

## Epoxyeicosatrienoic acids are involved in the C<sub>70</sub> fullerene derivative-induced control of allergic asthma

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### **Abstract:**

Background: Fullerenes are molecules being investigated for a wide range of therapeutic applications. We have shown previously that certain fullerene derivatives (FDs) inhibit mast cell (MC) function *in vitro*, and here we examine their *in vivo* therapeutic effect on asthma, a disease in which MCs play a predominant role.

Objective: We sought to determine whether an efficient MC-stabilizing FD (C<sub>70</sub>-tetraglycolate [TGA]) can inhibit asthma pathogenesis *in vivo* and to examine its *in vivo* mechanism of action.

Methods: Asthma was induced in mice, and animals were treated intranasally with TGA either simultaneously with treatment or after induction of pathogenesis. The efficacy of TGA was determined through the measurement of airway inflammation, bronchoconstriction, serum IgE levels, and bronchoalveolar lavage fluid cytokine and eicosanoid levels.

Results: We found that TGA-treated mice have significantly reduced airway inflammation, eosinophilia, and bronchoconstriction. The TGA treatments are effective, even when given after disease is established. Moreover, we report a novel inhibitory mechanism because TGA stimulates the production of an anti-inflammatory P-450 eicosanoid metabolites (*cis*-

epoxyeicosatrienoic acids [EETs]) in the lung. Inhibitors of these anti-inflammatory EETs reversed TGA inhibition. In human lung MCs incubated with TGA, there was a significant upregulation of *CYP1B* gene expression, and TGA also reduced IgE production from B cells. Lastly, MCs incubated with EET and challenged through Fc  $\epsilon$  RI had a significant blunting of mediator release compared with nontreated cells.

**Conclusion:** The inhibitory capabilities of TGA reported here suggest that FDs might be used a platform for developing treatments for asthma.

Abbreviations used: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BALF, Bronchoalveolar lavage fluid; EET, Cis-epoxyeicosatrienoic acid; FD, Fullerene derivative; MC, Mast cell; OVA, Ovalbumin; PPOH, 6-(2-Propargyloxyphenyl) hexanoic acid; TGA, C70-tetraglycolate

**Keywords:** Fullerene derivative | eicosanoids | asthma | airway inflammation | bronchoconstriction | allergy

### **Article:**

In asthmatic patients the massive influx of immune cells, particularly eosinophils, causes airway thickening and reduced airflow into the lungs.<sup>1</sup> Eicosanoids, including leukotrienes and prostaglandins, are rapidly produced by immune cells to initiate inflammation.<sup>2</sup> Cytokines maintain chronic inflammation because IL-4 and IL-13 stimulate B-cell class-switching to IgE, IL-13 also promotes goblet cell metaplasia and mucus overproduction, and IL-5 recruits and stimulates eosinophils.<sup>3</sup> The airways also become hyperreactive with smooth muscle contraction and mast cell (MC) activation. Breathing difficulty manifests as wheezing, which can become life-threatening in severe cases. Although the causative allergens are not always identified, in patients with allergic asthma, degranulated MCs are found in lung tissue, and patient serum contains increased antigen-specific IgE and tryptase levels.<sup>4</sup> MCs are thought to play an important role in pathogenesis because significant numbers are recruited to the airways and because MC degranulation products are found in the bronchoalveolar lavage fluid (BALF).<sup>5</sup> In the murine asthma models that most closely mimic human disease, MC-deficient mice have reduced airway inflammation and bronchoconstriction in response to allergen challenge.<sup>6 and 7</sup>

Fullerene derivatives (FDs) are nanospheres of carbon that have a unique ability to catalytically scavenge large numbers of oxygen free radicals, making them potentially useful for treating disease.<sup>8, 9 and 10</sup> These robust antioxidants can reduce cellular damage and inflammation, and their therapeutic value has been suggested for the treatment of neurodegenerative and inflammatory diseases.<sup>11 and 12</sup> Previous research has found that polyhydroxylated FDs can enter human lung MCs and suppress degranulation and inflammatory cytokine production after IgE cross-linking.<sup>13</sup> Further studies have demonstrated that the biological function of FDs depends on the structure of the chemical moieties added to the carbon cage.<sup>14</sup> Given that MCs play a role in the pathogenesis of allergic asthma and that FDs can stabilize MCs when challenged with

activating stimuli, we hypothesized that FDs could prevent or possibly reverse the mechanisms leading to asthma.

To test this hypothesis, we used an ovalbumin (OVA) challenge model of asthma to assess the *in vivo* functionality of a novel FD (C<sub>70</sub>-tetraglycolate [TGA]) previously demonstrated to be an efficient *in vitro* MC stabilizer.<sup>14</sup> We find that whether TGA is administered before or after pathogenesis, it can significantly dampen airway inflammation in mice. In addition to reductions in eosinophil recruitment, airway hyperresponsiveness, and overall airway inflammation, significant reductions in IL-4 and IL-5 and serum IgE levels were also observed. TGA also suppressed IgE production by activated B cells. Furthermore, we have discovered a novel mechanism of action for TGA through the upregulation of the anti-inflammatory eicosanoid 11,12-*cis*-epoxyeicosatrienoic acid (EET) and discovered these molecules can inhibit human MC mediator release in response to FcεRI challenge. TGA treatment causes no acute toxicity to mice because liver and kidney function are unaltered. Thus our results suggest that rationally designed FDs might provide an effective therapeutic option for the treatment of asthma and that induction of anti-inflammatory EETs represent a new strategy for asthma regulation.

## Methods

### Mice and reagents

Chicken egg OVA, decamethonium bromide, and acetyl-β-methylcholine chloride (methacholine) were purchased from Sigma-Aldrich (St Louis, Mo). Aluminum hydroxide (alum) was purchased from Pierce (Rockford, Ill). Fullerene TGA was synthesized and tested at Luna Innovations, Inc (Roanoke, Va), as described previously.<sup>14</sup> The EET inhibitor 14,15-EE-5(Z)-E (an EET-specific antagonist)<sup>15</sup> and EETs were obtained from Cayman Chemicals (Ann Arbor, Mich), and 6-(2-propargyloxyphenyl) hexanoic acid (PPOH; a selective inhibitor of epoxygenation catalyzed by CYP450 isozymes)<sup>16</sup> was obtained from Sigma-Aldrich. Female 8- to 12-week-old C57BL/6 and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Me).

