.26	1.94 ± 0.39
Neutrophil	K/uL	0.50 ± 0.07	0.77 ± 0.11	0.61 ± 0.09
Lymphocyte	K/uL	1.56 ± 0.47	1.42 ± 0.19	1.70 ± 0.33
Monocyte	K/uL	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
Eosinophil	K/uL	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Basophil	K/uL	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.00
Red blood cell	M/uL	9.03 ± 0.13	8.91 ± 0.16	8.98 ± 0.10
Hemoglobin	g/dL	14.96 ± 0.15	14.80 ± 0.17	14.28 ± 0.16
Hematocrit	%	42.60 ± 0.54	41.56 ± 0.75	41.72 ± 0.17
Mean corpuscular volume	fL	47.18 ± 0.38	46.58 ± 0.17	46.55 ± 0.39
Mean corpuscular hemoglobin	pg	16.54 ± 0.10	16.62 ± 0.14	15.9 ± 0.34
Mean corpuscular hemoglobin conc.	g/dL	35.12 ± 0.32	35.64 ± 0.30	34.24 ± 0.46
Red blood cell distribution width	%	17.36 ± 0.33	17.24 ± 0.28	17.74 ± 0.25
Platelet	K/uL	724.5 ± 56.85	743.2 ± 43.12	743.50 ± 49.82
Mean platelet volume	fL	5.17 ± 0.29	4.81 ± 0.06	4.99 ± 0.42
Kidney and Liver Toxicity Test	**Units**	**Saline**	**HDM**	**HDM / γ-T3**
Creatinine	mg/dl	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
AST	U/L	118.22 ± 9.98	116.64 ± 9.71	111.36 ± 11.15
ALT	U/L	32.58 ± 0.89	34.56 ± 4.17	32.74 ± 1.02