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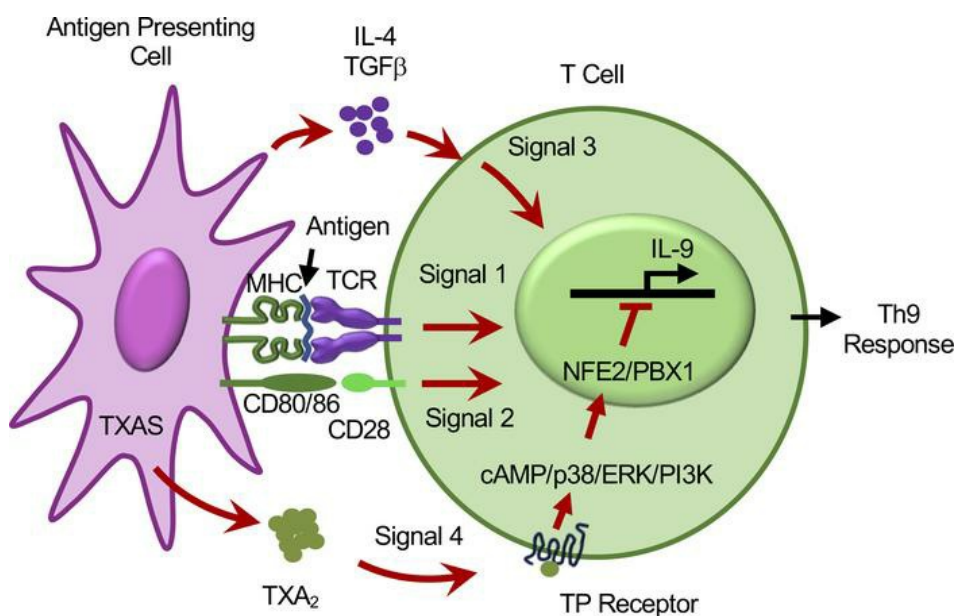
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Graphical abstract

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

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Running Title: TXA$_2$ attenuates Th9 differentiation

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Abstract

In lung, thromboxane A_2 (TXA$_2$) activates the TP receptor to induce pro-inflammatory and bronchoconstrictor effects. Thus, TP receptor antagonists and TXA$_2$ 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 TXA$_2$ reduces Th9 cell differentiation during allergic lung inflammation. Th9 cells were decreased ~2-fold and airway hyperresponsiveness was attenuated in lungs of allergic mice treated with TXA$_2$. Naïve CD4$^+$ T cell differentiation to Th9 cells and IL-9 production was inhibited dose-dependently by TXA$_2$ in vitro. TP receptor deficient mice had a ~2-fold increase in numbers of Th9 cells in lungs in vivo after OVA exposure compared to wild type (WT) mice. Naïve CD4$^+$ T cells from TP deficient mice exhibited increased Th9 cell differentiation and IL-9 production in vitro compared to CD4$^+$ T cells from WT mice. TXA$_2$ also suppressed Th2 and enhanced Treg differentiation both in vitro and in vivo. Thus, in contrast to its acute, pro-inflammatory effects, TXA$_2$ 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.

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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 (APCs) 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 naïve 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 three 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 (TCR) on the T cell. Signal 2 involves the interaction between co-stimulatory 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 naïve 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 (7-9). Interestingly, anti-IL-9 blocking antibodies inhibit allergic

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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 (COX-2)-derived prostaglandins (PGs), including PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2, regulate Th17 and Th9 cell differentiation in the allergic lung (12, 13). Thromboxane A_2 (TXA_2) is produced by the sequential actions of cyclooxygenases-1 (COX-1) or COX-2 and thromboxane synthase (TXAS, encoded by the *TBXAS1* gene) (14). TXA_2 is chemically unstable, with a biological half-life of approximately 30 seconds. TXA_2 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 TXA_2 production upon cell activation (18, 19). TXA_2 is elevated in bronchoalveolar lavage fluid (BALF) from allergic lungs (20); however, the role of TXA_2 in Th cell differentiation and function during allergic lung inflammation remains unknown.