### Murine asthma induction and tissue collection

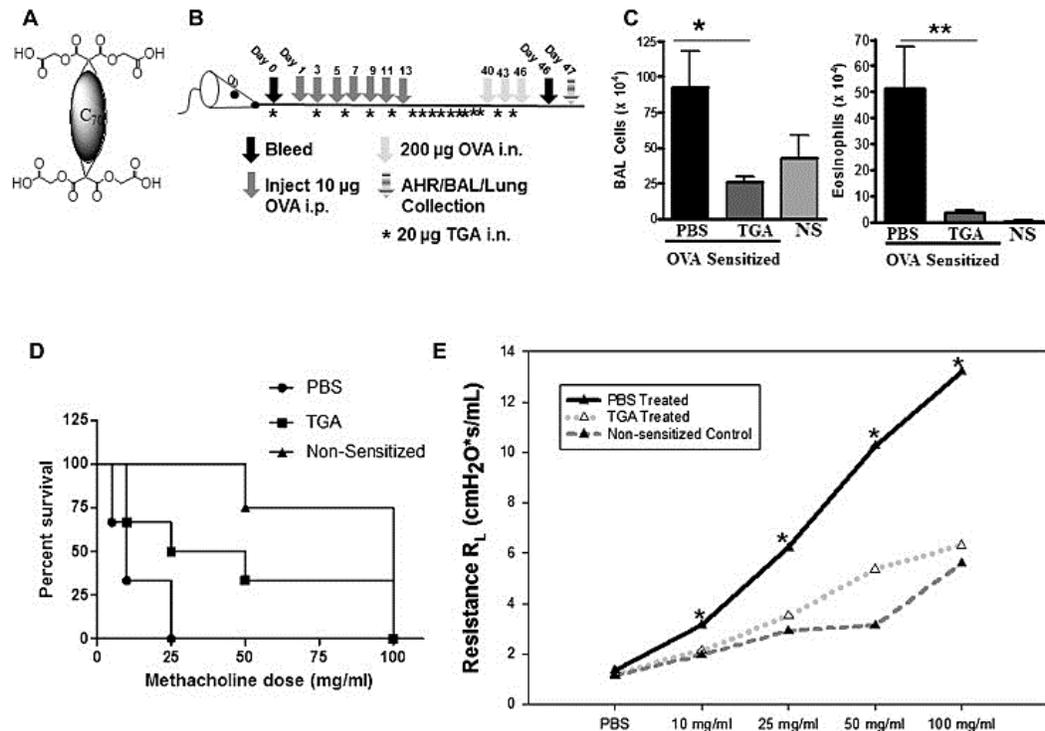
Acute asthma was induced as described by Williams and Galli<sup>7</sup> because MCs are known to be important for this model. Mice were administered 20 μg of TGA intranasally every 3 days throughout the experiment. In some experiments the EET inhibitors (14,15-EE-5[Z]-E and PPOH) were administered intranasally 15 minutes before TGA inhalation by using 0.128 mg/kg in 20 μL. PPOH was also administered 2 hours after TGA inhalation at the same dose. Mice were killed on day 47, and BALF was collected by flushing the lungs with 1 mL of PBS. Supernatants were saved for cytokine analysis. Pelleted cells were spun onto slides and stained with the Hema 3 stain set (Fisher Diagnostics, Middletown, Va). Percentages were determined by counting at least 100 leukocytes per slide. Lung tissue was fixed with 10% buffered formalin phosphate (Fisher Diagnostics) and embedded in paraffin at the VCU pathology core. Sections (5 μm) were

cut onto slides and stained with hematoxylin and eosin. A Nikon eclipse with a SPOT Flex Shifting Pixel Color Mosaic (Diagnostic Instrumental, Inc, Sterling Heights, Mich) camera was used to photograph lung sections. All mouse protocols were approved by the VCU Institutional Care and Use Committee.

A modified asthma model similar to that described by Williams and Galli<sup>7</sup> was used to assess bronchoconstriction. In this model mice were sensitized with 4 injections of 50  $\mu$ g of OVA and then challenged on days 22, 25, and 28 with 200  $\mu$ g of OVA. Mice were killed on day 29, and bronchoconstriction was assessed with the Flexivent system (Scireq, Montreal, Quebec, Canada). Mice were anesthetized, and a 19-gauge blunt-end cannula was inserted into the trachea; ventilation began immediately. Mice were paralyzed by means of intraperitoneal injection of 0.5 mg of decamethonium bromide. Lung function was assessed once it was determined that breathing was completely through mechanical ventilation. Responsiveness to methacholine was determined by exposing mice to aerosolized PBS and then to increasing doses (10, 25, 50, and 100 mg/mL) of methacholine. Maximum airway resistance in response to each methacholine dose was determined by averaging the 3 highest values.

#### Established asthma induction

Mice were sensitized intraperitoneally and challenged intranasally, as in Fig 1, B, to determine whether TGA could reverse established disease. Intranasal treatment with 20  $\mu$ g of TGA began on day 47 and continued every 3 days after. Mice were challenged again with 200  $\mu$ g of OVA administered intranasally on days 66, 69, 72, and 75. On day 76, mice were killed, and data on airway hyperresponsiveness, BALF, and lung tissues were collected and assessed as above.



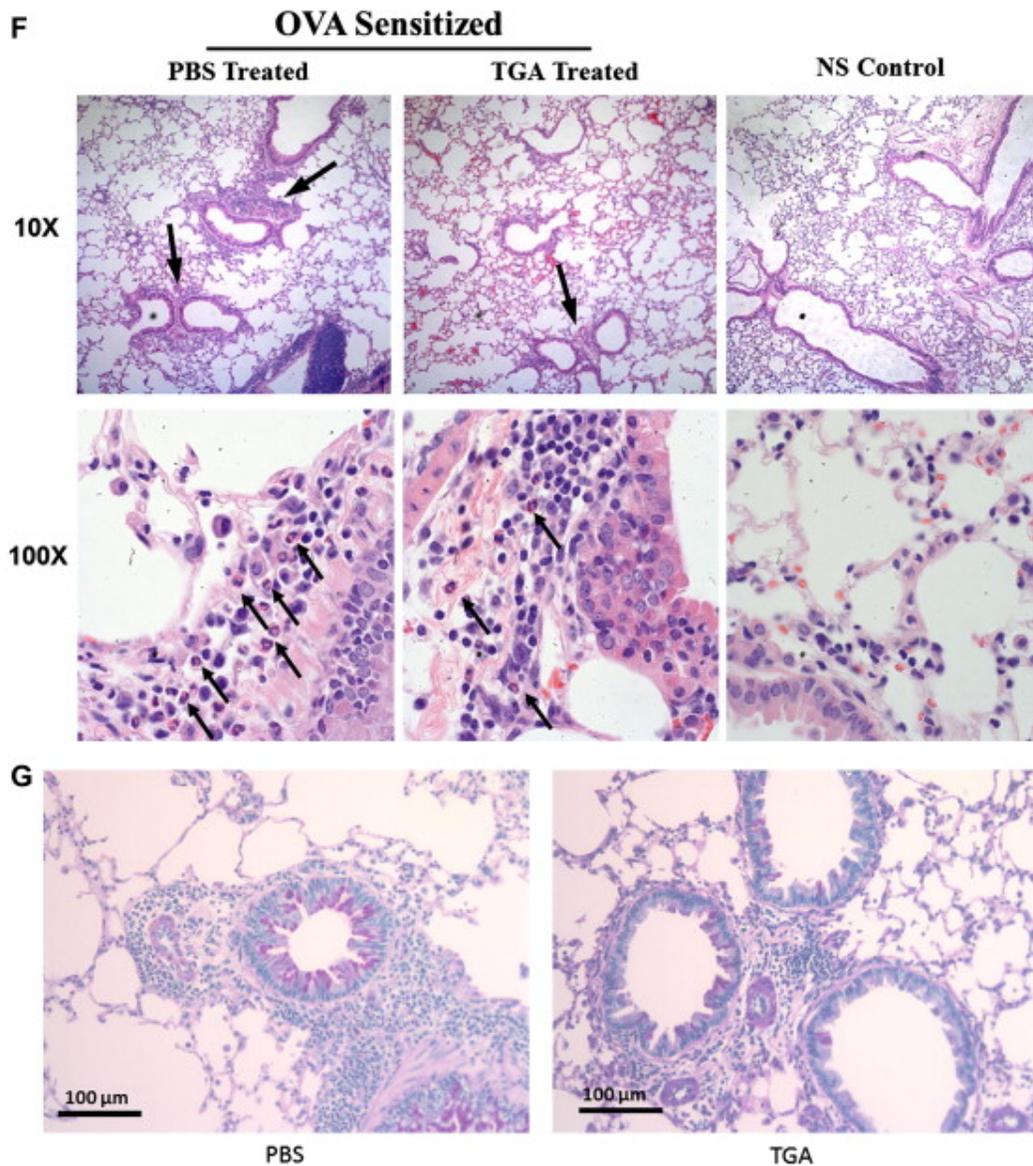


Fig 1. A novel FD, C<sub>70</sub>-tetraglycolate, inhibits airway inflammation and bronchoconstriction in a murine model of asthma. **A**, Fullerene TGA consists of 4 glycolic acids covalently bonded to the fullerene C<sub>70</sub> core, creating a water-soluble molecule. **B**, Schematic of the OVA challenge acute asthma model. Allergic lung inflammation was induced by means of intraperitoneal (*i.p.*) sensitization with OVA followed by 3 intranasal (*i.n.*) OVA challenges. Tetraglycolate was administered intranasally every 3 days starting on day -1. **C**, After OVA intranasal challenge, BALF and lung tissues were collected. The total number of cells (*left*) was counted with trypan blue and the total number of eosinophils (*right*) in BALF cells was counted by using Hema 3. Nonsensitized (*NS*) controls are shown for reference (n = 6-10 animals per group). \**P* < .05 or \*\**P* < .001, as determined by using the Student *t* test. **D** and **E**, TGA-treated mice survived to a higher dose of methacholine (Fig 1, *D*) and were less responsive to aerosolized methacholine compared with PBS-treated animals (Fig 1, *E*). **F** and **G**, In separate experiments using this model, lung tissues were fixed, sectioned, and stained with hematoxylin and eosin (Fig 1, *F*) and periodic acid-Schiff (Fig 1, *G*). Representative photographs show cellular infiltration into the airways of OVA-

sensitized mice treated with PBS or TGA. *Arrows* indicate areas of inflammation (magnification  $\times 10$ ) and eosinophils (magnification  $\times 100$ ).

#### Human lung MC preparation and mediator release

Human lung MCs were purified as described previously.<sup>17 and 18</sup> Tissue procurement and institutional review board approval were obtained from the Cooperative Human Tissue Network. Purified cells were incubated for 16 hours (a time point determined to be optimal for inhibition, data not shown) with or without 3 different EETs, washed, and incubated with optimal concentrations of anti-Fc $\epsilon$ RI stimuli (1  $\mu\text{g}/\text{mL}$ ). Mediator release (degranulation and cytokine production) was determined as previously described.<sup>19</sup>

#### B-cell IgE production and measurement

Naive B cells isolated from spleens of BALB/c mice<sup>20</sup> were treated with or without TGA (1  $\mu\text{g}/\text{mL}$ ) before challenge with IL-4 (20  $\text{ng}/\text{mL}$ ) and anti-CD40 antibodies (1  $\mu\text{g}/\text{mL}$ ) for 8 days, and IgE levels were measured by means of ELISA.

#### Quantitative analysis of eicosanoids by means of HPLC electrospray tandem mass spectrometry

Three hundred microliters of ethanol was added to 300  $\mu\text{L}$  of centrifuged BALF together with 10 ng of each deuterated standard. Ten microliters of this mixture was resolved in a 30 minute reversed-phase HPLC method. A Kinetex C18 column (100  $\times$  2.1 mm, 2.6  $\mu\text{m}$ ) was used to separate the eicosanoids at a flow rate of 200  $\mu\text{L}/\text{min}$  at 50°C. Before sample injection, the column was equilibrated with 100% Solvent A (acetonitrile/water/formic acid [40:60:0.02 vol/vol/vol]). One hundred percent Solvent A was used for the first minute of elution. Solvent B (acetonitrile/isopropanol [50:50 vol/vol]) was increased following a linear gradient to 25% Solvent B by 3 minutes, 45% Solvent B between 3 and 11 minutes, 60% Solvent B between 11 and 13 minutes, 75% Solvent B between 13 and 18 minutes, and 100% Solvent B between 18 and 20 minutes. The gradient was maintained at 100% Solvent B from 20 to 25 minutes and was then decreased to 0% by 26 minutes and held at 0% until 30 minutes. The eluting eicosanoids were analyzed by using an inline hybrid linear ion trap triple quadrupole tandem mass spectrometer (ABI 4000 Q-Trap; Applied Biosystems, Foster City, Calif) equipped with an electrospray ionization source operating in negative ion multiple-reaction monitoring mode. Eicosanoids were monitored by using relevant precursor  $\rightarrow$  product multiple reaction monitoring (MRM pairs). Additional mass spectrometric parameters used are as follows: curtain gas, 30 psi; charged aerosol detection, high; ion spray voltage,  $-3500$  V; temperature, 500°C; gas 1, 40 psi; gas 2, 60 psi; declustering potential, collision energy, and cell-exit potential vary per transition.

#### ELISA and multiplex cytokine assay

Mucin-5AC (Muc5AC) protein was measured by using ELISA, as previously described.<sup>21</sup> BALF was diluted, and 75  $\mu\text{L}$  was incubated with bicarbonate-carbonate buffer (75  $\mu\text{L}$ ) at 40°C in a 96-well plate (Nunc) for a total volume/well of 150  $\mu\text{L}$  until dry. Plates were washed 3 times with

PBS and blocked with 2% BSA (Sigma-Aldrich) for 1 hour at room temperature. Plates were again washed 3 times with PBS and then incubated with 50  $\mu$ L of murine Muc5AC mAb (1:100) and diluted with PBS containing 0.05% Tween 20 for 1 hour. Plates were washed 3 times with PBS, and 100  $\mu$ L of horseradish peroxidase–goat anti-mouse IgG conjugate (1:10,000) diluted in blocking solution was added for an hour and washed. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine peroxidase solution, stopped with 0.18 mol/L H<sub>2</sub>SO<sub>4</sub>, and measured at 450 nm. Cytokine levels in BALF were measured by using a multiplex cytokine assay (Bio-Rad Laboratories, Hercules, Calif). Total murine IgE levels were measured as described previously.<sup>22</sup> Levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as described previously.<sup>17</sup> Serum creatinine levels were measured according to the manufacturer's instructions (Arbor Assays, Ann Arbor, Mich).

## Results

### FD TGA inhibits airway eosinophilia and bronchoconstriction

A panel of FDs was developed with the objective of finding compounds capable of inhibiting MC responses.<sup>14</sup> The C<sub>70</sub>-based FD with 4 glycolic acids attached (TGA; Fig 1, A) was one of the most efficient inhibitors of MC degranulation and cytokine production *in vitro*, revealing its potential as an inhibitor of MC-driven diseases. Because asthma pathogenesis is strongly influenced by MC activation and mediator release, we examined the therapeutic potential of TGA in a murine model of asthma. To determine whether TGA could inhibit disease onset, we used an intranasal OVA challenge protocol, as described in Fig 1, B. TGA administered intranasally throughout OVA sensitization and challenge resulted in a reduction in both total inflammatory cell numbers and eosinophil infiltration of the airways (Fig 1, C). Eosinophil percentages decreased from approximately 50%  $\pm$  7% to 13%  $\pm$  4% ( $\pm$  SD) with TGA treatment. Additionally, TGA-treated animals were more resistant to methacholine-induced death ( Fig 1, D) because they were able to survive higher doses of methacholine than untreated mice. Furthermore, TGA-treated mice had significantly less methacholine-induced airway resistance compared with that seen in vehicle (PBS)–treated control mice ( Fig 1, E). In lung sections stained with hematoxylin and eosin, TGA-treated animals had only a few small areas of mild inflammation ( Fig 1, F, *top panel*). Within these areas, eosinophil infiltration is clearly reduced in TGA-treated animals ( Fig 1, F, *bottom, arrows* indicate eosinophils). In contrast, several large areas of inflammation are seen in PBS-treated mice ( Fig 1, F, *top*), and many eosinophils are found within this inflammation ( Fig 1, F, *bottom, arrows* indicate eosinophils). Periodic acid–Schiff staining is marginally reduced in TGA-treated animals ( Fig 1, G); however, no differences in Muc5 RNA or protein levels were seen (not shown). These data indicate that TGA is able to dampen multiple features of IgE-dependent asthma pathogenesis, leading us to further investigate its mechanism of action.

### TGA dampens established disease

Because asthma therapeutics are administered only after symptoms are established, we have developed a model to examine the effect of TGA on established disease. In this model (Fig 2, A) mice are sensitized and challenged with OVA before the initiation of TGA treatment. After disease initiation, mice are treated with TGA every 3 days for 20 days total. Mice then underwent a secondary OVA challenge and were subsequently sacrificed; BALF and lung tissues were assessed as before. Even after disease is established, TGA administration significantly reduced eosinophil infiltration of BALF and lung tissue ( Fig 2,B). Furthermore, IL-4 and IL-5 levels were significantly reduced in BALF, suggesting an overall reduction in disease pathogenesis ( Fig 2, C). Lung tissue sections show a dramatic reduction in cellular infiltration after TGA treatment ( Fig 2, D). Thus TGA has the ability to inhibit asthma pathogenesis, even after disease is established.

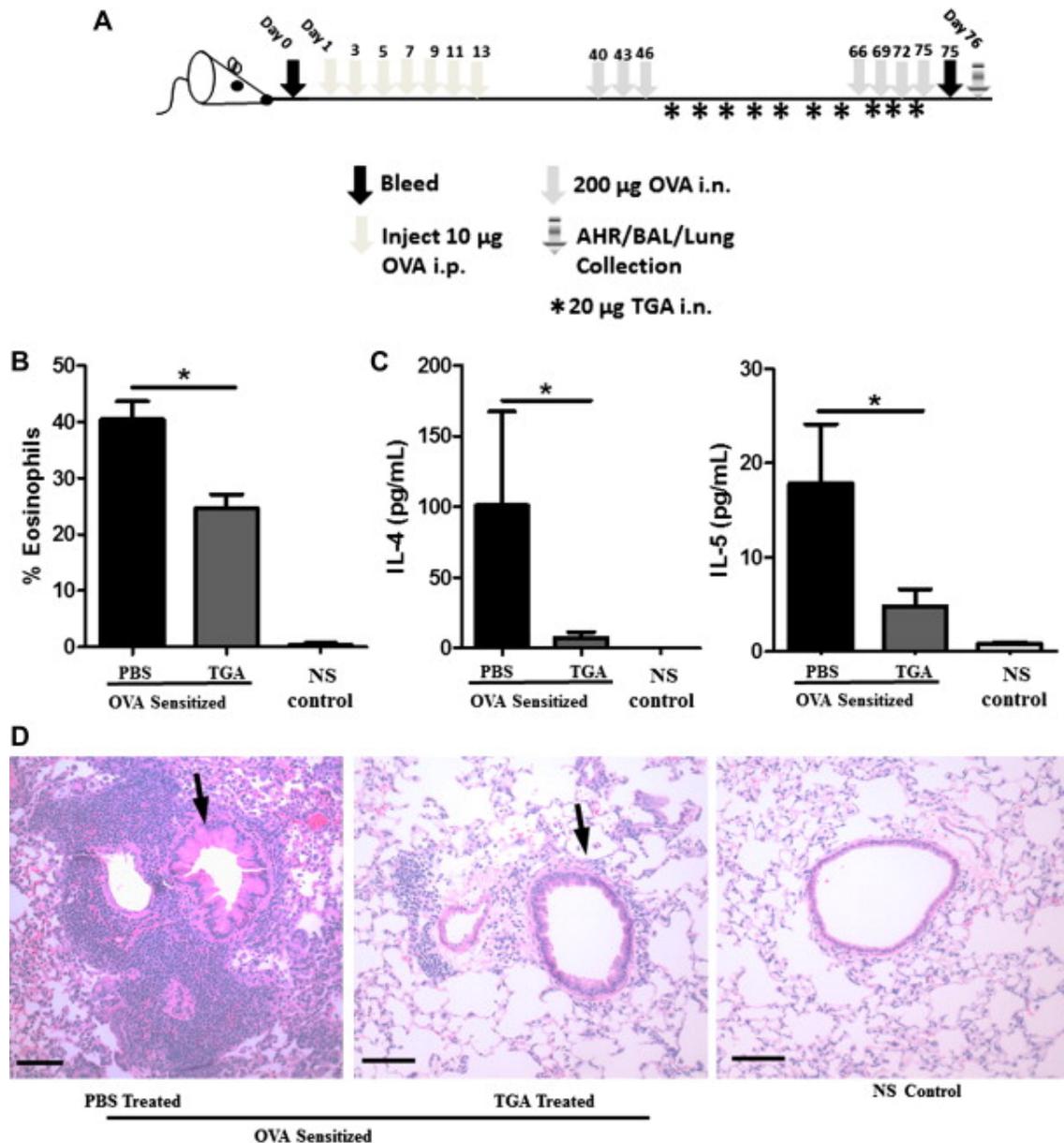


Fig 2. Tetraglycolate dampens cellular infiltration, eosinophilia, and inflammatory cytokine levels in a murine model of established asthma. **A**, An established asthma model was used, as described in the Methods section. After the first set of intranasal(*i.n.*) OVA challenges, mice began treatment with 20 µg of TGA every 3 days. *i.p.*, Intraperitoneal. **B**, Cells in BALF were stained with the Hema 3 stain set. Inflammatory cells in BALF were counted, and eosinophils are represented as a percentage of total inflammatory cells. **C**, Cytokine levels in BALF were measured with a multiplex cytokine assay. **D**, Lung tissue was sectioned and stained with hematoxylin and eosin. *Arrows* indicate airways. Representative photographs are shown (n = 6-12 mice per group). \**P* < .05, as determined by using the Student *t* test when variances were normal or the Mann-Whitney test in the case of unequal variances. *NS*, Nonsensitized.

TGA inhibits BALF cytokine and serum IgE levels and increases anti-inflammatory eicosanoid levels

We next assessed the ability of TGA to inhibit several mediators of asthma pathogenesis in the disease model described in Fig 1, *B*. In BALF levels of the asthma-promoting cytokines IL-4 and IL-5 were significantly reduced in TGA-treated mice to levels approaching those of nonsensitized animals ( Fig 3, *A*). Additionally, serum IgE levels were significantly reduced in TGA-treated animals ( Fig 3, *B*). Epoxyeicosatrienoic acids are anti-inflammatory derivatives of arachidonic acid.<sup>23</sup> We used mass spectrometry to quantify EET levels in the BALF of control and TGA-treated mice. Surprisingly, we found 11,12-EET levels to be significantly upregulated in the BALF of TGA-treated mice compared with those seen in PBS-treated control animals ( Fig 3, *C*). This finding is mirrored in human lung MCs, in which we find a significant increase in *CYP1B1* RNA, a cytochrome P450 capable of producing the hydroxyeicosatetraenoic acids and EETs,<sup>18</sup> after TGA treatment ( Fig 3, *D*). Further experiments with purified human lung MCs demonstrated that both 11,12-EET and 14,15-EET significantly stabilized MCs challenged through FcεRI, inhibiting both degranulation and cytokine production compared with non-EET-treated cells ( Fig 3, *E*). In contrast, 8,9-EET did not demonstrate any inhibition of MC mediator release (not shown). Although TGA-induced increases in 11,12-EET levels paralleled inhibition of *in vivo* asthma induction and stabilized human MC mediator release alone, other arachidonic acid derivatives were not upregulated *in vivo* (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Lastly, when B cells were incubated with TGA before challenge with IgE-producing stimuli, there was a significant inhibition of IgE levels in TGA-treated cells compared with those seen in non-TGA-treated cells ( Fig 3, *F, left*) that was not due to reduced B-cell viability ( Fig 3, *F, right*). Similar inhibition of IgE production was observed with EETs (data not shown). Together, these data indicate that TGA suppresses asthma by directly targeting lung MC activation or through reduced B-cell IgE production.

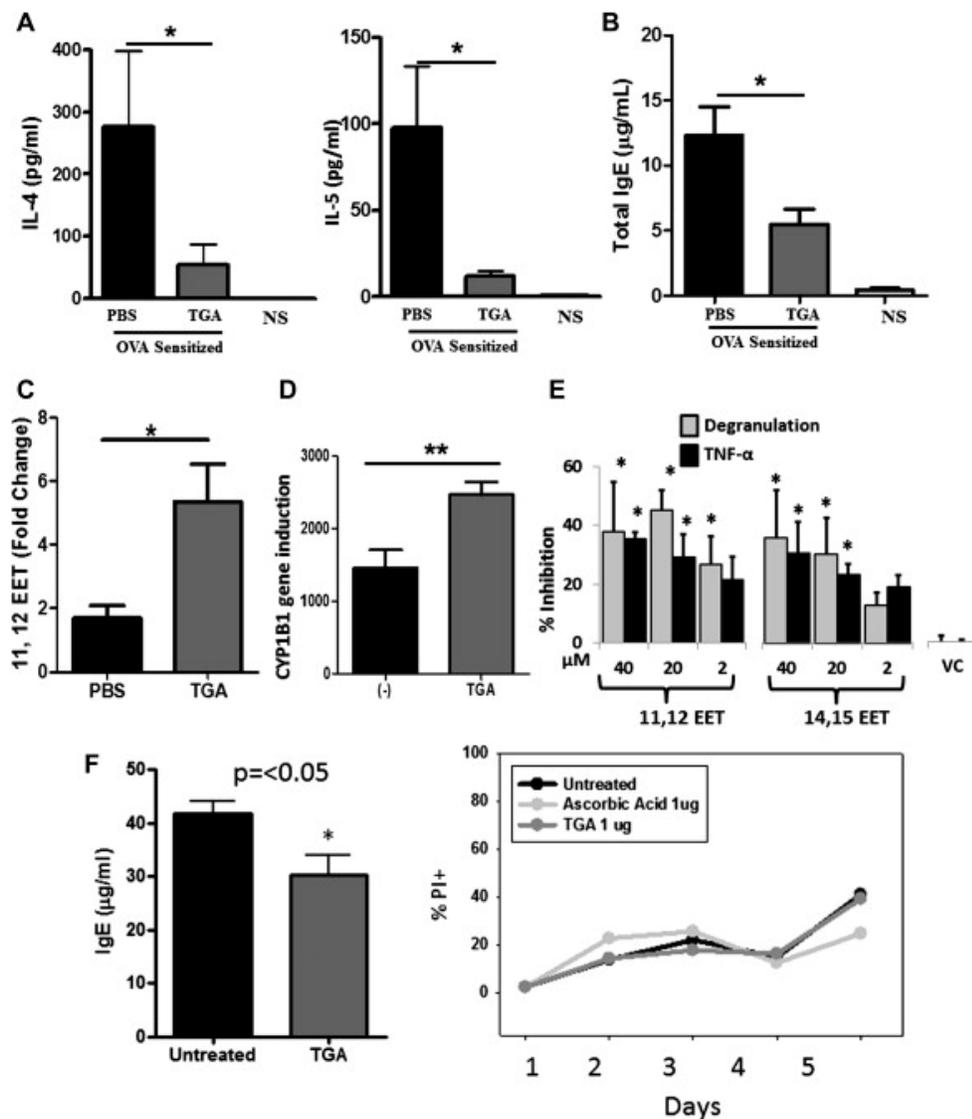


Fig 3. Tetraglycolate significantly reduces inflammatory cytokine, eicosanoid, and serum IgE levels. Asthma induction and fullerene treatment is described in Fig 1, *B*. **A**, Inflammatory cytokine levels were measured in BALF by using a multiplex cytokine assay. **B**, Serum was collected after OVA intranasal challenge and total IgE levels were measured by using ELISA. **C**, Levels of 11,12-EET were measured in BALF by means of mass spectrometry. These values were standardized by calculating the fold change relative to nonsensitized (*NS*) animals to account for variation between experiments ( $n = 6-9$  mice per group). **D**, Human lung MCs were pretreated with  $10 \mu\text{g/mL}$  TGA overnight and activated 16 hours later with anti-FcεRI antibodies ( $1 \mu\text{g/mL}$ ). Gene microarray was performed,<sup>14</sup> and relative gene expression of *CYP1B1* is shown. Each condition was performed in triplicate.  $*P < .05$ , as determined by using the Student *t* test when variances were normal or the Mann-Whitney test in the case of unequal variances. **E**, Epoxygenases reduce degranulation from human lung MCs after anti-FcεRI activation. Cells were cultured with fixed concentrations of the indicated EET or vehicle control (VC; ethanol,  $40 \mu\text{mol/L}$ ), washed, and stimulated for 20 minutes with optimal concentrations of anti-FcεRI antibodies (3B4;  $1 \text{mg/mL}$ ). Cells were centrifuged, and β-hexosaminidase release and cytokine production were determined. The data shown are the average of 2 separate experiments performed in triplicate, with means

± SEs. The *asterisk* indicates statistical significance compared with the vehicle control. **F**, TGA inhibits IgE production by cultured B cells. B cells were incubated with or without TGA before being activated with IL-4/anti-CD40 for 8 days. Cells were centrifuged, and IgE levels in the supernatants were measured by using ELISA. B-cell viability was assessed by using propidium iodide staining of dead cells. The percentage of propidium iodide-positive cells was assessed by using a flow cytometer for the first 5 days of incubation, after which viability was dramatically reduced in all groups. Experiments were performed at least 3 times in duplicate, and representative data are shown. There are no toxic effects on these cells compared with B cells that are left untreated or those treated with the nontoxic antioxidant ascorbic acid.

To confirm that the increase in EET levels was relevant to the reduced asthmatic response that was seen, we examined the effect of blocking the synthesis of EETs by using the inhibitor PPOH and the model described in Fig 1, *B*. The PPOH is a potent and selective inhibitor of arachidonic acid epoxidation<sup>16</sup> and is more selective for EET inhibition compared with other terminal acetylenic compounds. Additionally, we used an antagonist of EET activity, 14,15-EE-5(Z)E. As seen in Fig 4, there was a significant increase in BALF eosinophil numbers ( Fig 4, *A*) and airway constriction ( Fig 4, *B*) in TGA-treated mice given the EET inhibitor PPOH over those given TGA alone. Similar results were obtained with the EET receptor antagonist 14,15-EE-5(Z)-E (data not shown). Lung sections show increased cellular infiltration in PPOH-treated mice compared with that seen in those given TGA alone ( Fig 4, *C*). These data indicate that TGA-induced production of anti-inflammatory 11,12-EET is largely responsible for inhibition of the asthma phenotype seen in TGA-treated mice.

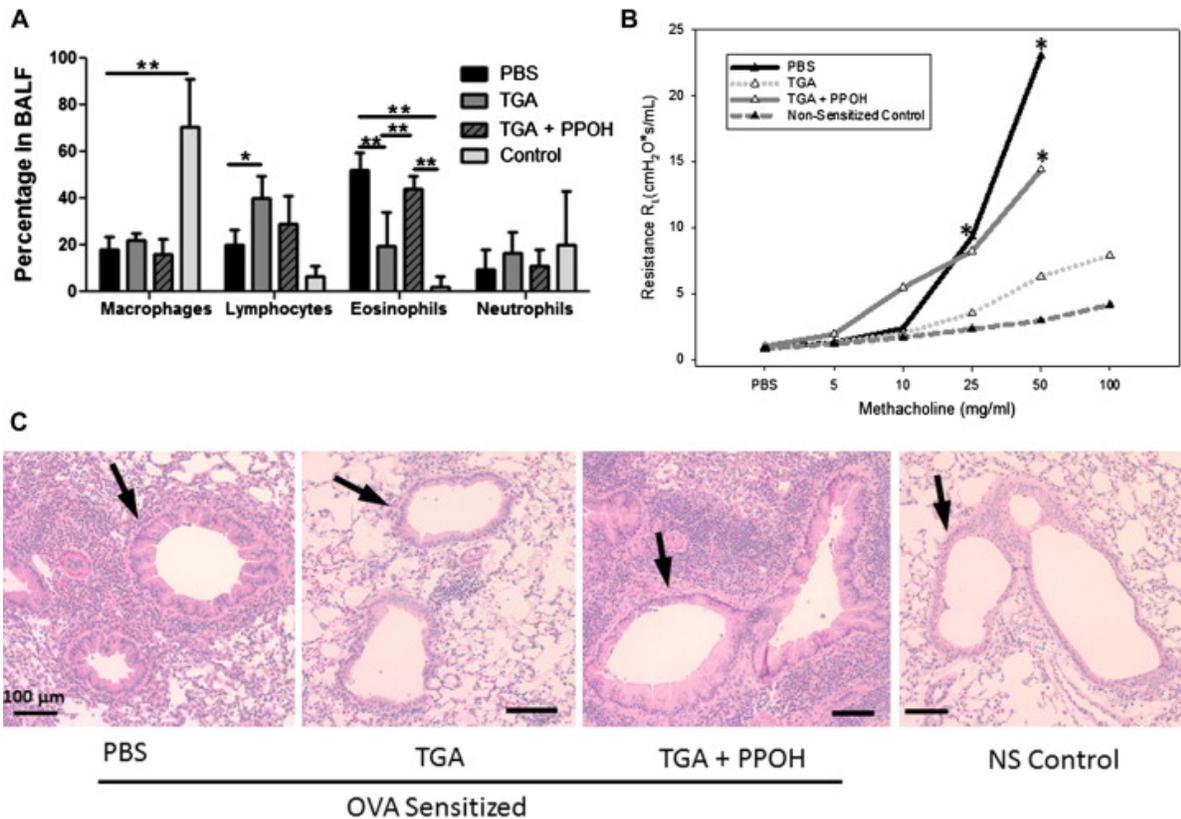


Fig 4. Inhibiting the induction of 11,12-EET by TGA restores the asthma phenotype. Effects of the inhibitor of EET, PPOH, on airway inflammation and bronchoconstriction were assessed. **A**, Cells in BALF were stained with the Hema 3 stain set. Inflammatory cells in BALF were counted, and each cell type is shown as a percentage of total inflammatory cells. **B**, Airway resistance (*RL*) was measured in response to increasing doses of methacholine. **C**, Representative lung sections are stained with hematoxylin and eosin. *Arrows* indicate airways. *Bar* = 100  $\mu$ m. N = 6 mice per group. \**P* < .05.

FDs do not accumulate in major organs or alter liver or kidney function

The toxicity of FDs is still widely debated and varies based on the specific moieties added to the fullerene core.<sup>24</sup> Therefore the *in vivo* toxicity of TGA was assessed through several methods. A TGA molecule containing gadolinium within the fullerene sphere<sup>25</sup> was administered intranasally to mice every 3 days for 30 days to assess accumulation. Twenty-four hours after the last dosage, gadolinium levels were measured in the serum and lung, spleen, liver, kidney, and brain tissue. Gadolinium was detected only in the lung tissue, where less than 10% of that injected remained, indicating the TGA is cleared from the lung. No gadolinium was detected in any other tissue examined (data not shown). These enzymes are present at low levels in healthy subjects, and large increases would suggest liver toxicity.<sup>26</sup> No significant differences in serum levels of AST/ALT were observed between treated, untreated, and nonsensitized control animals (see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). No significant differences in serum creatinine levels<sup>27</sup> were seen between treated, untreated, and nonsensitized animals (see Fig E1, B). These initial studies suggest that TGA does not accumulate within the body and is not acutely toxic to the liver or kidney.

## Discussion

There is a strong need for novel therapeutics to treat asthma; indeed, up to 55% of patients receiving treatment for asthma have uncontrolled symptoms.<sup>28</sup> In this study we have shown that TGA is able to suppress both disease onset and reverse established disease. The latter is especially important because human asthma treatment always involves established disease. MCs play an important role in human disease, and previous publications have shown that MC-deficient mice sensitized with OVA lacking adjuvant and challenged with OVA alone, as performed in this study, have diminished airway inflammation and bronchoconstriction.<sup>7 and 29</sup> Additionally, we developed a model of established asthma to further emulate the pathogenesis of human disease because therapeutics are administered after the initiation of symptoms. Although murine and human anatomy differ, these models were chosen to best represent human pathogenesis and thus suggest that TGA (or similar FD) could have therapeutic efficacy in the treatment of human disease.

Specifically, it is demonstrated that mice treated with TGA throughout OVA challenge have significantly less airway inflammation and bronchoconstriction compared with that seen in untreated animals. In fact, total inflammation and bronchoconstriction in TGA-treated animals is not only significantly reduced but is similar to that seen in nonsensitized control subjects. Thus

symptoms of disease were largely reversed in these animals. Note that these studies used a model previously shown to use MCs.<sup>29</sup> In studies not shown, a different asthma model that is IgE but not MC dependent<sup>30</sup> was also found to be inhibited by TGA. However, when a nonallergic airway inflammation model that develops in both MC- and IgE-deficient mice<sup>20</sup> was used, TGA had no effect on eosinophilia or cytokine production (data not shown). In the established disease model, as when mice were treated throughout disease development, we found that TGA dampens eosinophilia and cytokine levels significantly in BALF. Lung sections show massive cellular infiltration in untreated animals, whereas those receiving TGA have minimal cellular infiltration surrounding the airways. Airway hyperresponsiveness trended toward reduction in TGA-treated animals, but because of high variability between mice, significant differences were not observed.

Importantly, we have demonstrated that TGA activity has novel mechanisms of action. Although previously published *in vitro* studies suggested that MC inhibition might be the predominant mechanism of FD inhibition, these *in vivo* studies suggest multipotent effects of these unique compounds. TGA treatment reduced the levels of BALF T<sub>H</sub>2 proinflammatory cytokines and reduced lung inflammation. Although IL-4 stimulates IgE production by B cells, IL-5 both recruits and activates eosinophils at the site of inflammation. Tetraglycolate treatment significantly reduces both IL-4 and IL-5 levels in BALF. Additionally, serum IgE levels were significantly reduced after TGA treatment and TGA suppressed IgE production from B cells. In contrast, a nonallergic lung inflammation model was not influenced by TGA treatment.

Several eicosanoids derived from the cytochrome P450 pathway are relatively stable, and thus we measured levels of these molecules in BALF samples by using mass spectrometry. The EETs are consistently associated with relaxation of the bronchi and other anti-inflammatory actions *in vivo*.<sup>23, 31 and 32</sup> Intriguingly, 11,12-EET was consistently upregulated in BALF from TGA-treated mice. Further *in vivo* studies demonstrated that the EETs play a major role in dampening the asthma phenotype. Specifically, selective inhibitors of EET production, as well as inhibitors of EET activity, reversed the TGA-induced modulation of the OVA-induced asthma model.

The *in vivo* results with EETs led us to examine possible mechanisms *in vitro*. We show, for the first time, that EETs stabilize human lung MCs through the inhibition of FcεRI-induced mediator release. The actual concentration of 11,12-EET in the BALF of TGA-treated animals averaged 40 ng/mL ( Fig 3, C), which was determined after diluting approximately 4 to 5 times when PBS was used to flush out BALF. On the basis of this calculation, the concentrations of EETs observed in BALF (approximately 160-200 ng/mL) is approximately the same as that observed for maximal MC inhibition (20 μmol/L). The 11,12-EET also showed a small but significant decrease in IgE production from IL-4/CD40-stimulated B cells (data not shown). These results suggest a possible mechanism as to how EETs could block the asthmatic response. We are currently examining the mechanisms of how EETs could block both MC and B-cell responses focusing on common signaling molecules in these 2 cell types.

The cellular source of EETs is not certain at present. However, we found that TGA upregulates expression of human lung MC *CYP11B*, a gene involved in the production of EETs, suggesting MCs could be the source of 11,12-EET. Other studies have demonstrated that EETs can be produced by lung epithelial and endothelial cells and can relax histamine-precontracted guinea pig and human bronchi.<sup>31</sup> Furthermore, they can inhibit the upregulation of vascular cell adhesion molecule 1, E-selectin, and intercellular adhesion molecule 1, thus potentially limiting cellular infiltration of the lung.<sup>32</sup> Consequently, 11,12-EET upregulation is playing a significant role in dampening airway inflammation and bronchoconstriction in these models. Together, these results suggest that TGA/EETs suppress the asthmatic response through a variety of mechanisms, targeting both B cells and MCs. In addition, our results implicate EETs as a heretofore undiscovered mechanism for controlling asthma and suggest that strategies that induce the production of EETs might be a viable therapeutic strategy for treating asthmatic patients. We are currently identifying the cell types that produce EETs in response to TGA and are also exploring lung BALF from asthmatic patients to compare their EET levels with those of nonasthmatic patients. The antagonist data indicate that EETs induced by TGA are directly involved in the suppression seen in the asthmatic response. However, we recognize the possibility that TGA could have indirect effects by shunting arachidonic acid away from production of proinflammatory eicosanoids, such as the cysteinyl leukotrienes. Further studies will be required to determine whether the TGA also alters the relative levels of proinflammatory eicosanoids as well.

Levels of the liver enzymes AST and ALT, which are indicative of liver toxicity when present at high levels in the serum, were not different between treated and untreated animals. TGA-treated animals had creatinine levels similar to those seen in untreated animals, and all were within the normal range. Thus liver and kidney function appear to be unaffected by short-term local administration of TGA. In addition, a TGA-like compound containing gadolinium within the fullerene core was developed so that its presence in tissue could be detected by using inductively coupled plasma neutron bombardment. After 1 month of intranasal inhalations, followed by no challenge for up to a week, only lung tissue contained detectable amounts of gadolinium. Thus TGA does not appear to build up in the body tissues, limiting the possibility of toxicity. Further testing will be necessary to determine the safety of these compounds in human subjects.

In conclusion, these studies are the first to suggest the efficacy of FDs for the treatment of asthma through a previously undescribed mechanism involving the upregulation of anti-inflammatory EETs. Evidence presented here suggests that specific FD compounds have the potential to become novel therapeutics for the treatment of asthma and pave the way to new research efforts focusing on the role of EETs in asthma.

#### Key messages

- The fullerene compound TGA might represent a new therapeutic option for the treatment of asthma.

- Inhibition of asthma pathogenesis through the upregulation of 11,12-EET represents a novel inhibitory mechanism. Thus TGA might provide therapeutic benefit to patients with refractory asthma, and increased EET levels might be an efficacy marker for asthma therapies.

- EETs stabilize MCs in response to FcεRI challenge.

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#### Appendix

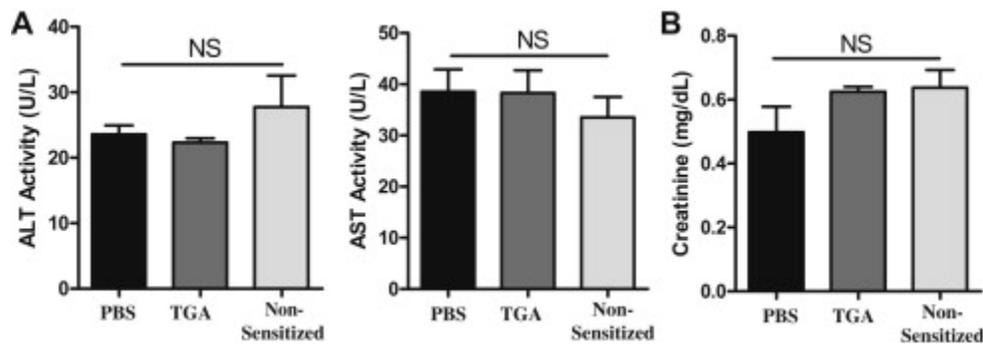


Fig E1. Mice have no evident toxicity after long-term treatment with TGA. **A**, Levels of the liver enzymes AST and ALT were measured in sera of TGA-treated animals by using ELISA. **B**, Creatinine levels were measured in sera of TGA-treated animals by using ELISA. N = 6 animals per group. NS, Not significant.

Table E1. Effect of TGA on other eicosanoids *in vivo*

Eicosanoid	PBS	TGA
8-HETE	4.0 ± 2.9	6.14 ± 4.13
12-HETE	2.58 ± 1.85	2.19 ± 1.62
15-HETE	4.37 ± 3.15	4.76 ± 3.23
PGE <sub>2</sub>	1.78 ± 0.99	1.55 ± 0.32
PGF <sub>2α</sub>	1.34 ± 0.18	1.35 ± 0.22
6-Keto-PGF <sub>1α</sub>	1.22 ± 0.23	1.43 ± 0.41
Arachidonic acid	1.66 ± 0.66	1.77 ± 1.04

Values are presented as the mean fold change (± SD) relative to nonsensitized animals.

Eicosanoid levels were measured in BALF by using mass spectrometry. TGA treatment does not significantly alter BALF levels of the noninhibitory eicosanoids listed. Fold change relative to nonsensitized animals was calculated to account for variation between experiments (n = 6 mice per group).

*HETE*, Hydroxyeicosatetraenoic acid; *PG*, prostaglandin.

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