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Supplemental and Highly Elevated Tocopherol Doses Differentially Regulate Allergic Inflammation: Reversibility of α -Tocopherol and γ -Tocopherol's Effects

Christine A. McCary, Hiam Abdala-Valencia, Sergejs Berdnikovs, and Joan M. Cook-Mills

We have reported that supplemental doses of the α - and γ -tocopherol isoforms of vitamin E decrease and increase, respectively, allergic lung inflammation. We have now assessed whether these effects of tocopherols are reversible. For these studies, mice were treated with Ag and supplemental tocopherols in a first phase of treatment followed by a 4-wk clearance phase, and then the mice received a second phase of Ag and tocopherol treatments. The proinflammatory effects of supplemental levels of γ -tocopherol in phase 1 were only partially reversed by supplemental α -tocopherol in phase 2, but were completely reversed by raising α -tocopherol levels 10-fold in phase 2. When γ -tocopherol levels were increased 10-fold (highly elevated tocopherol) so that the lung tissue γ -tocopherol levels were equal to the lung tissue levels of supplemental α -tocopherol, γ -tocopherol reduced leukocyte numbers in the lung lavage fluid. In contrast to the lung lavage fluid, highly elevated levels of γ -tocopherol increased inflammation in the lung tissue. These regulatory effects of highly elevated tocopherols on tissue inflammation and lung lavage fluid were reversible in a second phase of Ag challenge without tocopherols. In summary, the proinflammatory effects of supplemental γ -tocopherol on lung inflammation were partially reversed by supplemental levels of α -tocopherol but were completely reversed by highly elevated levels of α -tocopherol. Also, highly elevated levels of γ -tocopherol were inhibitory and reversible in lung lavage but, importantly, were proinflammatory in lung tissue sections. These results have implications for future studies with tocopherols and provide a new context in which to review vitamin E studies in the literature. *The Journal of Immunology*, 2011, 186: 3674–3685.

Vitamin E is an antioxidant lipid that has been used to regulate inflammatory disease. Vitamin E consists of multiple natural isoforms, including the natural α -, β -, γ -, and δ -tocopherols and the unsaturated α -, β -, γ -, and δ -tocotrienols, which differ in the number of methyl groups on the chromanol head (1, 2). The natural isoforms (*d*-form) of tocopherols are the R,R,R stereoisomers in the 2', 4', and 8' positions of the lipid tail, whereas synthetic tocopherols have R or S racemic conformations at these positions. Dietary tocopherols are taken up from the intestine, transported via chylomicrons in the lymph to the blood, and then to the liver. Subcutaneously administered tocopherols, which are used in our studies, are also transported through the lymph to the blood and then to the liver. Although γ -tocopherol is the most abundant vitamin E isoform in diet, it exists in tissue at only 10% the concentration of α -tocopherol due to the preferential uptake of α -tocopherol by the hepatic enzyme α -tocopherol transfer protein (α TTP) (3). Nevertheless, α TTP transfers significant quantities of γ -tocopherol to lipid particles, which then enter the circulation (3). Cells acquire tocopherols from plasma lipoproteins by plasma

phospholipid transfer protein, scavenger receptors, or the lipoprotein lipase pathway (4). After cell uptake, tocopherols are located in cell membranes. At equal molar concentrations in vitro, α -tocopherol, γ -tocopherol, and the tocotrienol isoforms have a similar capacity to scavenge reactive oxygen species during lipid oxidation (1, 5, 6). In addition to serving as antioxidants, vitamin E isoforms have been reported to have nonantioxidant functions (1, 7, 8).

It is reported that asthmatics have low levels of the vitamin E isoform α -tocopherol (9–12); however, there are seemingly contradictory reports regarding the effect of vitamin E on allergic/asthmatic inflammation (13–15). It is reported that α -tocopherol is beneficial in reducing asthma in some European countries (Finland and Italy) (16, 17). Disappointingly, clinical trials in the United States and the Netherlands using α -tocopherol supplements have failed to show benefit in asthma (17–20). The plasma levels of tocopherols in Americans and Europeans are reported to differ; several reports indicate that plasma levels of γ -tocopherol in Americans and people in the Netherlands are two to six times higher than most Europeans, whereas α -tocopherol plasma levels do not differ among these countries (reviewed in Ref. 21). These plasma tocopherol levels reflect differences in diet because γ -tocopherol is the major form of vitamin E in the diet of Americans but is not in abundance in most European diets (21), which use oils with no or very low γ -tocopherol. Moreover, we recently reported that supplemental levels of γ -tocopherol enhance inflammation, and supplemental levels of α -tocopherol reduce inflammation in a murine asthma model (8). It is not known whether the proinflammatory and anti-inflammatory effects of tocopherol isoforms are reversible. Therefore, we investigated the reversibility of the effects of tocopherols by determining whether the proinflammatory effect of supplemental γ -tocopherol during a phase of three OVA challenges can be reversed with a 4-wk clearance of tissue tocopherols/

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Abbreviations used in this article: BAL, bronchoalveolar lavage; α TTP, α -tocopherol transfer protein.

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inflammation, followed by administration of α -tocopherol during a second phase with OVA challenge.

Using purified natural isoforms of tocopherols at supplemental concentrations, we report in this study the novel findings that the differential effects of supplemental tocopherols on allergic inflammation are only partially reversible. In contrast, this inflammation is completely reversed and reduced by highly elevated amounts of α -tocopherol. In vivo, raising γ -tocopherol to equal molar concentrations as α -tocopherol inhibited lung lavage fluid inflammation. In vitro, these doses of γ -tocopherol partially blocked leukocyte transendothelial migration by a direct effect on endothelial cells. Unlike the partial reversibility of tocopherols' effects at supplemental treatment levels, the anti-inflammatory effects of highly elevated levels of tocopherols were completely reversible for inflammation in lung lavage. However, for highly elevated levels of γ -tocopherol, there were differential effects on inflammation in lung lavage and tissue sections because there was reduced inflammation in lung lavage but elevated inflammation in lung tissue sections.

Materials and Methods

Animals

BALB/c mice were from The Jackson Laboratory (Bar Harbor, ME). The studies are approved by the Northwestern University institutional review committee for animals.

Cells

The endothelial cell line mHEVa was cultured as previously described (22, 23). Spleen cells were prepared from freshly isolated male BALB/c mouse spleens (22), and spleen RBCs were lysed by hypotonic shock (22).

Tocopherol reagents

Ethoxylated castor oil was composed of polyethoxylated castor oil (BASF Pharma), 20% ethanol, and 1% benzyl alcohol. *d*- α -tocopherol (MP Biochemicals) was >98% pure. *d*- γ -tocopherol (Supelco) was 99.9% pure. We confirmed the purity of these tocopherols by HPLC with electrochemical detection as described below. No tocopherols were present in the ethoxylated castor oil, also measured by HPLC. The purified tocopherols were diluted in ethoxylated castor oil for the in vivo studies. It was determined that complete suspension of tocopherols in the ethoxylated castor oil required rolling and inverting mixture at room temperature for 20 min (data not shown). This is in contrast to our previous study in which the tocopherols were suspended for only a couple of minutes (8). As discussed in the Results section (Fig. 2), we determined that 0.2 mg s.c. tocopherol/d (supplemental tocopherol levels) is sufficient to achieve plasma tocopherol levels that were in our previous studies (8). Highly elevated dosing refers to treatment of 2 mg s.c. tocopherol/d.

OVA/tocopherol administration and inflammation

BALB/c female mice, 4–6 wk of age at the start of the experiment, were maintained on a chow diet. The mice were sensitized by i.p. injection (200 μ l) of OVA grade V (10 μ g)/alum or saline/alum on days 0 and 7 (24). Then the mice received either one phase or two phases of treatments with tocopherols during OVA challenge (Fig. 1) as indicated for each study.

Briefly, for studies with only one phase of tocopherol treatments and OVA challenges (Fig. 1A), on days 13–20, the mice received daily s.c. injections (50 μ l) of supplemental (0.2 mg) or highly elevated (2 mg) doses of tocopherols in 50 μ l ethoxylated castor oil (25). Vehicle mice were injected with ethoxylated castor oil alone. Mice treated with both isoforms of tocopherols at supplemental levels were injected with 0.2 mg α -tocopherol and 0.2 mg γ -tocopherol in 50 μ l vehicle; mice treated with both isoforms at highly elevated levels were injected with 2 mg α -tocopherol and 2 mg γ -tocopherol in 50 μ l vehicle. The tocopherol doses for each study are as indicated in the figures for the studies. On days 16, 18, and 20, the mice were challenged with 150 μ g intranasal OVA fraction VI (Sigma-Aldrich) in saline or saline alone (24). On day 21, mice were weighed and sacrificed for tissue collection.

In studies with two phases of tocopherol treatments and OVA challenges (Fig. 1B), after phase 1 OVA challenges, mice entered a clearance phase from days 21–50 during which tocopherol was cleared from tissues and lung inflammation receded. Starting on day 51, mice received daily vehicle

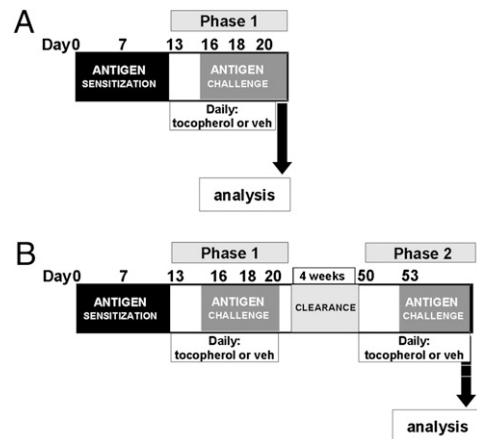


FIGURE 1. Timeline synopsis for tocopherol and OVA treatments. *A*, Tocopherol treatment during a single phase of OVA challenges. *B*, Tocopherol treatment during two phases of OVA challenges. veh, vehicle.

or tocopherol treatments. In the studies with supplemental levels of tocopherols, mice were challenged once on day 53 with 150 μ g intranasal OVA fraction VI (Sigma-Aldrich) in saline or saline alone to examine the initial response to rechallenge with Ag (Fig. 1B). In contrast, in the studies with highly elevated tocopherols in two phases of treatments, mice received three rechallenges (days 53, 55, and 57) with 150 μ g intranasal OVA fraction VI (Sigma-Aldrich) in saline or saline alone to examine the ability to regulate the response to repeated challenges with Ag (Fig. 1B).

Twenty-four hours after the last OVA challenge (Fig. 1), plasma and lung tocopherol levels were measured. Bronchoalveolar lavage (BAL) cells and blood eosinophils were stained and counted as previously described (24). Frozen lung tissue sections were stained with H&E. OVA-specific IgE was determined by ELISA as previously described (26). Lung lavage and lung tissue were examined for cytokines by ELISA and quantitative RT-PCR, respectively.

Tocopherol measurement

Lungs were perfused, weighed, and homogenized in absolute ethanol with 5% ascorbic acid on ice. The internal standard tocol is added to each lung to determine recovery. mHEVa cells, plasma, or homogenate were extracted with an equal volume of 0.37 weight percent butylated hydroxytoluene in hexane to prevent oxidation and increase recovery of tocopherol. The samples were vortexed and then centrifuged for 5 min at 2000 rpm at room temperature. The hexane layer was removed to a separate vial, and the hexane extraction step was repeated two more times for a total of three hexane extractions per sample. The hexane layer was dried under nitrogen and stored at -20°C . The samples were reconstituted in methanol, and then tocopherols were separated using a reverse-phase C_{18} HPLC column and HPLC (Waters Company, Milford, MA) with 99% methanol-1% water as a mobile phase with detection with an electrochemical detector (potential 0.7 V) (Waters Company).

Cytokine and chemokine measurement

The BAL were tested for levels of cytokines using Invitrogen's mouse Th1/Th2 multiplexing detection kit plus IL-13 singleplex kit (both from Life Technologies) using the Luminex 200 multiplexing system and xPONENT analysis software (Luminex, Austin, TX). CCL11 and CCL24 were determined by quantitative RT-PCR from lung tissue. Total RNA was isolated from 10–15 mg lung tissue using the Qiagen RNeasy Mini Kit. cDNA was prepared using Quanta's qScript cDNA synthesis kit and analyzed by PCR on an ABI 7300 Thermal Cycler (Applied Biosystems, Carlsbad, CA). TaqMan primers/probes and TaqMan Universal Master Mix were used as directed (Applied Biosystems).

In vitro cell association and migration assays with laminar flow

For endothelial cell pretreatment, 50–70% confluent endothelial cells (mHEVa cells) grown in a monolayer were treated with γ -tocopherol in DMSO overnight; migration assay was run the following day when cells reached 100% confluence. For leukocyte pretreatment, 4–6-wk-old male BALB/c mice were treated daily for 4 d with s.c. injections of highly

elevated γ -tocopherol or vehicle, as described above. Spleen cells were freshly prepared on day 5, and the migration assay was completed using untreated confluent mHEVa monolayers. A parallel plate flow chamber was used to examine leukocyte migration under conditions of laminar flow of 2 dynes/cm² as previously described (23, 27). Leukocyte transendothelial migration was examined at 15 min by fixing the cells and examination of phase dark cells by phase light microscopy (23, 27). The transendothelial migration of leukocytes in this assay is induced by the endothelial cell-derived chemokine MCP-1 (28).

Statistics

Data were analyzed by a one-way ANOVA followed by Tukey's or Dunn's multiple comparisons test (SigmaStat; Jandel Scientific, San Ramon, CA). Data are presented as means $\pm$ SEs.

Results

Reversibility of tocopherol regulation of allergic inflammation

We previously reported that α -tocopherol supplementation reduces and γ -tocopherol elevates leukocyte recruitment during allergic lung inflammation by, at least, direct effects of tocopherols on the endothelium (8). Additionally, when supplemental α -tocopherol and γ -tocopherol are administered together, an intermediate phenotype is observed such that inflammation is not significantly different from allergen-challenged vehicle-treated mice (8). Therefore, we determined whether these regulatory effects of purified natural *d*- α -tocopherol (α -tocopherol) and *d*- γ -tocopherol (γ -tocopherol) isoforms (Fig. 2A) were reversible. As in previous studies (8), ethoxylated castor oil was used as the vehicle for the tocopherols because it does not contain tocopherols or compounds that react with tocopherols and is used for pharmaceutical suspension of viscous lipids. A few days of s.c. administration of tocopherols was used in these studies rather than feeding for weeks with tocopherol-containing diets to change tissue tocopherol levels (25, 29) so that tissue tocopherol levels were altered in the few days before Ag challenge (Fig. 2C).

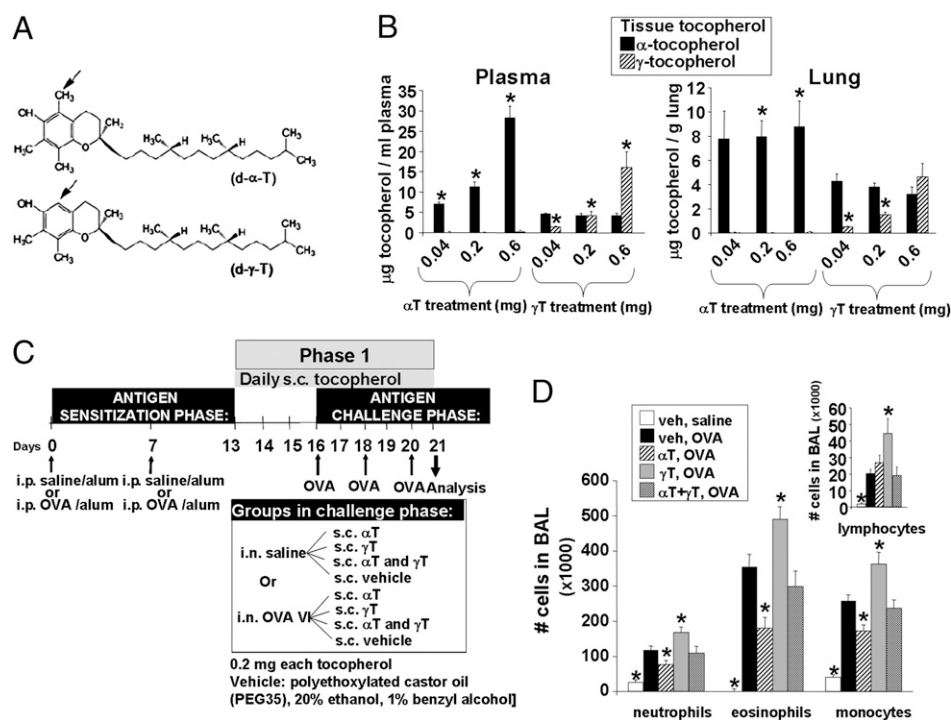
Before administering the tocopherols to the mice, purity (>98%) and concentration of tocopherols in the vehicle were determined by HPLC with electrochemical detection (data not shown). In contrast to our previous studies in which 2 mg/d doses of toco-

pherols were suspended for only a couple of minutes (8), we have determined that complete suspension of tocopherols in ethoxylated castor oil requires at least 20 min as determined by HPLC (data not shown). Therefore, a dose curve for s.c. tocopherol administration was used to determine the quantities of completely suspended tocopherols that will raise plasma concentrations to 10–12 μ g α -tocopherol/ml and 3–5 μ g γ -tocopherol/ml as in our previous report (8). Plasma and lung tocopherols for mice treated with 0.2 mg α -tocopherol or γ -tocopherol in 50 μ l ethoxylated castor oil per day for 8 d (Fig. 2B) are consistent with plasma and lung tocopherol levels in our previous report (8). The fold increase in plasma tocopherols in these studies is similar to the fold increase in human plasma levels from tocopherol supplementation (30, 31). Furthermore, the 0.2 mg dose of tocopherols administered daily during OVA Ag challenge (Fig. 2C) demonstrated opposing immune regulatory functions of α -tocopherol and γ -tocopherol (Fig. 2D), as we previously described (8). Tocopherols did not alter basal levels of leukocytes in saline-challenged mice (data not shown) (8). In these and previous reports of tocopherol supplementation (8), the body weights of the mice were unaltered by tocopherol treatments (data not shown). In summary, we define 0.2 mg tocopherol treatment/d as supplemental tocopherol treatment and the resulting tissue uptake of tocopherols as supplemental tissue tocopherol levels.

To examine the reversibility of supplemental tocopherol treatment in the OVA-asthma model (timeline in Fig. 3A), mice were sensitized with OVA/alum followed by s.c. treatment with daily supplemental α -tocopherol or γ -tocopherol and challenged three times with OVA intranasally in phase 1; next, these mice entered a 4-wk clearance phase without tocopherol or Ag administration; then, the mice entered phase 2 in which they received daily s.c. tocopherol treatment and were rechallenged once with OVA to examine the effects of tocopherols at the initiation of Ag rechallenge (Fig. 3A). In phase 2, the mice received the same tocopherol isoform or the other tocopherol isoform; the tocopherol treatment groups are shown in Fig. 3A. In addition to treatment groups that were given supplemental tocopherol treatments (0.2 mg/d) during phase 2, two treatment groups were administered α -tocopherol

FIGURE 2. Opposing functions of supplemental levels of natural *d*- α -tocopherol and *d*- γ -tocopherol.

A. Structure of natural *d*- α -tocopherol (*d*- α -T) and natural *d*- γ -tocopherol (*d*- γ -T), which differ by one methyl group (arrows). **B.** Tocopherol in plasma and perfused lung tissue after eight daily s.c. doses of 0.04, 0.2, or 0.6 mg α T or γ T. Tocopherol was measured by HPLC. **p* < 0.05 as compared to groups not treated with the tocopherol isoform. *n* = 4 to 5 animals per group. **C.** Allergic lung inflammation protocol with i.p. sensitization with chicken egg white albumin (OVA) fraction V/alum and tocopherol treatment starting after OVA sensitization. Intranasal OVA challenge was with fraction VI OVA. Inflammation was analyzed 24 h after the last OVA challenge. **D.** BAL neutrophils, eosinophils, monocytes, and lymphocytes were counted with a hemocytometer, and cytopun BAL cells were counted by standard morphological criteria. **p* < 0.05 as compared to veh, OVA group. veh, vehicle.



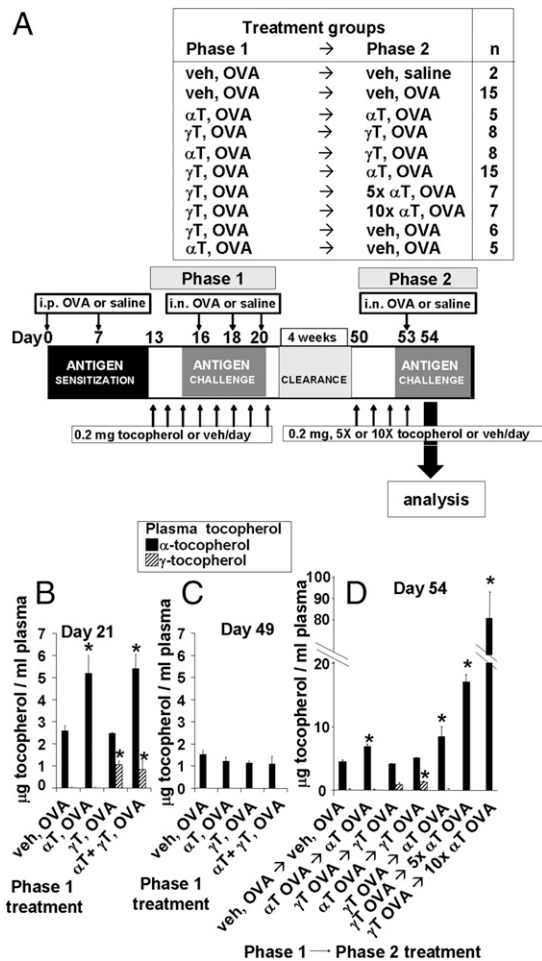


FIGURE 3. Timeline and plasma tocopherol levels during analysis of the reversibility of the regulatory effects of supplemental tocopherol on allergic lung inflammation. **A**, Mice were sensitized with OVA and then administered s.c. 0.2 mg tocopherol daily during OVA challenge in phase 1 of treatments. Following phase 1, mice entered a 4-wk clearance phase during which they were not treated with tocopherols or challenged with OVA. At the end of the clearance phase, mice were treated s.c. with 0.2 mg tocopherol and rechallenged with OVA in phase 2 of treatments. In phase 2, mice were again treated with daily doses of supplemental tocopherol (same isoform as phase 1, different isoform than phase 1, or vehicle) and rechallenged one time with OVA. In addition, two treatment groups were daily administered 5 $\times$ and 10 $\times$ supplemental *d*- α -tocopherol in phase 2. Inflammation was analyzed 24 h following this rechallenge. **B**, Tocopherol in plasma on day 21 as measured by HPLC. **C**, Tocopherol in plasma on day 49 as measured by HPLC. **D**, Tocopherol in plasma on day 54 as measured by HPLC. Note that on day 21, 49, or 54, plasma tocopherol levels in saline-treated groups receiving tocopherols were the same as those treated with OVA and tocopherols (data not shown). **p* < 0.05. i.n., intranasal; n, number of animals per group; α T, *d*- α -tocopherol; γ T, *d*- γ -tocopherol; veh, vehicle; veh, OVA $\rightarrow$ veh, OVA, indicates phase 1 treatments followed by phase 2 treatments as in **A**.

at 5 times (1 mg/d) and 10 times (2 mg/d) the supplemental tocopherol levels (Fig. 3A). Twenty-four hours following the end of phase 1 or phase 2 (days 21 and 54, respectively) (Fig. 3A), there was the expected elevation in plasma tocopherols after tocopherol treatment (Fig. 3B, 3D). A baseline of α -tocopherol but not γ -tocopherol is observed in groups that were not treated with tocopherols because α -tocopherol is present in standard rodent chow, whereas γ -tocopherol is low to not detected in rodent chow. The 4-wk clearance phase was sufficient time to clear tocopherol

from plasma (Fig. 3C) and resolve inflammation from the lung (32, 33).

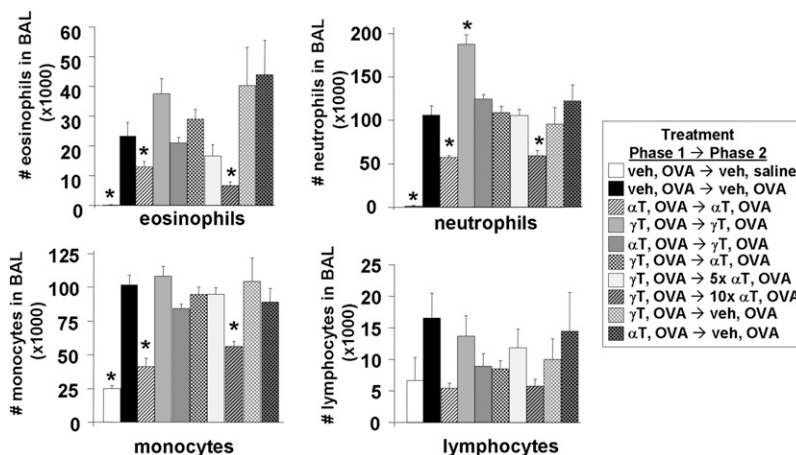
As expected, the single OVA rechallenge that was used to examine the initial response to Ag rechallenge induced an increase in leukocytes in the BAL including eosinophils, neutrophils, monocytes, and lymphocytes as compared with saline controls (Fig. 4). It is reported that at 24 h after a single OVA challenge, there is recruitment of several leukocyte cell types including eosinophils, neutrophils, monocytes, and lymphocytes, whereas during allergic inflammation, the peak accumulation in eosinophils occurs after several OVA challenges (24, 34). Supplemental α -tocopherol and γ -tocopherol treatments did not alter basal levels of the lung leukocytes in saline-treated mice (data not shown). Treatment with supplemental α -tocopherol in both phase 1 and 2 significantly reduced OVA-challenged recruitment of lung lavage eosinophils, neutrophils, and monocytes as compared with vehicle-treated OVA-challenged mice (Fig. 4). In contrast, treatment with supplemental γ -tocopherol in both phases raised OVA-induced lung lavage eosinophils and significantly increased OVA-induced neutrophils (Fig. 4). Groups that received phase 1 $\rightarrow$ phase 2 treatments of α -tocopherol, OVA $\rightarrow$ γ -tocopherol, OVA or received γ -tocopherol, OVA $\rightarrow$ α -tocopherol, OVA had an intermediate phenotype such that the number of lung lavage leukocytes was similar to that for vehicle-treated OVA-challenged mice (Fig. 4). Thus, switching tocopherol isoform in phase 2 did not completely reduce lung lavage inflammation. In addition, the lung leukocytes in the tocopherol, OVA $\rightarrow$ vehicle, OVA groups were not different from the vehicle, OVA $\rightarrow$ vehicle, OVA group (Fig. 4). However, raising α -tocopherol in phase 2 (the γ -tocopherol, OVA $\rightarrow$ 10 $\times$ α -tocopherol, OVA group) completely reduced the inflammation to the level in the α -tocopherol, OVA $\rightarrow$ α -tocopherol, OVA group (Fig. 4). We define these 10-fold higher levels of tocopherols (10 $\times$ 0.2 mg/d = 2 mg tocopherol/d) in phase 2 as highly elevated doses of tocopherols (Fig. 3).

The tocopherols also regulated the lung tissue inflammation. α -tocopherol was anti-inflammatory (Fig. 5C), and γ -tocopherol was proinflammatory (Fig. 5D). Switching the tocopherol isoform in phase 2 (Fig. 5E, 5F) or omitting tocopherol during phase 2 with OVA restimulation (Fig. 5G, 5H) resulted in an intermediate phenotype in which inflammation was similar to the levels in the vehicle-treated OVA-restimulated group (Fig. 5A). However, the γ -tocopherol, OVA $\rightarrow$ 10 $\times$ α -tocopherol, OVA group had reduced inflammation in the lung tissue (Fig. 5I). Tocopherols did not alter blood eosinophils (Fig. 5J). Consistent with OVA-specific IgE Ab production during the sensitization phase, the tocopherols that were administered after sensitization did not affect OVA-specific IgE (Fig. 6A).

Supplemental levels of tocopherols do not alter OVA-induced Th1/Th2 cytokines

Because tocopherols regulated inflammation in Figs. 4 and 5, we determined whether tocopherols affected regulatory cytokines and chemokines. The Th1 cytokines IFN- γ and IL-2 were not affected by treatment with supplemental tocopherols (Fig. 6I, 6J). The OVA-induced increases in IL-4, IL-5, CCL11, and CCL24 were not affected by the supplemental tocopherol treatments or the 10 $\times$ α -tocopherol treatment (Fig. 6B, 6C, 6G, 6H). OVA-induced IL-13 was not altered by tocopherols (Fig. 6F). This is consistent with reports that leukocyte infiltration can be regulated in Th2-driven models with no change in cytokine production (8, 35, 36). IL-12 and IL-10 were altered in two treatment groups. IL-12 expression was increased in the γ -tocopherol, OVA $\rightarrow$ γ -tocopherol, OVA group compared with the vehicle, OVA $\rightarrow$ vehicle, OVA group (Fig. 6E). IL-10 in the γ -tocopherol, OVA $\rightarrow$ 10 $\times$ α -tocopherol, OVA group

FIGURE 4. Reversibility of the regulatory effects of supplemental tocopherol levels on allergic BAL inflammation. Mice were treated with tocopherols and OVA as in Fig. 3. On day 54, BAL neutrophils, eosinophils, monocytes, and lymphocytes were counted with a hemocytometer, and cytospun BAL cells were counted by standard morphological criteria. $*p < 0.05$ as compared with veh,OVA→veh,OVA group. veh, OVA→veh,OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 3A; α T, d - α -tocopherol; γ T, d - γ -tocopherol.



was reduced compared with the vehicle, OVA→vehicle,OVA group (Fig. 6D). Overall, supplemental levels of tocopherols did not greatly impact expression of cytokines or chemokines.

Highly elevated γ -tocopherol reduces lung lavage inflammation

When equal amounts of α -tocopherol or γ -tocopherol (0.2 mg tocopherol/d) are administered to mice (Figs. 2, 3), 10 times more α -tocopherol than γ -tocopherol is acquired by tissues due to the preferential transfer of α -tocopherol to lipid particles in the liver by α -TTP (reviewed in Refs. 3, 37). To examine the functional effects of equal molar tissue levels of γ -tocopherol versus α -tocopherol, it was determined that 10 times more γ -tocopherol (2 mg γ -tocopherol/d) must be s.c. administered to achieve approximately equal molar lung tissue levels of γ -tocopherol as that for lung tissue levels of supplemental α -tocopherol (Fig. 7C versus Fig. 2B). We have defined 10 times the supplemental treatment levels as highly elevated tocopherol treatment and the resulting tissue levels from this highly elevated treatment as highly elevated tissue tocopherol levels. For these studies, mice were treated with highly elevated γ -tocopherol and three OVA rechallenges as in the timeline in Fig. 7A. After the three OVA rechallenges, the highly elevated γ -tocopherol treatments inhibited lung lavage eosinophilia as compared with vehicle controls (Fig. 7D). In addition, highly elevated γ -tocopherol treatment significantly reduced IL-5, IL-10, MIP-1 α , and MCP-1 but not IFN- γ , IL-2, CCL11, or CCL24 (Fig. 8). Thus, at equal molar lung tissue levels of α -tocopherol and γ -tocopherol, α -tocopherol (Fig. 2D) and γ -tocopherol (Fig. 7D) both function to inhibit lung lavage inflammation.

Highly elevated γ -tocopherol in endothelial cells partially inhibits leukocyte transendothelial migration in vitro

We have previously reported that supplemental levels of γ -tocopherol enhance leukocyte transendothelial migration in vitro by a direct effect on endothelial cells (8). Because the highly elevated γ -tocopherol reduced lung lavage inflammation (Fig. 7D), we determined whether highly elevated levels of γ -tocopherol modulated endothelial cell function during MCP-1-induced leukocyte transendothelial migration in vitro. Exogenous γ -tocopherol was loaded into endothelial cells overnight to generate tocopherol levels equivalent to highly elevated lung tissue γ -tocopherol (Figs. 7C, 9A); this required addition of exogenous 20 μ M γ -tocopherol overnight followed by three washes (Fig. 9A). Highly elevated levels of γ -tocopherol in endothelial cells in vitro inhibited leukocyte transendothelial migration by 40% under physiological laminar flow (Fig. 9B). To examine effects of highly elevated γ -tocopherol on leukocyte function during transmigration, mice were treated with highly elevated levels of γ -tocopherol for 4 d, spleen leukocytes were isolated and examined in vitro for transendothelial migration. Preloading leukocytes in vivo for 4 d with

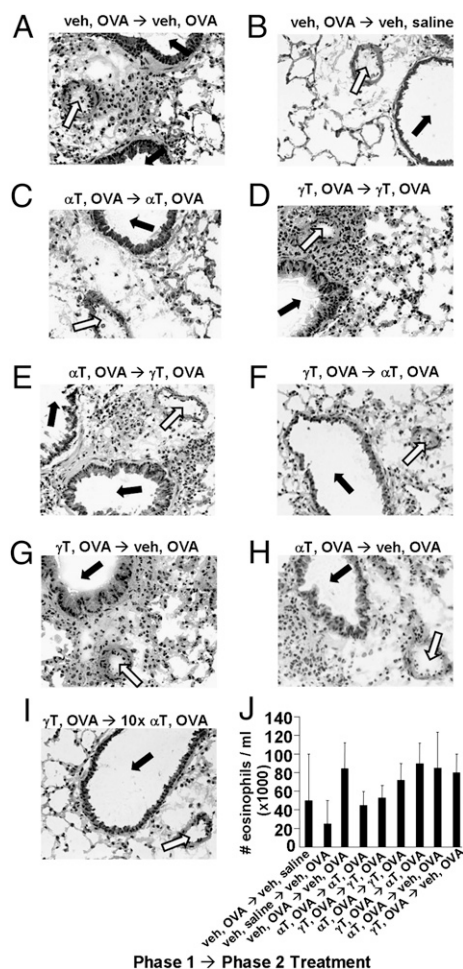


FIGURE 5. Reversibility of the regulatory effects of supplemental tocopherol on allergic lung tissue inflammation. Mice were treated with tocopherols as in Fig. 3A. A–I, Representative micrographs (original magnification $\times 40$) of perivascular regions in the lung tissue from day 54 as in Fig. 3A. Lung tissues were stained with H&E. J, Number of blood eosinophils on day 54 of treatments as in Fig. 3A. White arrow, vessel lumen; black arrow, bronchial airway. α T, d - α -tocopherol; γ T, d - γ -tocopherol; veh, vehicle; veh,OVA→veh,OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 3A.

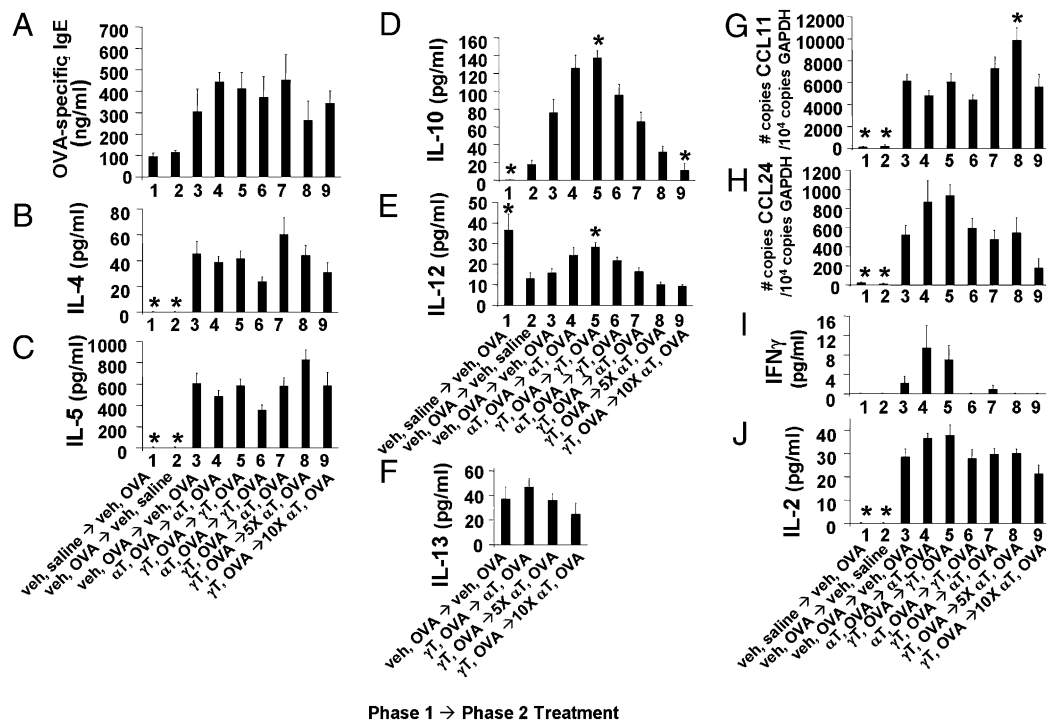


FIGURE 6. Switching tocopherol isoforms at supplemental levels in phase 2 did not alter OVA-specific IgE, lung cytokines, or lung chemokines. Mice were treated with supplemental levels of tocopherols as in Fig. 3A. A, Serum OVA-specific IgE as measured by ELISA. B–F, I, and J, BAL supernatants were examined for cytokines using the Th1/Th2 mouse cytokine multiplexing kit with IL-13 (Life Technologies). G and H, Lung tissue was placed in RNA later, then examined for eotaxin 1 (CCL11) and eotaxin 2 (CCL24) expression by real-time PCR. * $p < 0.05$ as compared with veh, OVA → veh, OVA group. αT, α -tocopherol; γT, γ -tocopherol; veh, vehicle; veh, OVA → veh, OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 3A.

highly elevated γ -tocopherol had no effect on leukocyte transendothelial migration in vitro (Fig. 9C). Thus, highly elevated levels of γ -tocopherol partially inhibited endothelial cell function but not leukocyte function during leukocyte transendothelial migration.

Regulation of lung inflammation by highly elevated tocopherols is reversible

It was determined whether the regulatory effects of highly elevated levels of tocopherols on lung lavage inflammation are reversible when tocopherols are withdrawn before Ag rechallenge. Highly elevated levels of tocopherols (2 mg/d) were administered, and the mice were treated with three OVA rechallenges as in the timeline in Fig. 10A. At the end of phase 1 (day 21), significantly more tocopherol was present in the plasma of mice that received highly elevated levels of tocopherols (Fig. 10B) as compared with the supplemental (0.2 mg/d) tocopherol-treated mice (Fig. 2B). At the completion of the clearance phase, the tocopherols were cleared from the plasma (Fig. 10C). At the end of phase 2 (day 58), there were significant increases in tocopherols in plasma and lung tissue as compared with vehicle controls (Fig. 10D, 10E).

The vehicle, OVA → vehicle, saline group had the same baseline numbers of lung lavage cells as the vehicle, saline → vehicle, saline group (Fig. 11), indicating that OVA treatment from phase 1 did not alter background lung lavage leukocytes during phase 2. Regardless of whether the OVA-challenged mice were treated in phase 1 with tocopherol or vehicle, in phase 2 the highly elevated α -tocopherol or γ -tocopherol reduced lung lavage eosinophils (Fig. 11) as compared with mice that received OVA, vehicle in both phase 1 and 2. Interestingly, the effects of highly elevated tocopherols in phase 1 are reversible when tocopherol is withdrawn in phase 2 because the lung lavage eosinophil numbers in the α -tocopherol, OVA → vehicle, OVA group and the γ -tocopherol, OVA → vehicle,

OVA group were not different from the lung lavage eosinophil numbers in the vehicle, OVA → vehicle, OVA group (Fig. 11). Blood eosinophils were not significantly altered by the highly elevated tocopherols, except for the γ -tocopherol, OVA → γ -tocopherol, OVA group (Fig. 12F).

Surprisingly, although highly elevated γ -tocopherol reduced leukocyte numbers in the lung lavage (Fig. 11), the γ -tocopherol, OVA → γ -tocopherol, OVA treatment elevated lung tissue leukocytes and induced lung tissue remodeling (Fig. 12C) as compared with the vehicle, OVA → vehicle, OVA treatment (Fig. 12A). In contrast, α -tocopherol, OVA → α -tocopherol, OVA treatment reduced lung tissue inflammation (Fig. 12B). These regulatory effects of highly elevated levels of tocopherols in lung tissue were reversible because the γ -tocopherol, OVA → vehicle, OVA group and the α -tocopherol, OVA → vehicle group (Fig. 12D, 12E) were not different from the vehicle, OVA → vehicle, OVA group (Fig. 12A). In summary, in the lung lavage and lung tissue, highly elevated α -tocopherol significantly reduces OVA-induced inflammation. However, highly elevated γ -tocopherol elevates lung tissue inflammation but reduces lung lavage inflammation. These effects of highly elevated tocopherols were reversible.

Highly elevated tocopherols reduce expression of IL-13

Because lung tissue and lung lavage inflammation were significantly affected by the highly elevated tocopherol treatments, regulatory Th1/Th2 cytokines, chemokines, and OVA-specific IgE were examined. The OVA-specific IgE Abs at the end of phase 2 were not affected by the tocopherol treatments (Fig. 13A). As expected, Th1 cytokine IFN- γ (not detected) and the low levels of IL-2 (Fig. 13H) were not affected by tocopherol treatments. Highly elevated levels of α -tocopherol or γ -tocopherol treatments in phase 1 and/or 2 did affect OVA-restimulated IL-4, IL-5, IL-10, or IL-12 (Fig. 13B–E), except one group (α -tocopherol, OVA → γ -

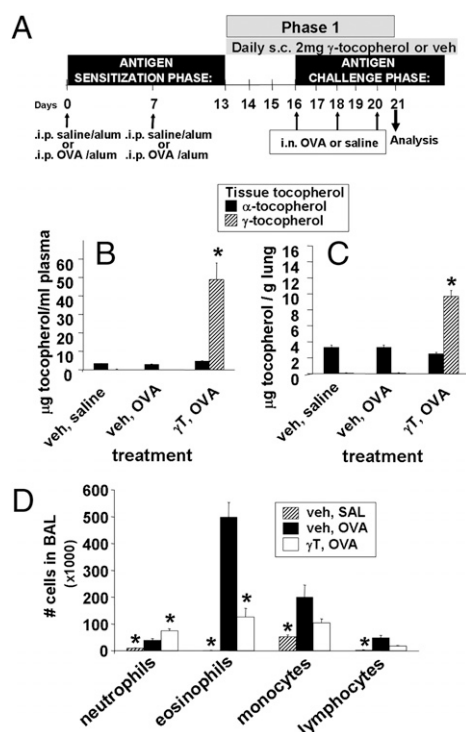


FIGURE 7. Highly elevated γ -tocopherol decreases lung BAL inflammation. **A**, Timeline for treatments with OVA and highly elevated γ -tocopherol (γ T) during allergic lung inflammation (only 1 phase of OVA treatments). On day 21 (**A**), plasma was collected and lungs were perfused free of blood. Tocopherol was measured by HPLC. Lung lavage leukocytes were examined on day 21. **B**, Plasma tocopherol. **C**, Lung tocopherols. **D**, BAL neutrophils, eosinophils, monocytes, and lymphocytes were counted with a hemocytometer, and cytospun BAL cells were counted by standard morphological criteria. $n = 4$ mice per group for the saline group and 8 mice per group for the OVA-stimulated groups. * $p < 0.05$ as compared with veh,OVA group. γ T, d - γ -tocopherol; veh, vehicle.

tocopherol, OVA), which had reduced IL-5 and IL-10 production (Fig. 13C, 13D). The OVA-induced lung tissue chemokine CCL11 was not reduced by the tocopherols (Fig. 13F), and there was a trend in reduced CCL24 with phase 2 tocopherol treatments that did not reach significance (Fig. 13G). In contrast, OVA-induced IL-13 production was reduced by highly elevated tocopherols in phase 2, and this was reversed with Ag rechallenge in the absence of tocopherols in phase 2 (i.e., γ -tocopherol,OVA→vehicle,OVA and α -tocopherol,OVA→vehicle,OVA groups) (Fig. 13I). In summary, highly elevated tocopherols reduced lung lavage inflammation and lung lavage IL-13, but highly elevated γ -tocopherol increased lung tissue inflammation.

Discussion

The opposing effects of supplemental α - and γ -tocopherol are partially reversible

We previously reported that supplemental levels of α -tocopherol and γ -tocopherol have anti-inflammatory and proinflammatory effects, respectively, during experimental asthma in mice (8). In addition, tocopherols regulate infiltration of all of the leukocyte cell types in response to Ag challenge by a direct effect on the endothelium (8). In the studies in this paper, the regulatory effects of tocopherols on inflammation were partially reversed when tocopherol-treated mice were cleared of plasma tocopherol for 1 mo and then supplemented with the opposing tocopherol in a second phase of tocopherol and Ag treatments. There was only partial reversal despite the mild inflammation upon a single

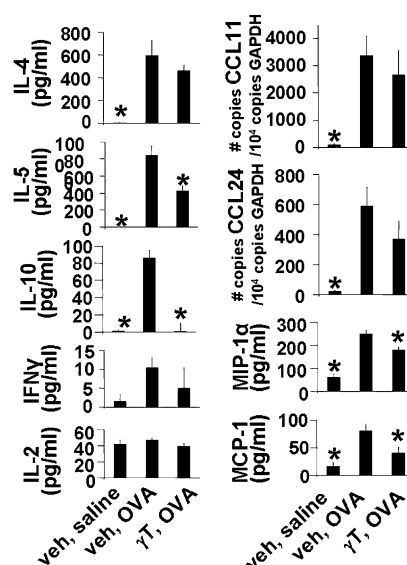


FIGURE 8. Highly elevated γ -tocopherol treatment decreases Th2 cytokines and chemokines in the lung. Mice were treated with highly elevated γ -tocopherol as in Fig. 7A. BAL supernatants were examined for cytokines (IL-4, IL-5, IL-10, IFN- γ , IL-2, MIP-1 α , MCP-1) using a mouse cytokine multiplexing assay (Millipore). Lung tissue was placed in RNA later and then examined for eotaxin 1 (CCL11) and eotaxin 2 (CCL24) expression by quantitative real-time PCR. * $p < 0.05$ as compared with veh,OVA group. γ T, d - γ -tocopherol; veh, vehicle.

rechallenge with Ag. In contrast, the proinflammatory effects of supplemental levels of γ -tocopherol in phase 1 were completely reversed by treatment with highly elevated α -tocopherol (10 times supplemental amounts) in the second phase with a single Ag-rechallenge. At 24 h after the single Ag rechallenge, there was the expected early neutrophil and monocyte infiltrate (34) as well as the beginning of eosinophil accumulation in the lung in the OVA-challenged vehicle-treated control group, because eosinophil

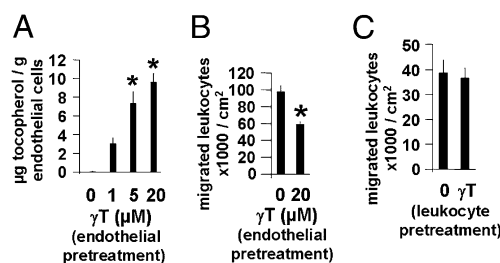


FIGURE 9. Highly elevated γ -tocopherol reduces leukocyte trans-endothelial migration through direct regulation of endothelial cells. **A**, At 90% confluence, endothelial cell monolayers were treated overnight with a dose curve of γ -tocopherol. After washing, cells were harvested, and tocopherol was measured by HPLC. **B**, At 90% confluence, endothelial cell monolayers were treated overnight with 20 μ M γ -tocopherol. Cells were washed five times before the start of the leukocyte migration assay with physiological laminar flow as detailed in *Materials and Methods*. **C**, Mice were treated with highly elevated (2 mg/d) γ -tocopherol or vehicle (ethoxylated castor oil) for 4 d as we previously reported for in vivo loading of tocopherols in leukocytes (8). Spleens were collected, and RBCs were lysed by hypotonic lysis. Spleen leukocytes from vehicle mice or spleen leukocytes from mice treated with highly elevated γ -tocopherol were added to untreated endothelial cell monolayers and examined for transendothelial migration under physiological laminar flow conditions as detailed in *Materials and Methods*. Leukocytes in each treatment group were not pooled; leukocytes from each mouse were processed individually. $n = 3$ migration assays per treatment. * $p < 0.05$ as compared with vehicle-treated group. γ T, d - γ -tocopherol; veh, vehicle.

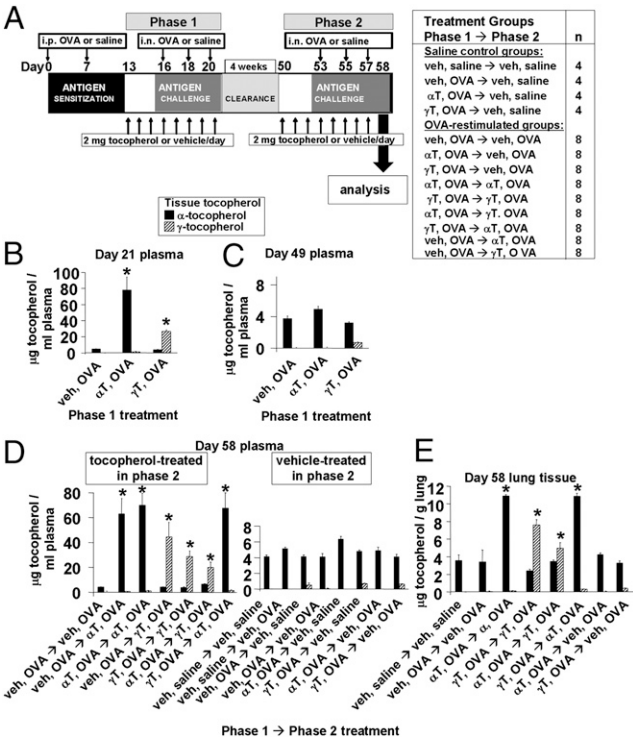


FIGURE 10. Timeline and plasma tocopherol levels during analysis of the reversibility of the regulatory effects of highly elevated tocopherol levels on allergic lung inflammation. *A*, Mice were sensitized with OVA and then administered s.c. highly elevated (2 mg) tocopherol daily during OVA challenge in phase 1 of treatments. Following phase 1, mice entered a 4-wk clearance phase during which they were not treated with tocopherols or challenged with OVA. At the end of the clearance phase, mice were treated s.c. with highly elevated (2 mg) tocopherols and rechallenged with OVA in phase 2 of treatments. In phase 2, mice were again treated with daily doses of supplemental tocopherol (same isoform as phase 1, different isoform than phase 1, or vehicle) and rechallenged three times with OVA. All treatment groups are listed. *B*, Plasma tocopherol on day 21. *C*, Plasma tocopherol on day 49. *D*, Plasma tocopherol on day 58. *E*, Tocopherol in perfused lung on day 58. **p* < 0.05 as compared with veh, OVA on days 21 or 49 or as compared with veh, OVA → veh, OVA group on day 58 where indicated. n, number of mice per group; αT, d-α-tocopherol; γT, d-γ-tocopherol; veh, vehicle; veh, OVA → veh, OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 10A.

infiltration peaks after several Ag challenges (24). The tocopherol effects were not accompanied by differences in expression of cytokines or chemokines. This is consistent with reports by us and

others demonstrating that leukocyte recruitment to the lung, in response to Ag challenge, can be regulated in the absence of changes in cytokine or chemokine expression when there is a functional effect on the endothelium (8, 24, 35).

Tocopherol isoforms and doses differentially regulate lung lavage inflammation, leukocyte transendothelial migration, and lung tissue inflammation

Interestingly, the opposing regulatory effects of supplemental α-tocopherol and γ-tocopherol on lung inflammation occur when γ-tocopherol is present in tissues at just 10% the concentration of α-tocopherol. Physiological levels of γ-tocopherol are lower than α-tocopherol in vivo because of the selective transfer of α-tocopherol to lipid particles by the hepatic enzyme αTTP (3). However, when we increased tissue γ-tocopherol levels to that of supplemental α-tocopherol tissue levels (10 µg/g lung) by administration of 10-fold higher levels of γ-tocopherol, the lung lavage inflammation was decreased after three Ag challenges despite the very high eosinophil levels that normally occur after three Ag challenges in phase 2. Importantly, highly elevated γ-tocopherol elevated the lung tissue inflammation and airway remodeling after three Ag challenges.

The different regulatory effects of supplemental versus highly elevated γ-tocopherol are consistent with tocopherols having both antioxidant and nonantioxidant properties (38). Moreover, the nonantioxidant properties of tocopherols can be specific to the tocopherol isoform (39). In our previous report (8), supplemental levels of γ-tocopherol exerted nonantioxidant effects by enhancing VCAM-1 activation of endothelial cell protein kinase Cα, resulting in increased VCAM-1-dependent leukocyte recruitment. In contrast, α-tocopherol at supplemental levels blocked VCAM-1-induced oxidative activation of protein kinase Cα and thus likely functioned as an antioxidant (8). Administration of supplemental levels of α-tocopherol and γ-tocopherol results in a 10-fold higher level of α-tocopherol than γ-tocopherol in the tissues as expected (38). Therefore, because α-tocopherol and γ-tocopherol possess approximately equal antioxidant activity, the higher concentration of α-tocopherol in vivo results in a greater total antioxidant capacity by α-tocopherol than γ-tocopherol. In our studies in this paper, when γ-tocopherol was highly elevated in the tissues such that γ-tocopherol tissue concentrations were equivalent to the tissue concentrations of supplemental α-tocopherol, the highly elevated γ-tocopherol would have approximately equal total antioxidant capacity to that for α-tocopherol. Nevertheless, in vivo, highly elevated levels of γ-tocopherol increased accumulation of leukocytes in the tissue but decreased lung lavage inflammation at 24 h after the third Ag challenge.

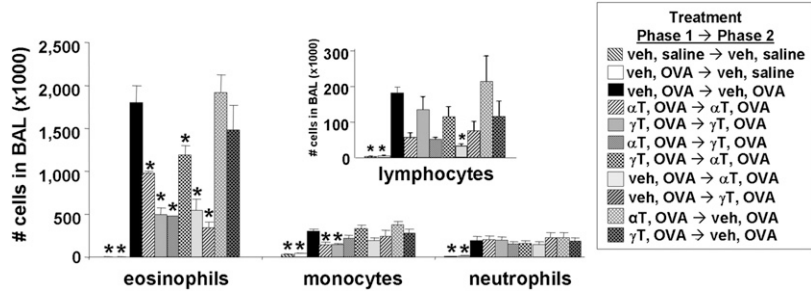


FIGURE 11. Reversibility of the regulatory effects of highly elevated tocopherol levels on allergic BAL inflammation. Mice were treated with highly elevated tocopherols as in Fig. 10A. On day 58, BAL neutrophils, eosinophils, monocytes, and lymphocytes were counted with a hemocytometer. Cytospun BAL cells were counted by standard morphological criteria. Not shown are two saline groups: leukocytes in the BAL of the αT, OVA → veh, saline and γT, OVA → veh, saline groups had BAL cell infiltrate not significantly different from veh, saline → veh, saline group. **p* < 0.05 as compared with veh, OVA → veh, OVA group on day 58. αT, d-α-tocopherol; γT, d-γ-tocopherol; veh, vehicle.

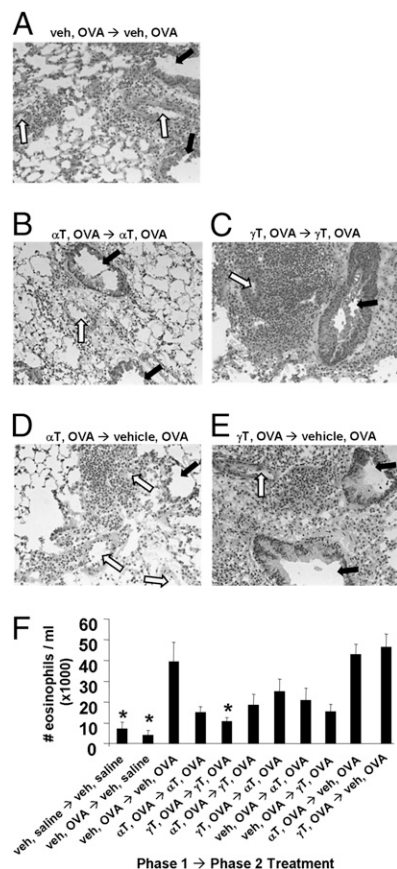


FIGURE 12. Reversibility of the regulatory effects of highly elevated tocopherol on allergic lung tissue inflammation. Mice were treated with highly elevated tocopherols as in Fig. 10A. A–E, Representative micrographs (original magnification $\times 20$) of perivascular regions in the lung tissue from day 58 as in Fig. 10A. Lung tissue was stained with H&E. F, Number of blood eosinophils on day 58 of treatments as in Fig. 10A. White arrow, vessel lumen; black arrow, bronchial airway. * $p < 0.05$ as compared with veh, OVA → veh, OVA group. αT, α -tocopherol; γT, γ -tocopherol; veh, vehicle; veh, OVA → veh, OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 10A.

Thus, even though *in vitro* transendothelial migration was partially inhibited at 15 min by highly elevated levels of γ -tocopherol, the *in vivo* data suggest a continued recruitment of leukocytes in lung tissue. This regulatory effect of highly elevated γ -tocopherol is likely a result of both its antioxidant functions and its nonantioxidant enhancement of protein kinase C α activity in lung tissue cells.

Because the highly elevated γ -tocopherol reduced leukocytes in the lung lavage despite the high numbers of leukocytes in the lung tissue, it suggests that highly elevated γ -tocopherol reduces recruitment of the leukocytes across the epithelium. It is reported that CCL11 has a role in transendothelial recruitment of eosinophils, whereas CCL24 has a greater role in the transepithelial recruitment of eosinophils (40). Therefore, a reduction in leukocyte migration across the epithelium is consistent with the trend in reduction in CCL24 but not CCL11 in the OVA-rechallenged high γ -tocopherol group as compared with OVA-rechallenged vehicle treatment group. In addition, in the lung tissues, there was airway remodeling with epithelial hyperplasia and narrowing of the airways. This may also contribute to reduced leukocyte migration through the epithelium.

Although highly elevated tissue levels of γ -tocopherol were achieved in our studies by s.c. tocopherol treatments, lower levels

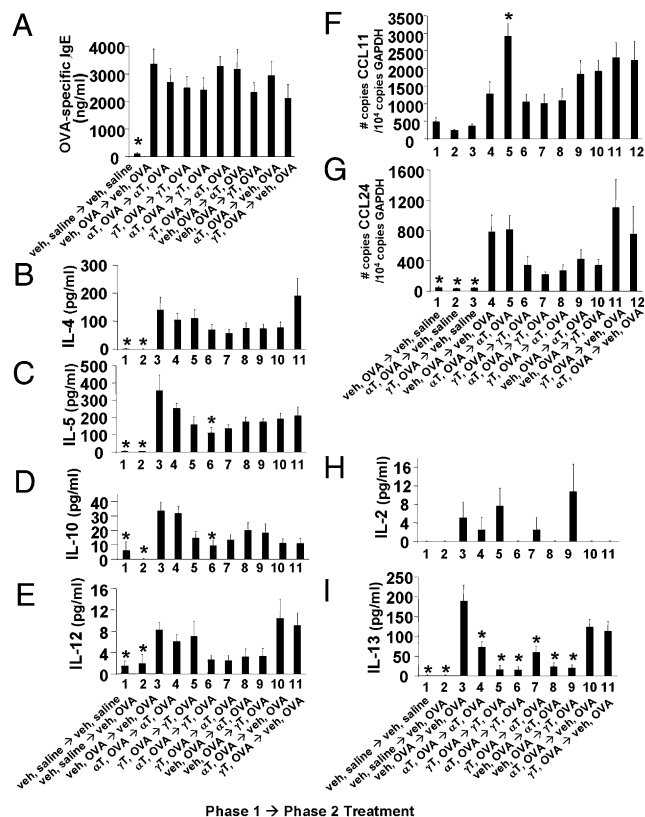


FIGURE 13. Switching tocopherol isoforms at highly elevated levels in phase 2 decreased IL-13 but not other lung cytokines, lung chemokines, or OVA-specific IgE. Mice were treated with highly elevated levels of tocopherols as in Fig. 10A. A, Serum OVA-specific IgE was measured by ELISA. B–E, H–I, BAL supernatants were examined for cytokines using the Th1/Th2 mouse cytokine multiplexing kit plus IL-13 singleplex supplement (Life Technologies). F and G, Lung tissue was placed in RNA later, then examined for eotaxin 1 (CCL11) and eotaxin 2 (CCL24) expression by quantitative real-time PCR. * $p < 0.05$ as compared with veh, OVA → veh, OVA group. αT, α -tocopherol; γT, γ -tocopherol; veh, vehicle; veh, OVA → veh, OVA, indicates phase 1 treatments followed by phase 2 treatments as described in Fig. 10A.

of tissue γ -tocopherol are reported to be achieved by dietary means. In a report in which mice were fed a diet containing 1150 mg α -tocopheryl acetate per kilogram diet for 15 d, they achieved ~ 13 μ g/ml α -tocopherol in plasma (41); if dietary γ -tocopherol had been administered at this dose, the plasma level of γ -tocopherol would be lower than 13 μ g/ml. In our studies with s.c. administration of tocopherols, the excretion is likely lower than that for dietary tocopherols, and thus equal molar lung tissue levels of α -tocopherol and γ -tocopherol (10 μ g tocopherol/g lung) were achieved by s.c. administration of supplemental levels of α -tocopherol (0.2 mg/d for 8 d) and highly elevated levels of γ -tocopherol (2 mg/d for 8 d). The supplemental α -tocopherol elevated plasma α -tocopherol to 12 μ g/ml, and the highly elevated γ -tocopherol elevated plasma γ -tocopherol to 25 μ g/ml. In addition, s.c. administration of highly elevated α -tocopherol treatment (2 mg/d for 8 d) results in 75 μ g/ml α -tocopherol in plasma. This very high α -tocopherol plasma level that we observed through s.c. tocopherol administration has not been reported for animal or human studies and may not be achievable through dietary means. Nevertheless, our studies indicate that the proinflammatory physiological tissue levels of γ -tocopherols function differently than the anti-inflammatory physiological levels of α -tocopherol; however, at equal molar tissue levels of these tocopherols, the isoforms can

have a similar function for inhibition of lung lavage inflammation, but high levels of γ -tocopherol elevate tissue inflammation. Although high doses of α -tocopherol reversed the proinflammatory effects of supplemental γ -tocopherol in our studies, reports indicating that high doses of tocopherol can significantly increase the incidence of hemorrhagic stroke, elevate blood pressure, and increase all-cause mortality (42–45) suggest that administration of high-dose α -tocopherol may be a potentially risky approach for reversing the proinflammatory effects of supplemental levels of γ -tocopherol. Nevertheless, in our studies, there was an isoform-specific and dose-dependent regulation of inflammation by tocopherols.

Anti-inflammatory effects of highly elevated α -tocopherol and γ -tocopherol in the BAL are reversible

In contrast to the partial reversibility of the regulatory effects of supplemental levels of tocopherols, the anti-inflammatory effects of highly elevated α -tocopherol and γ -tocopherol in the BAL were reversible to the levels of the Ag-challenged vehicle controls when tocopherols were cleared from plasma during a 4-wk phase, and mice were rechallenged three times with Ag in the absence of tocopherol. In contrast to the anti-inflammatory effects of highly elevated γ -tocopherol in the lung lavage, inflammation in the lung tissue was increased. This increase in tissue inflammation by highly elevated γ -tocopherol is consistent with reports of some adverse cardiovascular effects that can occur when elevating α -tocopherol or γ -tocopherol (15, 42–46). The increase in tissue inflammation by highly elevated γ -tocopherol, in our studies, was reversed in a second phase with three Ag challenges without tocopherol administration.

Highly elevated levels of γ -tocopherol reduced the Ag-induced proinflammatory mediators IL-5, IL-13, MIP-1 α , and MCP-1 in the lung lavage, whereas supplemental levels of tocopherols did not alter cytokine or chemokine expression. The Th1 cytokines IFN- γ and IL-2 were not induced by highly elevated tocopherol treatments during OVA challenges. There were some alterations in IL-10 in the studies with supplemental or highly elevated tocopherols. IL-10 is a cytokine with broad anti-inflammatory properties (47–49). However, IL-10 can also be elevated in the presence of increased inflammation (50). Enhanced IL-10 with concomitant increases in inflammation is not limited to allergic Th2-type disease as it also occurs in Th1/IFN- γ -driven celiac disease (51). Therefore, IL-10 can be elevated to limit further inflammation. In Figs. 6, 8, and 13, there is a trend of elevated IL-10 during elevated lung lavage inflammation and reduced IL-10 during reduced lung lavage inflammation, although some groups do not reach significance. In addition to IL-10, inflammation is controlled by a combination of multiple regulators in the microenvironment.

Alternative interpretations for previous studies of tocopherol regulation of inflammation

Interpretations of animal studies with conflicting effects of tocopherols on allergic disease are influenced by our studies demonstrating opposing functions of supplemental levels of tocopherols and partial reversibility of tocopherol immunoregulation. In a report by Suchankova et al. (52), Ag-sensitized rats were treated by oral gavage with α -tocopherol in soy oil for 10 d and then challenged with Ag. These α -tocopherol-treated rats did not exhibit changes in bronchoconstriction or lung inflammation when compared with controls. Because the soy oil vehicle in these studies would contain significant amounts of γ -tocopherol, the results are consistent with the interpretation that the γ -tocopherol in the vehicle ablated the anti-inflammatory benefit of α -tocopherol. In

a report by Wagner et al. (53), inhibition of lung lavage inflammation was observed when OVA-sensitized rats were treated daily by oral gavage with 100 mg γ -tocopherol/kg body weight and administered two OVA challenges. In their report, perivascular areas of the lung were not shown, and therefore lung tissue inflammation is not known. In contrast, in our current and previous report (8), supplemental and highly elevated γ -tocopherol levels increased lung tissue inflammation. Consistent with our current study in which highly elevated levels of γ -tocopherol inhibited lung lavage inflammation after three OVA challenges, the report by Wagner et al. (53) showed reduced lung lavage inflammation after γ -tocopherol treatment. However, in their studies, the lung lavage inflammation was predominantly neutrophils rather than the expected predominance of eosinophils at 48 h post second challenge with OVA (53).

The outcomes of clinical studies that focused on α -tocopherol were likely influenced by tissue γ -tocopherols. It is reported that the average baseline human plasma concentration of α -tocopherol (10 μ g/ml or 23 μ M α -tocopherol) is the same among various countries (21, 54). In contrast, the average human plasma γ -tocopherol level is two to five times higher in the United States (2.4 μ g/ml or 5.8 μ M γ -tocopherol) and The Netherlands (1 μ g/ml or 2.3 μ M γ -tocopherol) than in European and Asian countries (0.6 μ g/ml or 1.6 μ M γ -tocopherol) including Italy (0.5 μ g/ml or 1.2 μ M γ -tocopherol) (21, 54). These differences in human plasma tocopherol are consistent with dietary consumption of tocopherols (21, 38, 54, 55). Although it is acknowledged that species differ in basal levels of tocopherols and metabolism, it is interesting that the outcomes and the fold increase in human plasma γ -tocopherol in the United States compared with other countries is similar to that in our animal studies on the reversibility of the proinflammatory effects of supplemental γ -tocopherol. The clinical studies indicate that α -tocopherol supplementation of asthmatic patients is beneficial in Italy and Finland, but α -tocopherol is not beneficial for asthmatic patients in studies in the United States or The Netherlands (16–20). Because we report that the proinflammatory effects of γ -tocopherol are only partially reversible, elevated human plasma γ -tocopherol in the United States may have influenced the outcomes of α -tocopherol on allergic inflammation in the clinical studies. In addition, in one study, asthmatics were given an α -tocopherol dietary supplement (or soy oil placebo, which is rich in γ -tocopherol) for 6 wk, and there was no beneficial effect of α -tocopherol on lung function (56). In their study, the γ -tocopherol in the vehicle may have ablated the benefit of α -tocopherol supplementation. It is acknowledged that although there are many other differences regarding the environment and genetics of the people in the clinical studies, the data are, at least, consistent with our animal studies.

In summary, the regulatory effect of tocopherols on leukocyte recruitment to the lung tissue or lung lavage in Ag-sensitized mice depends on: 1) the isoform of the tocopherol used to treat the mice (natural α -tocopherol versus natural γ -tocopherol); 2) the concentration of tocopherol in tissues (supplemental versus highly elevated); and 3) the previous levels of tocopherol isoforms in tissues. α -tocopherol at highly elevated but not supplemental levels, during a second phase of Ag challenges, overcomes the proinflammatory effects of supplemental γ -tocopherol in the lung lavage and lung tissue. Changes in inflammation due to supplemental levels of tocopherol treatment are not accompanied by changes in inflammatory modulators. In contrast, highly elevated γ -tocopherol reduces lung lavage leukocytes but elevates lung tissue inflammation; these effects of γ -tocopherol are accompanied by modest changes in cytokines and chemokines. During a second phase of Ag challenge without highly elevated tocopherol

treatment, inflammation returns back to the levels of inflammation in the vehicle-treated Ag-challenged control. These results have important implications for interpretations of previous studies using supplemental or highly elevated levels of tocopherol isoforms. Although we have demonstrated the importance of tocopherol dose and isoform in the context of allergic inflammation, tocopherol doses and isoforms may also play a significant role in other inflammatory diseases (38, 54).

Disclosures

The authors have no financial conflicts of interest.

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