Like other arachidonic acid-derived signaling molecules, TXA_2 exerts its actions through a specific G-protein-coupled receptor termed the thromboxane receptor (TP receptor encoded by the *TBXA2R* gene) (21). TXA_2 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 naïve $CD4^+$ T cells (24, 25). In the lung, the TP receptor is expressed in bronchial airway smooth muscle cells and other cell types (26). TXA_2

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induces expression of adhesion molecule expression in vascular endothelial cells, eotaxin-1 (CCL-11) by bronchial smooth muscle and stimulates monocyte formation of TNFα, IL-1β, IL-2, IL-5 and IFNγ (27, 28). TXA$_2$ induces bronchoconstriction, mucin secretion, plasma extravasation, vascular smooth muscle constriction, vascular smooth muscle proliferation and exacerbates airway hyperresponsiveness (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/antagonists and TP receptor-deficient mice (TP$^{-/-}$) to investigate the role of TXA$_2$ in Th9 cell differentiation and function in vitro and during allergic lung inflammation in vivo. We found that DCs express TXAS and produce TXA$_2$ which signals through the TP receptor on differentiating T cells. To further elucidate the signaling cascade through which TXA$_2$ 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 TXA$_2$-TP receptor signaling suppresses Th9 cell differentiation through recruitment of the NFE2 and PBX1 transcriptional repressors to the *Il9* promotor. Thus, while TXA$_2$ is well known for inducing inflammation and bronchoconstriction, 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 TXA$_2$/TP receptor signaling pathway may lead to the development of novel asthma treatments.

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Results

TXA$_2$ attenuates Th9 cell responses to allergen exposure in vivo

To examine the role of TXA$_2$ 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 carbocyclic TXA$_2$ (cTXA$_2$), a biologically stable TXA$_2$ 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 cTXA$_2$-treated mice compared to vehicle-treated controls. (Figure 1B). Histological sections scored by a blinded pathologist revealed decreased inflammation in lungs from cTXA$_2$-treated mice compared to 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 to mice treated with vehicle, mice implanted with cTXA$_2$-containing osmotic mini-pumps exhibited a significant decrease in the percentage of Th9 cells in the lung following OVA exposure (Figure 1E, Supplemental Figure 1). Similar results were obtained in BALF, blood and lymph nodes, but not spleen (Figure 1E, Supplemental Figures 1 and 2). Immunofluorescent microscopy provided independent confirmation of the FACS results. Lung tissue sections from allergic mice treated with vehicle or cTXA$_2$ 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$^+$IL10$^+$; white overlay) in lungs of cTXA$_2$-treated mice compared to vehicle-treated controls (Figure 1, F and G). While cTXA$_2$ treatment reduced the overall number of Th9 cells in OVA-exposed mice, it did

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not reduce the mean fluorescence intensity of IL-9$^+$ T cells (Supplemental Figure 3A). This data suggests that cTXA$_2$ regulates Th9 cell differentiation, not IL-9 expression in differentiated Th9 cells. The density of mast cells in allergic lungs were not significantly different between vehicle and cTXA$_2$-treated mice (Supplemental Figure 4, A and B). Similarly, the number of IL9$^-$IL10$^+$CD4$^+$ T cells were not significantly different between vehicle and cTXA$_2$-treated mice (Supplemental Figure 5A). Together, these results indicate that cTXA$_2$ 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, cTXA$_2$, the TP receptor antagonist iodophenyl sulfonyl amino pinane TXA$_2$ (ISAP), or cTXA$_2$ + 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, Supplemental Figure 6). Similarly, OVA/cTXA$_2$+ISAP and OVA/ISAP treated mice also displayed AHR significantly above that of non-allergic mice. Importantly, cTXA$_2$ reduced airway responsiveness to levels that were not different from non-OVA sensitized/exposed controls. The reduction in AHR by cTXA$_2$ was not significantly less than OVA/vehicle-treated mice p=0.06 but was significantly less than cTXA$_2$ and ISAP-treated mice (p<0.05). Together, these results suggest that cTXA$_2$ 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/alum sensitization primarily induces lung

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eosinophilia, OVA/LPS sensitization results in lung inflammation characterized by increased numbers of macrophages, neutrophils and lymphocytes (33). Compared to vehicle, cTXA$_2$ treatment significantly reduced BALF neutrophil numbers, but did not reduce overall lung inflammation following sensitization with OVA/LPS and airway exposure to OVA (Supplemental Figures 7, A and B). However, significant reductions in Th9 cells were observed in lungs of cTXA$_2$-treated mice relative to vehicle-treated controls (Supplemental Figure 7C). cTXA$_2$ treatment also significantly reduced Th9 cells in BALF, blood and lymph nodes, but not spleen (Supplemental Figure 7C). Thus, cTXA$_2$ suppressed Th9 cells in both the OVA/alum 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 G$_{\alpha q}$ and G$_{12/13}$) and participates in the activation of multiple signaling cascades (34). To confirm that endogenously produced TXA$_2$ regulates Th9 cells, we compared Th9 cell numbers between TP$^{+/+}$ and TP$^{-/-}$ mice during OVA-induced allergic lung inflammation. After OVA/alum sensitization and airway OVA exposure, BALF total cells, macrophages, neutrophils, lymphocytes and eosinophils were not significantly different between TP$^{+/+}$ and TP$^{-/-}$ mice (Figure 2A). Likewise, tissue sections showed no significant differences in overall inflammation between allergic TP$^{-/-}$ and TP$^{+/+}$ lungs (Figure 2, B and C). However, FACS analysis indicated that TP$^{-/-}$ mice had significantly more Th9 cells compared to TP$^{+/+}$ mice in lung, BALF, lymph nodes and blood, but not in spleen (Figure 2D, Supplemental Figure 8 and 9). Consistent with these results, immunofluorescent microscopy revealed that lungs

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from TP$^{-/-}$ mice had significantly more Th9 cells compared to lungs from TP$^{+/+}$ mice (Figure 2, E and F). TP$^{+/+}$ and TP$^{-/-}$ mice sensitized with OVA/LPS and exposed to OVA via the airway produced similar results to those observed in the OVA/alum model; BALF cell numbers and lung inflammation were not significantly different between allergic TP$^{-/-}$ and TP$^{+/+}$ lungs (Supplemental Figure 10, A and B). However, lungs, BALF and lymph nodes from allergic TP$^{-/-}$ mice had significantly more Th9 cells than corresponding tissues from allergic TP$^{+/+}$ mice (Supplemental Figure 10C). TP disruption did not alter the mean fluorescence intensity of lung or BALF IL-9^{+} cells compared to wild type (Supplemental Figure 3B). Neither mast cell numbers nor IL9^{-}IL10^{+}CD4^{+} T cell numbers were significantly different between lungs from allergic TP$^{-/-}$ vs. TP$^{+/+}$ mice (Supplemental Figures 4C and 5B). Together, these results suggtest that endogenous TXA$_2$ signaling through its canonical TP receptor attenuates Th9 cell numbers in both the OVA/alum and the OVA/LPS models of allergic lung inflammation in vivo.

TXA$_2$ *inhibits Th9 cell differentiation in vitro*

To examine the role of TXA$_2$ in Th9 cell differentiation in vitro, we isolated naïve CD4^{+}CD62L^{+} T cells by magnetic activated cell sorting (FACS, >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 cTXA$_2$, 500 nM TXB$_2$ (stable TXA$_2$ metabolite) or 300 nM U-46619 (TP receptor agonist). As shown in Figure 3A, only 0.6 ± 0.1% of untreated naïve T cells differentiated to Th9 cells after 5 days in culture. Treatment with TGF-β and IL-4 induced 4.0 ± 0.3% of naïve T cells to differentiate into Th9 cells. Interestingly, cTXA$_2$, TXB$_2$ and U-46619 significantly inhibited Th9 cell differentiation (Figure 3A and Supplemental Figure 11). cTXA$_2$ suppressed Th9 cell differentiation in a dose-

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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 cTXA$_2$ and U-46619 (Figure 3C). Moreover, TP$^{-/-}$ naïve T cells exhibited significantly increased Th9 cell differentiation compared to TP$^{+/+}$ naïve T cells as evidenced by increased *Il9, Il10* and *Irf4* mRNA levels (Figure 3D). Importantly, both cTXA$_2$ and U-46619 also inhibited Th9 cell differentiation of naïve T cells isolated from peripheral blood of healthy human volunteers as determined both by FACS (Figure 4A) and mRNA analyses (Figure 4B, Supplemental Figure 12). Therefore, in both mouse and human, differentiation of naïve T cells to Th9 cells was significantly attenuated by cTXA$_2$ and a TP receptor agonist in vitro.

TXA$_2$ suppresses DC-mediated promotion of Th9 cell differentiation in vitro

DCs are central to the orchestration of various forms of immunity. DCs modulate T cell differentiation through cytokine signaling and membrane receptors (35). DCs can mature into functionally different effector cells that potentiate Th cell subset differentiation through the specific cytokines they secrete (36-41). TXA$_2$ is known to suppress low avidity interactions between DCs with T cells and reduce DC-mediated T cell migration and proliferation (42, 43); however, it remains unknown whether TXA$_2$ also inhibits DC-mediated promotion of Th9 cell differentiation from naïve T cells. To address this question, we differentiated FACS-purified, naïve CD4$^+$CD62L$^+$ T cells with or without CD11c$^+$F4/80$^-$ DCs in the presence or absence of cTXA$_2$. Similar to the data in Figure 3, differentiation of isolated naïve T cells to Th9 cells was inhibited by cTXA$_2$ (Figure 5A, white circles). Co-culture of DC cells with naïve T cells significantly

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potentiated Th9 cell differentiation and this differentiation was still suppressed by cTXA$_2$ (Figure 5A, black circles).

Myeloid cells secrete TXA$_2$ 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 naïve CD4$^+$CD62L$^+$ T cells by FACS. None of the cell types produced significant amount of TXB$_2$ (the stable TXA$_2$ metabolite) under unstimulated conditions (Figure 5B, white circles). After activating them with LPS for 24 hours, we observed that only monocytes/interstitial macrophages (F4/80$^+$), dendritic cells (CD11c$^+$) and alveolar macrophages (CD11c$^+$F4/80$^+$) produced significant amounts of TXB$_2$, but not naïve T cells (Figure 5B, black circles). Consistent with the pattern of TXB$_2$ formation, LPS induces the thromboxane synthase (*Tbxas1*) in CD11c$^+$ cells but not naïve 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 naïve T cells treated with TGFβ and IL-4. Treatment of isolated naïve T cells with TGFβ and IL-4 increased *Tbxar2* mRNA levels and suppressed *Tbxas1* mRNA levels (Supplemental Figure 13). In mixed cultures, TGFβ and IL-4 induced expression of both *Tbxa2r* and *Tbxas1* mRNAs but cTXA$_2$ treatment did not significantly alter the expression of either gene (Figure 5D). In cells co-cultured with TGFβ and IL-4 but separated using transwells, CD4$^+$ T cells expressed higher levels of *Tbxa2r* than CD11c$^+$ cells, while CD11c$^+$ cells expressed higher levels of *Tbxas1* than CD4$^+$ cells (Figure 5E). Taken together, this data suggests that CD11c$^+$ DCs can be stimulated to

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produce TXA$_2$ which activates the TP receptor on CD4$^+$ T cells to modulate differentiation.

TXA$_2$ regulates both Th2 and Th9 cell differentiation

To determine whether TXA$_2$ 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 cTXA$_2$-loaded minipumps as depicted in Figure 1A. Forty-eight hours after the last OVA exposure, lungs were collected and the percentages of CD4$^+$IFNγ$^+$ (Th1), CD4$^+$IL-4$^+$ (Th2), CD4$^+$IL-9$^+$ (Th9), CD4$^+$IL-17$^+$ (Th17) and CD4$^+$FOXP3$^+$ (Treg) cells were determined by FACS. Lungs from cTXA$_2$-treated mice had a significantly reduced percentage of Th2 and Th9 cells compared to lungs from vehicle-treated mice (Figure 6A). In contrast, the Th1 and Th17 cell percentages were not affected by cTXA$_2$. Interestingly, lungs from cTXA$_2$-treated mice had a significantly increased percentage of Treg cells compared to lungs from vehicle-treated mice (Figure 6A). The cTXA$_2$-induced reduction of Th2 and Th9 cells in allergic mice was reversed by the TP receptor antagonist ISAP (Supplemental Figure 14). cTXA$_2$ 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 two pathways (45); they can directly differentiate to Th9 cells in the presence of IL-4 and TGFβ, or they can differentiate to Th9 cells in a two-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 TGFβ (Figure 6B). TXA$_2$ may regulate Th9 differentiation at either step of the process, or it may regulate all three steps. To determine if TXA$_2$ can

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regulate Th2 cell differentiation, we incubated naïve T cells (MACS, >95% pure) with IL-4 alone in the presence or absence of cTXA$_2$. IL-4 induced the canonical Th2 transcription factor *Gata3* and the Th2 cytokine *Il4* (Figure 6C). Expression of both *Gata3, Il4* and *Il13* were significantly inhibited by cTXA$_2$ suggesting that cTXA$_2$ regulates Th2 cell differentiation from naïve T cells (Figure 6C and 6D). To determine if TXA$_2$ can regulate Th9 cell differentiation, we incubated naïve T cells with IL-4 and TGFβ together in the presence or absence of cTXA$_2$. Incubation of naïve T cells with TGFβ and IL-4 induced expression of the Th9 cell markers *Irf4* and *Il9* (Figure 6D) with minimal induction of the Th2 cell markers *Gata3* and *Il4* (Figure 6C). cTXA$_2$ attenuated differentiation of naïve T cells directly to Th9 cells. Finally, to determine whether TXA$_2$ 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 TGFβ in the presence or absence of cTXA$_2$. Incubation of naïve T cells with IL-4 initially, then IL-4 and TGFβ together induced expression of the Th9 cell markers *Irf4* and *Il9* (Figure 6, D and E). cTXA$_2$ attenuated differentiation of Th2 cells to Th9 cells. Therefore, cTXA$_2$ 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, cTXA$_2$ 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 TGFβ + IL-4; however, this was not a point of regulation by cTXA$_2$.

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Thromboxane also regulates differentiation of naïve 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 TGFβ increased Th9 cells (IL4$^+$CD4$^+$) and induced *Irf4* and *Il9* mRNAs, effects that were significantly attenuated by cTXA$_2$ treatment (Figure 7A). Similarly, IL-4 increased Th2 cells (IL4$^+$CD4$^+$) and induced *Gata3* and *Il4 mRNAs*, effects that were also significantly attenuated by cTXA$_2$ (Figure 7B). TGFβ increased Treg cells (FOXP3$^+$CD4$^+$) and induced *Fox3p* mRNA (Figure 7C). Interestingly, cTXA$_2$ treatment resulted in a non-significant increase in Treg cells and caused a further significant induction of *Fox3p* mRNA (Figure 7C). These data suggest that cTXA$_2$ regulates differentiation of naïve T cells to T2, Th9 and Treg cell subsets in both mice and humans.

Involvement of cAMP/PKA and p38 signaling cascades in Th9 differentiation

TXA$_2$ 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 TXA$_2$-TP receptor signaling pathways involved in Th9 cell differentiation, we first examined the effect of cTXA$_2$ treatment and TP receptor knockout on intracellular cAMP levels in vitro. As shown in Figure 8A, cTXA$_2$ increased cAMP levels by approximately 50% compared to vehicle in naïve T cells treated with TGFβ and IL-4 to induce Th9 cell differentiation. