TXA$_2$ attenuates allergic lung inflammation through regulation of Th2, Th9, and Treg differentiation

Hong Li, …, Thomas M. Coffman, Darryl C. Zeldin

*J Clin Invest.* 2024;134(9):e165689. [https://doi.org/10.1172/JCI165689](https://doi.org/10.1172/JCI165689).

**Graphical abstract**
TXA₂ attenuates allergic lung inflammation through regulation of Th2, Th9, and Treg differentiation

Hong Li, J. Alyce Bradbury, Matthew L. Edin, Artiom Gruzdev, Huiling Li, Joan P. Graves, Laura M. DeGraff, Fred B. Lih, Chiguang Feng, Erin R. Wolf, Carl D. Bortner, Stephanie J. London, Matthew A. Sparks, Thomas M. Coffman, and Darryl C. Zeldin

Division of Intramural Research, National Institute of Environmental Health Sciences/NIH, Research Triangle Park, North Carolina, USA. Department of Nephrology, Duke University Medical Center, Durham, North Carolina, USA. Program in Cardiovascular and Metabolic Disorders, Duke-NUS Medical School, Singapore.

In lung, thromboxane A₂ (TXA₂) activates the TP receptor to induce proinflammatory and bronchoconstrictor effects. Thus, TP receptor antagonists and TXA₂ synthase inhibitors have been tested as potential asthma therapeutics in humans. Th9 cells play key roles in asthma and regulate the lung immune response to allergens. Herein, we found that TXA₂ reduces Th9 cell differentiation during allergic lung inflammation. Th9 cells were decreased approximately 2-fold and airway hyperresponsiveness was attenuated in lungs of allergic mice treated with TXA₂. Naive CD4⁺ T cell differentiation to Th9 cells and IL-9 production were inhibited dose-dependently by TXA₂ in vitro. TP receptor-deficient mice had an approximately 2-fold increase in numbers of Th9 cells in lungs in vivo after OVA exposure compared with wild-type mice. Naive CD4⁺ T cells from TP-deficient mice exhibited increased Th9 cell differentiation and IL-9 production in vitro compared with CD4⁺ T cells from wild-type mice. TXA₂ also suppressed Th2 and enhanced Treg differentiation both in vitro and in vivo. Thus, in contrast to its acute, proinflammatory effects, TXA₂ also has longer-lasting immunosuppressive effects that attenuate the Th9 differentiation that drives asthma progression. These findings may explain the paradoxical failure of anti-thromboxane therapies in the treatment of asthma.

Introduction

Development of allergic lung inflammation is a complex process involving both immune and inflammatory events. In the immune phase, allergens are taken up and processed by antigen-presenting cells such as dendritic cells (DCs), which then migrate toward regional lymph nodes. During migration, DCs become activated and undergo maturation (1, 2). Antigen-loaded mature DCs encounter naive T cells in the lymph nodes and make a physical contact referred to as an immunological synapse, through which antigen presentation and associated signaling occur (3). The strength, duration, and efficiency of this interaction determine the extent of T cell activation and differentiation (3). The interaction of T cells with DCs is known to involve 3 distinct signals (4). Signal 1 involves the interaction between major histocompatibility complex (MHC) molecules containing peptide fragments on the DC and the T cell receptor on the T cell. Signal 2 involves the interaction of costimulatory molecules (e.g., CD80/86) on the surface of the DC with ligands (e.g., CD28) on the T cell surface. Signal 3 involves the secretion of cytokines by the DC that drive T cell differentiation to unique T cell subsets.

A T helper subset called Th9 cells can differentiate either from naive T cells or from Th2 cells in the presence of both TGF-β and IL-4. TGF-β and IL-4 act through the PU.1 and interferon regulatory factor 4 (IRF4) transcription factors to induce Th9 cells to produce IL-9 and IL-10 (5, 6). IL-9 plays a pivotal role in the pathogenesis of asthma by promoting eosinophil activation and enhancing IgG/IgE production by B cells. Like Th2 cells, Th9 cells also produce IL-5 and IL-13, which induce airway hyperresponsiveness (AHR) (7–9). Interestingly, anti-IL-9 blocking antibodies inhibit allergic airway inflammation and hyperresponsiveness in mouse models and have been examined in clinical trials for treatment of humans with asthma (10, 11).

We previously demonstrated that several cyclooxygenase-2-derived (COX-2-derived) prostaglandins (PGs), including PGD₂, PGE₂, PGF₂α, and PGI₄, regulate Th17 and Th9 cell differentiation in the allergic lung (12, 13). Thromboxane A₂ (TXA₂) is produced by the sequential actions of cyclooxygenase-1 (COX-1) or COX-2 and thromboxane synthase (TXAS; encoded by the TBXAS1 gene) (14). TXA₂ is chemically unstable, with a biological half-life of approximately 30 seconds. TXA₂ was initially identified in platelets and has potent prothrombotic and vasoconstrictive properties; as such it has been mainly studied in the cardiovascular system (15, 16). While TXAS is most abundant in platelets, it is also highly expressed in other bone marrow-derived immune cells including mast cells, granulocytes, monocytes, and macrophages (17). TXAS expression in both monocytes and macrophages results in TXA₂ production upon cell activation (18, 19). TXA₂ is elevated in bronchoalveolar lavage fluid (BALF) from allergic lungs (20); however, the role of TXA₂ in Th cell differentiation and function during allergic lung inflammation remains unknown.

Like other arachidonic acid-derived signaling molecules, TXA₂ exerts its actions through a specific G protein–coupled receptor termed the thromboxane receptor (TP receptor, encoded by the
TBXA2R gene) (21). TXA₂ and its receptor are present in many cell types, including cortical epithelial cells and DCs in the thymus (22, 23). Among immune cells, the TP receptor is predominantly expressed in immature CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes and naive CD4⁺ T cells (24, 25). In the lung, the TP receptor is expressed in bronchial airway smooth muscle cells and other cell types (26). TXA₂ induces expression of adhesion molecules in vascular endothelial cells and of ecto-5'-nucleotidase (E5NT)-1 by bronchial smooth muscle and stimulates monocyte formation of TNF-α, IL-1β, IL-2, IL-5, and IFN-γ (27, 28). TXA₂ induces bronchoconstriction, mucin secretion, plasma extravasation, vascular smooth muscle constriction, and vascular smooth muscle proliferation and exacerbates AHR (15, 16, 29). As a result, TP receptor antagonists and TXAS inhibitors have been developed as potential asthma therapeutics in humans (30, 31).

In this study, we used TP receptor agonists and antagonists and TP receptor-deficient (TP⁻/⁻) mice to investigate the role of TXA₂ in Th9 cell differentiation and function in vitro and during allergic lung inflammation in vivo. We found that DCs express TXAS and produce TXA₂ that signals through the TP receptor on differentiating T cells. To further elucidate the signaling cascade through which TXA₂ regulates Th9 cell differentiation, we examined cAMP and MAPK signaling pathways and interrogated the Il9 promoter using luciferase assays and transcription factor–specific ChIP analyses. Our results show that TXA₂/TP receptor signaling suppresses Th9 cell differentiation through recruitment of the NFE2 and PBX1 transcriptional repressors to the Il9 promoter. Thus, while TXA₂ is well known for inducing inflammation and suppressing Th9 cell differentiation, our studies reveal what we believe to be a novel immunosuppressive role of this eicosanoid. These results help explain the failure of anti-thromboxane therapies and suggest that targeting the TXA₂/TP receptor signaling pathway may lead to the development of novel asthma treatments.

Results

TXA₂ attenuates Th9 cell responses to allergen exposure in vivo. To examine the role of TXA₂ in regulating lung Th9 cell responses during allergic lung inflammation, mice were sensitized to OVA with aluminum hydroxide (alum) adjuvant, and then exposed to OVA via the airway for 4 days in the presence of vehicle or carboxylic TXA₂ (cTXA₂), a biologically stable TXA₂ analog. Fluids and tissues were collected for analysis 48 hours after the final OVA exposure (Figure 1A). OVA sensitization and exposure induced pronounced BALF eosinophilia, which was significantly attenuated in cTXA₂-treated mice compared with vehicle-treated controls (Figure 1B). Histological sections scored by a blinded pathologist revealed decreased inflammation in lungs from cTXA₂-treated mice compared with vehicle-treated controls (Figure 1, C and D). IL-9⁺CD4⁺ T cells (Th9 cells) from lung, BALF, lymph nodes, blood, and spleen were quantified by FACS. Compared with mice treated with vehicle, mice implanted with cTXA₂-containing osmotic minipumps exhibited a significant decrease in the percentage of Th9 cells in the lung following OVA exposure (Figure 1E and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI165689DS1). Similar results were obtained in BALF, blood, and lymph nodes, but not spleen (Figure 1E and Supplemental Figures 1 and 2). Immunofluorescence microscopy provided independent confirmation of the FACS results. Lung tissue sections from allergic mice treated with vehicle or cTXA₂ were stained with immunofluorescently labeled antibodies against CD4 (green), IL-9 (red), and IL-10 (blue). There were significantly fewer Th9 cells (CD4⁺IL-9⁺IL-10⁻; white overlay) in lungs of cTXA₂-treated mice compared with vehicle-treated controls (Figure 1, F and G). While cTXA₂ treatment reduced the overall number of Th9 cells in OVA-exposed mice, it did not reduce the mean fluorescence intensity of IL-9⁺ T cells (Supplemental Figure 3A). These data suggest that cTXA₂ regulates Th9 cell differentiation, not IL-9 expression in differentiated Th9 cells. The density of mast cells in allergic lungs was not significantly different between vehicle- and cTXA₂-treated mice (Supplemental Figure 4, A and B). Similarly, the number of IL-9⁺IL-10⁺CD4⁺ T cells was not significantly different between vehicle- and cTXA₂-treated mice (Supplemental Figure 5A). Together, these results indicate that cTXA₂ significantly reduces Th9 cell numbers during OVA-induced allergic lung inflammation in vivo.

We evaluated airway responsiveness to inhaled methacholine via flexiVent in non-allergic control mice and allergic OVA-sensitized/exposed mice treated with either vehicle, cTXA₂, the TP receptor antagonist iodophenyl sulfonyl amino pinane TXA₂ (ISAP), or cTXA₂ plus ISAP by minipump (Figure 1A). Vehicle-treated OVA-sensitized/exposed mice displayed increased airway responsiveness to methacholine as determined by measurement of resistance (R) and other flexiVent parameters (Figure 1H and Supplemental Figure 6). Similarly, OVA/cTXA₂+ISAP–treated and OVA/ISAP–treated mice also displayed AHR significantly above that of non-allergic mice. Importantly, cTXA₂ reduced airway responsiveness to levels that were not different from those in non-OVA-sensitized/exposed controls. The reduction in AHR by cTXA₂ was not significantly less than that in OVA/vehicle–treated mice (P = 0.06) but was significantly less than that in cTXA₂– and ISAP-treated mice (P < 0.05). Together, these results suggest that cTXA₂ reduces AHR during OVA-induced allergic lung inflammation in vivo through activation of the TP receptor.

Bacterial lipopolysaccharide (LPS) induces maturation of DCs and promotes T cell differentiation (32). While OVA/albumin sensitization primarily induces lung eosinophilia, OVA/LPS sensitization results in lung inflammation characterized by increased numbers of macrophages, neutrophils, and lymphocytes (33). Compared with vehicle, cTXA₂ treatment significantly reduced BALF neutrophil numbers but did not reduce overall lung inflammation following sensitization with OVA/LPS and airway exposure to OVA (Supplemental Figure 7, A and B). However, significant reductions in Th9 cells were observed in lungs of cTXA₂-treated mice relative to vehicle-treated controls (Supplemental Figure 7C). cTXA₂ treatment also significantly reduced Th9 cells in BALF, blood, and lymph nodes, but not spleen (Supplemental Figure 7C). Thus, cTXA₂ suppressed Th9 cells in both the OVA/albumin and the OVA/LPS models of allergic lung inflammation in vivo.

TP-deficient mice have increased lung Th9 cells after allergen exposure. T cells express the TP receptor, which is functionally coupled to distinct heterotrimeric G proteins (including Galphaq and G12/13) and participates in the activation of multiple signaling cascades (34). To confirm that endogenously produced TXA₂ regulates Th9 cells, we compared Th9 cell numbers between TP⁺/⁺ and TP⁻/⁻ mice during OVA-induced allergic lung inflammation. After OVA/albumin sensitit-
FACS analysis indicated that TP−/− mice had significantly more Th9 cells compared with TP+/+ mice in lung, BALF, lymph nodes, and blood, but not in spleen (Figure 2D and Supplemental Figures 8 and 9). Consistent with these results, immunofluorescence microscopy revealed that lungs from TP−/− mice had significantly more Th9 cells than those from TP+/+ mice (Figure 2D). Additionally, immunohistochemistry and histological analysis showed a reduction in the number of Th9 cells in TP−/− lungs compared to TP+/+ lungs (Figure 2D). These findings suggest that the absence of TP promotes Th9 cell differentiation and airway inflammation in response to allergen exposure.

**Figure 1. cTXA2 attenuates Th9 cell responses to allergen exposure in vivo.**

(A) Mice were sensitized with OVA/alum and exposed to OVA in the presence of vehicle or cTXA2, and/or ISAP (delivered by osmotic minipumps) as indicated. (B) Total cell number and cell differentials in BALF were analyzed 48 hours after the last airway OVA exposure. n = 6 per group, *P < 0.05. (C) H&E-stained lung sections from vehicle- and cTXA2-treated mice after OVA sensitization/exposure. Scale bars: 100 μm. Images are shown at original magnification ×40. (D) Scoring of lung sections revealed decreased inflammation in lungs from cTXA2-treated mice compared with vehicle-treated controls. n = 6 per group, *P < 0.05. Note that the lack of an error bar in the vehicle group is because all vehicle-treated lungs received a score of 3, i.e., 30%–50% of the lung involved in inflammation. (E) IL-9−/−CD4+ T cells, as a percentage of CD4+ cells in the lung and BALF after OVA-induced allergic lung inflammation. n = 12–13 mice per group, *P < 0.05. (F and G) Th9 cells in mouse lung tissue sections were visualized by immunofluorescence staining using anti–IL-9, anti–IL-10, and anti-CD4 antibodies. Quantitation of the number of IL-9−/−IL-10−/−CD4+ T cells per high-power field (HPF). (F) and representative ×40 images (G). n = 7 lungs per group, 5 HPFs per lung, *P < 0.05. Images are shown at original magnification ×40. (H) Airway resistance (R) to increasing doses of methacholine (MCH; left) and at the 25 mg/mL MCH dose (right) of non-allergic mice (vehicle) and OVA-sensitized/exposed mice treated with either vehicle, cTXA2, cTXA2+ISAP, or ISAP alone. n = 15–20 per group, *P < 0.05 vs. non-allergic (vehicle), #P < 0.05 vs. OVA-sensitized/exposed cTXA2-treated mice. Significance was evaluated by multiple t tests for B, t test for D–F, and 1-way ANOVA for H.

FACS analysis indicated that TP−/− mice had significantly more Th9 cells compared with TP+/+ mice in lung, BALF, lymph nodes, and blood, but not in spleen (Figure 2D and Supplemental Figures 8 and 9). Consistent with these results, immunofluorescence microscopy revealed that lungs from TP−/− mice had significantly more Th9 cells than those from TP+/+ mice (Figure 2D). Additionally, immunohistochemistry and histological analysis showed a reduction in the number of Th9 cells in TP−/− lungs compared to TP+/+ lungs (Figure 2D). These findings suggest that the absence of TP promotes Th9 cell differentiation and airway inflammation in response to allergen exposure.
The Journal of Clinical Investigation

RESEARCH ARTICLE

J Clin Invest. 2024;134(9):e165689  https://doi.org/10.1172/JCI165689

The Journal of Clinical Investigation

RESEARCH ARTICLE

J Clin Invest. 2024;134(9):e165689  https://doi.org/10.1172/JCI165689

4

>99% pure) from mouse spleens and induced them to the Th9 cell phenotype using TGF-β and IL-4 in the presence of vehicle, 300 nM cTXA2, 500 nM TXB2 (stable TXA2 metabolite), or 300 nM U-46619 (TP receptor agonist). As shown in Figure 3A, only 0.6% ± 0.1% of untreated naive T cells differentiated to Th9 cells after 5 days in culture. Treatment with TGF-β and IL-4 induced 4.0% ± 0.3% of naive T cells to differentiate into Th9 cells. Interestingly, cTXA2, TXB2, and U-46619 significantly inhibited Th9 cell differentiation (Figure 3A and Supplemental Figure 11). cTXA2 suppressed Th9 cell differentiation in a dose-dependent manner with significant effects at concentrations as low as 7.5 nM, which are physiologically relevant (Figure 3B). Consistent with these findings, Il9, Il10, and Irf4 mRNA levels were significantly reduced by cTXA2 and U-46619 (Figure 3C). Moreover, TP−/− naive T cells exhibited significantly increased Th9 cell differentiation compared with TP+/+ naive T cells as evidenced by increased Il9, Ii10, and Irf4 mRNA levels (Figure 3D). Importantly, both cTXA2 and U-46619 also inhibited Th9 cell differentiation of naive T cells isolated from peripheral blood of healthy human volunteers as determined by both FACS (Figure 4A) and mRNA analyses (Fig-
Myeloid cells secrete TXA₂ and Th9 cells express the TP receptor.
Specific cell signature markers can identify myeloid cell subsets in murine lungs, including alveolar macrophages, interstitial macrophages, monocytes, and DCs (44). We isolated different lung myeloid cell subsets using CD11c and F4/80 markers and also isolated naive CD4⁺CD62L⁺ T cells by FACS. None of the cell types produced significant amount of TXB₂ (the stable TXA₂ metabolite) under unstimulated conditions (Figure 5B, open circles). After activating them with LPS for 24 hours, we observed that only monocytes/interstitial macrophages (F4/80⁺), DCs (CD11c⁺), and alveolar macrophages (CD11c⁺F4/80⁺) produced significant amounts of TXB₂, but not naive T cells (Figure 5B, filled circles). Consistent with the pattern of TXB₂ formation, LPS induced thromboxane synthase (Tbxas1) in CD11c⁺ cells but not naive T cells (Figure 5C). Conversely, LPS induced the TP receptor (Tbxa2r) in naive T cells but not CD11c⁺ cells, similar to a previous report (43). Similar responses were observed in CD11c⁺ DCs and naive T cells treated with TGF-β and IL-4. Treatment of isolated naive T cells with TGF-β and IL-4 increased Tbxa2r mRNA levels and suppressed Tbxas1 mRNA levels (Supplemental Figure 13).
mixed cultures, TGF-β and IL-4 induced expression of both *Tbx2a* and *Tbxas1* mRNAs, but cTXA₂ treatment did not significantly alter the expression of either gene (Figure 5D). In cells cocultured with TGF-β and IL-4 but separated using Transwells, CD4⁺ T cells expressed higher levels of *Tbx2a* than CD11c⁺ cells, while CD11c⁺ cells expressed higher levels of *Tbxas1* than CD4⁺ cells (Figure 5E). Taken together, these data suggest that CD11c⁺ DCs can be stimulated to produce TXA₂, which activates the TP receptor on CD4⁺ T cells to modulate differentiation.

**TXA₂ regulates both Th2 and Th9 cell differentiation**. To determine whether TXA₂ regulates other Th cell subsets in vivo, mice were sensitized with OVA/alum and then exposed to OVA via the airway in the presence of vehicle- or cTXA₂-load ed minipumps as depicted in Figure 1A. Forty-eight hours after the last OVA exposure, lungs were collected, and the percentages of CD4⁺IL-4-γ (Th1), CD4⁺IL-4⁺ (Th2), CD4⁺IL-9⁺ (Th9), CD4⁺IL-17⁺ (Th17), and CD4⁺FOXP3⁺ (Treg) cells were determined by FACS. Lungs from cTXA₂-treated mice had a significantly reduced percentage of Th2 and Th9 cells compared with lungs from vehicle-treated mice (Figure 6A). In contrast, the Th1 and Th17 cell percentages were not affected by cTXA₂. Interestingly, lungs from cTXA₂-treated mice had a significantly increased percentage of Tregs compared with lungs from vehicle-treated mice (Figure 6A). The cTXA₂-induced reduction of Th2 and Th9 cells in allergic mice was reversed by the TP receptor antagonist ISAP (Supplemental Figure 14). cTXA₂ also attenuated the increase in BALF levels of the Th2 cytokine IL-4 in vivo in allergic mice (Supplemental Figure 15).

Naïve T cells can differentiate to Th9 cells via 2 pathways (45); they can directly differentiate to Th9 cells in the presence of IL-4 and TGF-β, or they can differentiate to Th9 cells in a 2-step process that involves differentiation of naïve T cells to Th2 cells in the presence of IL-4, and then differentiation of Th2 cells to Th9 cells with the addition of TGF-β (Figure 6B). TXA₂ may regulate Th9 differentiation at either step of the process, or it may regulate all 3 steps. To determine whether TXA₂ can regulate Th2 cell differentiation, we incubated naïve T cells (MACS, >95% pure) with IL-4 alone in the presence or absence of cTXA₂. IL-4 induced the canonical Th2 transcription factor *Gata3* and the Th2 cytokine IL-4 (Figure 6C). Expression of *Gata3*, *Il4*, and *Il13* was significantly inhibited by cTXA₂, suggesting that cTXA₂ regulates Th2 cell differentiation from naïve T cells (Figure 6, C and D). To determine whether TXA₂ can regulate Th9 cell differentiation, we incubated naïve T cells with IL-4 and TGF-β together in the presence or absence of cTXA₂. Incubation of naïve T cells with TGF-β and IL-4 induced expression of the Th9 cell markers *Il4* and *Il19* (Figure 6D) with minimal induction of the Th2 cell markers *Gata3* and *Il4* (Figure 6C). cTXA₂ attenuated differentiation of naïve T cells directly to Th9 cells. Finally, to determine whether TXA₂ also can inhibit differentiation of Th2 cells to Th9 cells, we first differentiated naïve T cells to Th2 cells using IL-4, and then treated the Th2 cells with IL-4 and TGF-β in the presence or absence of cTXA₂. Incubation of naïve T cells with IL-4 initially, then IL-4 and TGF-β together, induced expression of the Th9 cell markers *Il4* and *Il19* (Figure 6D). cTXA₂ attenuated differentiation of Th2 cells to Th9 cells. Therefore, cTXA₂ regulates differentiation of naïve T cells to Th2 cells, naïve T cells to Th9 cells, and Th2 cells to Th9 cells. Th9 cell differentiation is also dependent on the transcription factors BATF and STAT6 (46, 47). Interestingly, cTXA₂ suppressed *Batf* and *Stat6* induction only after naïve T cells were differentiated toward the Th2 phenotype with IL-4 or after Th2 to Th9 cell differentiation (Supplemental Figure 16). *Batf* and *Stat6* were both induced during Th9 cell differentiation by TGF-β plus IL-4; however, this was not a point of regulation by cTXA₂.

Thromboxane also regulates differentiation of naive human CD4⁺ T cells to multiple T helper subsets. Naïve T cells isolated from human peripheral blood were differentiated to Th2, Th9, and Treg cell subsets in vitro and analyzed by FACS and mRNA analyses. In an independent replication of the human T cell responses shown in Figure 4, IL-4 and TGF-β increased Th9 cells (IL-4⁺CD4⁺) and induced *Il4* and *Il19* mRNAs, effects that were significantly attenuated by cTXA₂ treatment (Figure 7A). Similarly, IL-4 increased Th2 cells (IL-4⁺CD4⁺) and induced *Gata3* and *Il4* mRNAs, effects that were also significantly attenuated by cTXA₂ (Figure 7B). TGF-β increased Tregs (FOXP3⁺CD4⁺) and induced *Foxp3* mRNA (Figure 7C). Interestingly, cTXA₂ treatment resulted in a non-significant increase in Tregs and caused a further significant induction of *Foxp3* mRNA (Figure 7C). These data suggest that cTXA₂ regulates differentiation of naïve T cells to Th2, Th9, and Treg cell subsets in both mice and humans.

**Involvement of cAMP/PKA and p38 signaling cascades in Th9 differentiation**. TXA₂ can signal through TP receptors to activate a variety of signaling cascades, including cAMP/PKA (cAMP-dependent protein kinase) and p38 MAPK pathways (48), which can interact with a variety of transcription factors, including NFE2 and PBX1, to regulate cell differentiation (49). To identify the downstream TXA₂/TP receptor signaling pathways involved in Th9 cell differentiation, we first examined the effect of cTXA₂ treatment and TP receptor knockout on intracellular CAMP levels in vitro. As shown in Figure 8A, cTXA₂ increased CAMP levels by approximately 50% compared with vehicle in naïve T cells treated with TGF-β and IL-4 to induce Th9 cell differentiation. Moreover, treatment of TP⁻/⁻ naïve T cells with TGF-β and IL-4 resulted in increased intracellular CAMP levels; however, this increase did not occur when TP⁻/⁻ naïve T cells were treated with TGF-β and
IL-4 (Figure 8B). Next, we examined the effect of cTXA2 on phosphorylation of p38 MAPK. cTXA2 enhanced p38 MAPK phosphorylation in naive T cells treated with TGF-β and IL-4 (Figure 8C and Supplemental Figure 17). Consistent with these data, phosphorylation of p38 MAPK appeared reduced in TP−/− naive T cells treated with TGF-β and IL-4 relative to TP+/+ naive T cells (Figure 8C and Supplemental Figure 17). The importance of cAMP/PKA and p38 MAPK in the cTXA2-mediated inhibition of Th9 cell differentiation was further examined using specific inhibitors of these signaling pathways. While protein kinase A inhibitory peptide (PKAi) modestly enhanced Th9 cell differentiation in vitro as measured by levels of Il9, Il10, and Irf4 mRNAs, it did not signifi-

Figure 5. TXA2 inhibits promotion of Th9 cell differentiation by DCs in vitro. (A) Coculture of purified naive CD4+CD62L+ T cells with CD11c+ DCs (from lung) enhanced Th9 cell differentiation compared with naive T cells alone. Treatment with 300 nM cTXA2 significantly impaired Th9 cell differentiation of naive T cells, whether cultured alone or in the presence of DCs. n = 10 per group, *P < 0.05. (B) Purified naive CD4+CD62L+ T cells or CD11c+, CD11c+F4/80+, and F4/80+ myeloid cells were treated with vehicle or LPS (1 mg/mL) in vitro, and supernatants were assayed for TXB2 by liquid chromatography-tandem mass spectrometry. (C) Purified CD11c+ or naive CD4+ T cells were treated with vehicle or LPS (1 mg/mL) in vitro. LPS treatment increased TP receptor (Tbxa2r) mRNA levels in CD4+ T cells and TXA2 synthase (Tbxas1) mRNA levels in CD11c+ cells. n = 3 per group, *P < 0.05. (D and E) Mixed cultures of CD11c+ and CD4+ T cells (D) or Transwell-separated CD11c+ and CD4+ T cells (E) were incubated with vehicle, cTXA2, and TGF-β plus IL-4 as indicated and assayed for Tbxa2r and Tbxas1 mRNA levels. n = 9, *P < 0.05. Significance was evaluated by 1-way ANOVA for A and D and multiple t tests for B, C, and E.
TXA₂ represses IL-9 production through PBX1 and NFE2. Ultimately, Th9 cell differentiation requires upregulation of Il9 gene transcription. The mouse Il9 proximal promoter contains consensus binding sites for the PBX1, PU.1, IRF4, NFE2, and CREB transcription factors (Figure 9A). Prior work has shown that PU.1 and IRF4 are key transcription factors involved in Th9 cell differentiation, IL-9 production, and allergic inflammation (50). In contrast, significantly alter the ability of cTXA₂ to inhibit Th9 cell differentiation (Figure 8D). We were unable to confirm the role of p38 MAPK in the cTXA₂ effect, as the p38 MAPK inhibitor SB203580 (p38i) alone abolished Th9 cell differentiation (Figure 8E). Thus, while activation of cAMP/PKA and p38 MAPK signaling pathways may be important in Th9 cell differentiation, we cannot conclude that they are definitively involved in the TXA₂ effect.

**Figure 6. TXA₂ inhibits Th2 cell differentiation in vivo and in vitro.** Mice were sensitized with OVA/alum and exposed to OVA via the airway as depicted in Figure 1A. (A) Forty-eight hours after the last OVA exposure, the percentages of Th1, Th2, Th9, Th17, and Treg cells were determined by FACS analysis. n = 8, *P < 0.05. (B) Th9 cells can differentiate directly from naive T cells or from Th2 cells. (C) IL-4 alone, but not TGF-β and IL-4, induced Th2 cell differentiation from naive T cells as determined by mRNA levels of Th2 markers Gata3 and Il4. cTXA₂ treatment attenuated Th2 cell differentiation from naive T cells. (D) TGF-β and IL-4, but not IL-4 alone, induced Th9 cell differentiation from naive T cells as determined by mRNA levels of Th9 markers Irf4, Il9, and Il13. cTXA₂ attenuated Th9 cell differentiation directly from naive T cells. Naive T cells were treated with IL-4 alone to generate Th2 cells, and then the Th2 cells were treated with TGF-β and IL-4 to differentiate them to Th9 cells. cTXA₂ treatment attenuated differentiation of Th2 cells to Th9 cells. n = 5, *P < 0.05. Significance was determined by t tests for A and 1-way ANOVA for C and D.
The Journal of Clinical Investigation

RESEARCH ARTICLE

little is known about the role of PBX1, NFE2, or CREB in regulation of Th9 cell differentiation or function. Interestingly, p38 MAPK and PKA have been reported to regulate activation and DNA binding of PBX1, NFE2, and CREB transcription factors (51–53).

We first determined the expression of these transcription factors during Th9 cell differentiation of mouse naive T cells in vitro (Figure 9B). Consistent with the role of IRF4 in Th9 cell differentiation, treatment of naive T cells with TGF-β and IL-4 increased Il9 mRNA expression. cTXA2 suppressed the induction of Ifr4 mRNA by approximately 50%. Notably, cTXA2 suppression of Il9 mRNA was less pronounced than cTXA2 suppression of Irf4 mRNA, suggesting that cTXA2 does not act solely through IRF4 to regulate Th9 cell differentiation. Pbx1 and Nfe2 mRNAs are both abundant in naive T cells, and treatment with TGF-β and IL-4 to induce Th9 cell differentiation decreased both Pbx1 and Nfe2 expression. This suggests that induction of IL-9 during Th9 cell differentiation may be through reduced expression of these known transcriptional repressors. Importantly, treatment with cTXA2 increased Pbx1 expression and restored Nfe2 expression close to that in naive T cells. Thus, suppression of IL-9 by cTXA2 may, at least in part, be due to restoration of the basal repression of the Il9 promoter by PBX1 and NFE2. Treatment of naive T cells with TGF-β and IL-4 to induce Th9 cell differentiation increased expression of Cref mRNA; however, cTXA2 had no significant effect on Cref expression. Expression of Ptu1 mRNA was low or undetectable in mouse naive T cells and was not changed during Th9 cell differentiation or by cTXA2 treatment (data not shown).

Since cTXA2 increased expression of Pbx1 and Nfe2 during Th9 cell differentiation, we examined whether it also influenced binding of these 2 transcription factors to the Il9 promoter. We performed chromatin immunoprecipitation using PBX1- or NFE2-specific antibodies followed by quantitative PCR (qPCR) and direct sequencing of PCR products (Figure 9C and Supplemental Figure 18). cTXA2 significantly increased binding of both transcription factors to the immunoprecipitated chromatin. Sequencing confirmed that this binding mapped to the respective DNA sites on the Il9 promoter.

To further study the transcription factors involved in repression of Il9 expression by TXA2, we made a series of luciferase reporter constructs containing varying lengths of the mouse Il9 promoter (Figure 10A and Supplemental Table 1). All of these constructs were transfected into 293T cells, and luciferase activity was measured after correction for transfection efficiency. The V2 construct, which has a 700 bp promoter sequence containing the consensus binding sites for the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factors, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B). As shown in Figure 10C, the intact V2 construct had strong promoter activity, which could be significantly inhibited by cTXA2. This indicates that the TXA2-sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factor binding sites, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B). As shown in Figure 10C, the intact V2 construct had strong promoter activity, which could be significantly inhibited by cTXA2. This indicates that the TXA2-sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factors, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B). As shown in Figure 10C, the intact V2 construct had strong promoter activity, which could be significantly inhibited by cTXA2. This indicates that the TXA2-sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factors, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B). As shown in Figure 10C, the intact V2 construct had strong promoter activity, which could be significantly inhibited by cTXA2. This indicates that the TXA2-sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factors, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B). As shown in Figure 10C, the intact V2 construct had strong promoter activity, which could be significantly inhibited by cTXA2. This indicates that the TXA2-sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2, CREB, PU.1, and IRF4 transcription factors, showed the strongest luciferase activity and was used in subsequent studies (Figure 10B).
Our findings add to a growing body of evidence supporting the role of TXA₂ in regulation of immune cell function. Mouse thymus and spleen have the highest expression of TP receptors compared with other organs (55). Leung and Mihich first demonstrated that TXA₂ had immunoregulatory; suppression of TXA₂ production inhibited splenocyte proliferation (56). Similarly, others have shown that TXA₂ inhibitors and TP receptor antagonists inhibit splenocyte proliferation but lack an additive or synergistic effect (57). Both mitogen- and antigen-induced splenocyte proliferation responses are impaired in TP–/– spleen cells (58). More recent studies indicated that TXA₂/TP receptor signaling dampens acquired immunity by suppressing the interactions between DCs and T cells (43). In our studies, cTXA₂ suppressed differentiation of purified naive T cells to Th9 cells in the presence or absence of costimulatory DCs, suggesting that it directly suppresses Th9 cell differentiation and IL-9 production, with little or no effect on DCs. TXA₂ appears to function in a paracrine fashion rather than an autocrine fashion to inhibit T cell differentiation. Multiple myeloid cell types are present in the lung and have been shown to regulate lung inflammation.

**Discussion**

The well-described role of TXA₂ as a potent bronchoconstrictor suggested a novel therapeutic approach for allergic lung disease; however, TXA₂ inhibitors and TP receptor antagonists have shown little efficacy in the treatment of asthma patients (54). In this study, we report several findings that may help to explain this apparent paradox: (a) cTXA₂ decreases lung inflammation, airway hyperresponsiveness, and numbers of Th2, Th9, and Treg cells in the allergic mouse lung in vivo; (b) allergic TP receptor–knockout mice have increased numbers of Th9 cells in vivo; (c) cTXA₂ suppresses and TP receptor knockout enhances differentiation of naive T cells to Th9 cells in vitro; (d) cTXA₂ is produced by myeloid cells and Th9 cells express the TP receptor; (e) cTXA₂ enhances and TP receptor knockout suppresses activation of p38 MAPK and cAMP/PKA signaling pathways; and (f) cTXA₂ induces NFE2 and PBX1 transcription factor binding to, and repression of, the Il9 promoter. Thus, although anti-TXA₂ therapies attenuate bronchoconstriction, they may exacerbate the asthma phenotype by promoting Th9-mediated inflammation.
TXA2/TP receptor signaling regulates cell function through multiple signaling pathways (60, 61). The precise signaling mechanisms through which TXA2/TP receptor activation suppresses Th9 cell differentiation remain unknown. Our data suggest that TXA2/TP receptor signaling can increase cAMP levels and activate p38 MAPK during Th9 cell differentiation. Interestingly, PKA inhibition modestly enhanced Th9 cell differentiation, but it did not significantly alter the ability of cTXA2 to inhibit Th9 cell differentiation. Likewise, p38 MAPK inhibition alone abolished Th9 cell differentiation. Thus, while activation of cAMP/PKA and p38 MAPK may be important in Th9 cell differentiation, we cannot conclude that these signaling pathways are involved in the TXA2 effect.

Figure 9. TXA2 alters expression/binding of PBX1 and NFE2 to the Il9 promoter. (A) The locations of transcription factor binding sites in the mouse proximal Il9 promoter. Conservation analysis and motif prediction were used to identify unique binding sites for NFE2 (−150 bp), PBX1 (−481 bp), CREB (−101 bp), PU.1 (−450 bp; −192 bp), and IRF4 (−237 bp) transcription factors relative to the transcription start site (TSS). (B) Naive T cells were treated with TGF-β and IL-4 to induce Th9 cell differentiation in the presence or absence of cTXA2, and expression of Irf4, Pbx1, Nfe2, Creb, and Il9 mRNAs was determined by qPCR. n = 9, *P < 0.05. (C) ChIP-qPCR assays of PBX1 and NFE2 binding to genomic DNA from naive T cells during Th9 cell differentiation with or without treatment with cTXA2. DNA fragments were pulled down with anti-NFE2, anti-PBX1, or IgG control antibodies, and NFE2- and PBX1-bound DNA was amplified using specific primers by qPCR. The percentage pull-down by NFE2 or PBX1 relative to input DNA is shown. The locations of ChIP-qPCR primers relative to the TSS (+1) and PBX1 or NFE2 binding sites are shown. n = 3, *P < 0.05. Significance was determined by 1-way ANOVA for B and multiple t tests for C.
Increased IL-9 production is a critical marker of Th9 cell differentiation; however, the network of transcription factors that mediate induction of IL9 mRNA is incompletely understood. PU.1 (5) and IRF4 (62) transcription factors are most commonly associated with Th9 cell differentiation, although other transcription factors, including BATF (47), STAT6 (46), FOXP1 (63), ID3 (64), SIRT1 (65), and BCL6 (66), may also be involved. We examined the proximal mouse Il9 promoter and found multiple transcription factor binding sites that could be involved in the induction of Il9. We focused on NFE2 and PBX1 because they were abundantly expressed in naive T cells and downregulated during Th9 cell differentiation and were known transcriptional repressors (67, 68). Interestingly, ChIP-qPCR analysis showed that cTXA2 induced binding of NFE2 and PBX1 to their respective sites in the Il9 promoter. Moreover, disruption of the NFE2 and PBX1 binding motifs using site-directed mutagenesis enhanced Il9 promoter activity and abolished the suppressive effect of cTXA2. Together, these data suggest that TXA2 inhibits IL9 transcription, at least in part, through activation of NFE2 and PBX1.

It is noteworthy that in addition to regulating Th9 cell differentiation/function, we observed that TXA2 also regulates Th2 and Treg cell differentiation/function both in vitro and in vivo in both mice and humans. Th2 cells produce IL-4, IL-5, and IL-13, which are key players in the lung immune response to allergen (69). Other COX-derived eicosanoids have been shown to play important roles in modulating Th2 immunity. For example, PGE2 can shift the balance of CD4+ Th cells toward a Th2-type immune response through regulation of DCs and altering the local cytokine microenvironment (70). In contrast, others have shown that COX-2 inhibition reduces PGE2 formation in vivo and increases Th2-mediated lung inflammation (71). PGD2 has been reported to stimulate chemotaxis of Th2 cells (72). PGI2 analogs suppress Th2 cytokine production in an antigen-specific manner through the IP receptor (73). Together with our work, these published studies suggest that the effects of COX-derived eicosanoids on Th2 responses are complex.

The effects of TP disruption and cTXA2 treatment on Th9 cell differentiation and function were consistent across different in vitro and in vivo models; however, not all variables tracked well with changes in Th9 cell numbers. For example, cTXA2 suppressed inflammation in the OVA/alum model but not in the OVA/LPS model. Similarly, increased Th9 cells in TP-null mice did not exacerbate lung inflammation. One possible explanation for these apparent discrepancies may be that histological scoring is less sensitive and more variable than other measures of inflammation. Alternatively, it is well established that selection of the adjuvant is determinative with regard to the characteristics of the allergic response. Our data are consistent with those of others who find that LPS induces a lower level of lung inflammation than other adjuvants (33). In our experiments, OVA/alum (inflammation scores ~3; Figure 1D and Figure 2C) induced more inflammation than OVA/LPS (inflammation scores ~1; Supplemental Figure 7B and Supplemental Figure 10B). OVA/LPS also may skew allergic responses more toward Th1 or Th17 responses (74, 75). For example, in our experiments, OVA/LPS induced a neutrophilic- and lymphocytic-predominant lung inflammatory response compared with a pronounced eosinophilic response induced by OVA/alum (Supplemental Figure 7 vs. Figure 1, respectively). Our study is also limited in that it cannot elucidate whether the major suppressive effects of cTXA2 on airway responsiveness are due to effects on Th2, Th9, Treg, and/or other cell types such as epithelial cells. Despite these limitations, the reproducible immunomodulatory effects of cTXA2 across multiple in vitro and in vivo models, the consistent findings in both mice and humans, and the suppression of airway responsiveness in a clinically relevant eosinophilic-predominant allergic model give us confidence that cTXA2 plays an important role in the development of allergic lung inflammation.

We believe that our findings are clinically relevant since we observed similar effects of TXA2 in human naive T cells isolated from peripheral blood of healthy volunteers. Indeed, both cTXA2.
and the TP receptor agonist U-46619 significantly attenuated Th9 cell differentiation of human naive T cells ex vivo, while the TP receptor antagonist ISAP was able to reverse the effects of cTXA₂. These findings suggest the use of TP receptor agonists and/or TXAS activators for the treatment of Th9 allergic inflammation in the asthmatic lung. In addition, these observations raise the possibility that polymorphisms in the TBXAS1 or the TXA2R gene may be associated with altered asthma risk in humans. Functional polymorphisms in these 2 genes have been reported in the literature, and several published studies have examined their contribution to asthma risk. A recent meta-analysis of 7 studies concluded that the TXA2R 924C/T polymorphism is associated with asthma risk and the TH2XAR 795C/T polymorphism may be a risk factor for aspirin-intolerant asthma (76). Likewise, a rare allele (rs6962291) in the TBXAS1 gene was associated with lower cat risk and the TBXA2R 924C/T polymorphism is associated with asthma risk. A recent meta-analysis of 7 studies concluded that the TH2XAR 924C/T polymorphism is associated with asthma risk and the TH2XAR 795C/T polymorphism may be a risk factor for aspirin-intolerant asthma (76). Likewise, a rare allele (rs6962291) in the TBXAS1 gene was associated with lower cat risk and the TBXA2R 924C/T polymorphism is associated with asthma risk. Further work is necessary to determine whether these or other polymorphisms in TXAS/TP receptor pathway genes are associated with allergic airway inflammation or asthma in other populations.

In summary, TXA₂/TP receptor signaling attenuates lung Th9 cell differentiation during OVA-induced allergic lung inflammation in vitro and in vivo. Within the immune synapse, TXA₂ is produced by activated DCs and detected by differentiating T cells that express the TP receptor. We believe that this novel pathway represents a fourth signal whereby antigen-presenting cells interact with T cells to influence Th9 cell differentiation (Figure 11). Within T cells, TXA₂ induces p38 MAPK activation, binding of NFE2 and PBX1 transcription factors to the IL9 promoter, and suppression of IL9 transcription. Thus, in contrast to its prothrombotic, proinflammatory, and spasmodic effects, thromboxane exerts immunosuppressive effects that attenuate Th9 cell differentiation and IL-9 secretion during allergic lung inflammation.

Methods
Sex as a biological variable. Our study exclusively examined male mice to limit variability in phenotype. Human samples were obtained from both male and female subjects. It is unknown whether the findings in mice are relevant to female mice.

Reagents and animals. Antibodies were purchased from BD Biosciences, eBioscience, BioLegend, and Cell Signaling Technology. Eicosanoids and inhibitors were purchased from Cayman Chemical. Other chemicals and buffers were purchased from Sigma-Aldrich. Tissue culture media and supplements were from Gibco/Thermo Fisher Scientific. Male and female C57BL/6J mice (6–10 weeks of age) were purchased from The Jackson Laboratory. Male TPΔ−/− and TPΔ mice (6–10 weeks old) on a pure C57BL/6 background (backcrossed >10 generations) were provided by Thomas Coffman (Duke University).

OVA-induced allergic airway inflammation model in vivo. Mice were sensitized with 20 μg OVA using either 0.2 mL aluminum hydroxide (alum) or 1 μg bacterial LPS (from Pseudomonas aeruginosa 10, Sigma-Aldrich) as an adjuvant by intraperitoneal injection on days 0 and 1; 14–21 days later, mice were exposed to 1% OVA (≥98% pure by agarose gel electrophoresis) in saline (or saline only) via inhalation for 30 minutes per day for 4 consecutive days. Vehicle (15% ethanol in PBS), cTXA₂, and/or iodophenyl sulfonyl amino pinane TXA2 (ISAP) (1 μmol/mouse/day) were delivered 1 week before airway OVA exposure via subcutaneously implanted osmotic minipumps (model 1001D, Alzet). Mice were euthanized for assessments 48 hours after the last OVA exposure.

Lung function assessment. Invasive lung function analysis was performed on mice with a flexiVent FX2 (SCIREQ) according to the manufacturer’s instructions as previously described (78).

Histology and immunofluorescence staining. Lungs were intratracheally instilled with 50% Sakura Tissue-Tek OCT compound (International Medical Equipment) at 25 cm H₂O and frozen on dry ice. Lung sections were fixed in methanol with 0.3% H₂O₂ for 10 minutes at 4°C and permeabilized with Triton X-100 (0.8%) for 10 minutes at room temperature. After lung sections were blocked with 5% BSA in PBS, they were simultaneously immunostained with anti-IL-9, anti-IL-10, and anti-CD4 antibodies for 1 hour at room temperature (BioLegend, catalog 514104, 505016, and 100414). Mast cells were stained with DAPI, anti-CD45, and antibodies for 1 hour at room temperature (BioLegend, catalog 514104, 505016, and 100414). Mast cells were stained with DAPI, anti-CD45, and anti-CD117 (BioLegend 160303 and 105831). Lung sections were imaged using an Axioscan 2 fluorescence microscope (Carl Zeiss) with a digital camera (AxioCam MRC or MRM, Carl Zeiss) or a confocal microscope (LSM 710, Carl Zeiss). Lungs were scored by a blinded pathologist based on the percentage of lung involved in inflammation: 0 = no inflammation; 1 = 1%–10%; 2 = 11%–30%; 3 = 31%–50%; 4 = >50%. Cell fluorescence intensity was quantified with ImageJ software (NIH).
In vitro differentiation of naive T cells. Lung and spleen CD4+ T cells were isolated by magnetic-activated cell sorting (MACS). Naive CD4+ T cells were isolated by using the CD4+CD62L- isolation kit (Miltenyi Biotech; ≥95% purity) or by fluorescence-activated cell sorting (FACS; ≥99% purity). Human CD4+ T cells were isolated by MACS from peripheral blood. Cells were cultured in the presence of anti-CD3 (BioLegend 317302; 3 μg/mL) and anti-CD28 (BioLegend 302992; 1 μg/mL) and differentiated to Th1 (with IL-12, 20 ng/mL), Th2 (IL-4, 20 ng/mL), and TGF-β to Th1 (with IL-12, 20 ng/mL), Th2 (IL-4, 20 ng/mL), and TGF-β (2 ng/mL), and Tregs (TGF-β, 10 ng/mL). In some experiments, cells were treated with cTXA2 (7.5–500 nM), TXB2 (500 nM), or TP receptor agonist U-46619 (300 nM) or corresponding vehicle.

Flow cytometry analysis. Single-cell suspensions from lung, spleen, and peritoneal lymph nodes were prepared by mechanical disruption. Analysis of Th9 cells was performed using anti-IL-9, anti-IL-10, and anti-CD4 antibodies (BioLegend 514104, 505016, and 100414). Anti-rat IgG was used as the negative control. Surface staining was performed by incubation of samples at 4°C for 20 minutes. 7-Aminoactinomycin D (EMD Millipore) was used to discriminate dead cells. Intracellular staining was performed with the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) using the PE-conjugated anti-IL-9 and APC-conjugated anti-IL-10 mAbs. Analysis of other T helper subsets was performed using anti-CD4 with PE-conjugated anti–IL-9 and APC-conjugated anti–IL-10 mAbs. Analysis of other T helper subsets was performed using anti-CD4 with PE-conjugated anti–IL-9 and APC-conjugated anti–IL-10 mAbs. Analysis of other T helper subsets was performed using anti-CD4 with PE-conjugated anti–IL-9 and APC-conjugated anti–IL-10 mAbs. Analysis of other T helper subsets was performed using anti-CD4 with PE-conjugated anti–IL-9 and APC-conjugated anti–IL-10 mAbs. Analysis of other T helper subsets was performed using anti-CD4 with PE-conjugated anti–IL-9 and APC-conjugated anti–IL-10 mAbs.

Naive CD4+ T cell purification. Lungs, lymph nodes, and spleens from C57BL/6J, TP+/+, or TP–/– mice were pooled and homogenized using a 70 μm cell strainer. Red blood cells were lysed in 1 mL of a Tris-HCl pH 7.5/0.83% ammonium chloride buffer for 3 minutes. After washing with 0.2% BSA/PBS, cells were placed in a medium containing RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μM streptomycin, 100 μM sodium pyruvate, l-glutamine, and nonessential amino acids. We used EasySep Mouse T Cell Negative Selection kit or Mouse CD4+ T Cell Enrichment kit as described in the manufacturer’s instructions (STEMCELL Technologies). Naive CD4+ T cells were sorted based on staining by anti-mouse CD4–APC–Cy7 (BioLegend 100414) and anti-mouse CD62L–PE (BioLegend 161204). Sorted naive CD4+ T cell purities were greater than 99%.

Lung myeloid cell isolation. Mouse lung tissue was cut into small pieces, then digested with collagenase II (0.5 mg/mL; Worthington) and DNase I (20 μg/mL; Sigma-Aldrich) at 37°C for 1 hour for preparation of single lung cells. Lung myeloid cells were enriched from the lung single-cell suspension with EasySep Mouse Pan-DC Enrichment Kit (STEMCELL Technologies). F4/80+ cells (monocytes/interstitial macrophages), CD11c+ cells (monocyte-derived DCs), and CD11c+/ F4/80+ cells (alveolar macrophages) were sorted with anti–mouse F4/80–FITC (BioLegend 123108) and anti–mouse CD11c–PE (BioLegend 117307) antibodies from the enriched lung myeloid cells using a BD FACSAria II instrument.

Eicosanoid analysis of myeloid cells and naive CD4+ T cells. Purified myeloid cell subsets (CD11c+, F4/80+, and CD11c+F4/80−) and naive CD4+ T cells were either unstimulated or stimulated with LPS (1 mg/mL) at 37°C for 4 hours. Eicosanoid levels in the supernatants were analyzed by liquid chromatography–tandem mass spectrometry as previously described (12).

Lung DC-T cell cocultures. Lung DC-T cell cocultures were performed in 24-well flat-bottom culture plates. Briefly, purified DCs (CD11c+) from lung were treated with LPS (1 mg/mL) overnight, after which the culture supernatants were removed, and the cells were extensively washed and resuspended in RPMI 10% FCS. Naive CD4+ T cells (1 × 10^6 per well) were then cocultured with the DCs (1 × 10^5 per well) in the presence of anti-CD3 (2 μg/mL), anti-CD28 (1 μg/mL), IL-4 (20 ng/mL), and TGF-β (2 ng/mL) for 5 days in 1 mL of complete culture medium. Some experiments were performed with DCs and T cells separated in 24-well Transwell cultures (Corning Costar 3470; 0.4 μm pores).

Measurement of intracellular cAMP concentrations. Isolated naive T cells (2 × 10^6 per mL) from C57BL/6, TP+/+, and TP–/– mice were treated with vehicle, cTXA2, and cytokines as described. cAMP levels were determined using a cAMP enzyme immunoassay kit following the manufacturer’s instructions (Cayman Chemical, catalog 581001).

IL-9 promoter luciferase reporter assay. The IL9 gene transcription start site (TSS) is located on the negative strand of chromosome 13 at position 56,630,060 (mm39). Five different lengths of the proximal IL9 promoter were PCR amplified from the C57BL/6J genome: 4.2 kb, V0 (TSS, 56,634,261); 3.2 kb, V4 (TSS, 56,633,269); 2.4 kb, V3 (TSS, 56,632,508); 1.2 kb, V1 (TSS, 56,631,308); and 0.7 kb, V2 (TSS, 56,630,769). Samples were cloned into the promoterless pGL4.10 luciferase reporter vector (Promega E6651) and transfected into Jurkat T cells (clone E6-1, ATCC TIB-152). Twenty-four hours after transfection, cells were treated with 300 nM cTXA2 or vehicle for 4 hours. Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).

ChIP-qPCR. Naive CD4+ T cells were either untreated or treated with TGF-β and IL-4 in the presence of vehicle or 300 nM cTXA2. Chromatin immunoprecipitation (ChIP) assays were performed following the manufacturer’s instructions (Agarose ChIP kit 26156, Pierce/Thermo Fisher Scientific). The fragmented DNA samples obtained from the ChIP with anti-NFE2 (Abcam ab140598), anti-PBX1 (Thermo Fisher Scientific PA5-17223), and anti-IG antibodies (Thermo Fisher Scientific PA5-31160) and input samples were amplified by qPCR with specific primers.

qPCR. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems). All qPCR reagents were purchased from Life Technologies. The following oligonucleotides (Applied Biosystems) were used to amplify IL9, I10, If4, Pu1, and Gapdh TaqMan primers: IL9, Mm00434304_m1; I10, Mm01288386_m1; If4, Mm00516431_m1; Pu1 (Sp1), Mm00488428_m1; Tbx2a, Mm00436917_m1; Pbx1, Mm04207617_m1; Nfe2, Mm00801891_m1; Crtc2, Mm01219960_m1; Gata3, Mm00484683_m1; Gapdh, Mm99999915_g1.

PKA and p38 MAPK inhibitor studies. Inhibitors of PKA (PKAi, Cayman Chemical; 100 nM) and p38 MAPK (SB203580, Cayman Chemical; 1 mg/mL) were used in Th9 cell differentiation studies in vitro.

Statistics. Data are presented as means ± SEM. Statistical comparisons among treatment groups were performed by randomized-design, 2-way ANOVA, followed by the Newman-Keuls post hoc test for more than 2 groups, or by unpaired 2-tailed Student’s t test for 2 groups using Prism software (GraphPad Inc.), as appropriate. Statistical significance was defined as a P value of less than 0.05.

Study approval. All animal experiments were performed according to NIH guidelines and were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee, Research Triangle Park, North Carolina, USA (95-18). Peripher-
al blood was collected following written informed consent under a protocol approved by the National Institute of Environmental Health Sciences Institutional Review Board, Research Triangle Park, North Carolina, USA (10-E-0063).

Data availability. Values for all data points in graphs are reported in the Supporting Data Values file.

Author contributions
Hong Li designed research studies, conducted experiments, analyzed data, and wrote the manuscript. JAB and MLE performed data acquisition and data analysis and revised the manuscript. AG designed research studies, generated reagents. Huiling Li and JPG performed data acquisition and data analysis. LMD acquired data and conducted experiments. FBL, CF, ERW, CDB, and SJL performed data acquisition and data analysis. MAS acquired data and conducted experiments. TMC provided reagents. DCZ designed research studies, analyzed data, and revised the manuscript.

Acknowledgments
This work was supported by the Division of Intramural Research, National Institute of Environmental Health Sciences, NIH (Z01-ES025034 to DCZ).

Address correspondence to: Darryl C. Zeldin, Division of Intramural Research, NIH/NIAMS, 111 T.W. Alexander Drive, Building 101, Room A214, Research Triangle Park, North Carolina 27709, USA. Phone: 984.287.3641; Email: zeldin@niehs.nih.gov.
55. Li X, Tai IH. Activation of thromboxane A2 receptor (TP) increases the expression of monocyte chemoattractant protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2) and recruits macrophages to promote invasion of lung cancer cells. PLoS One. 2013;8(1):e54073.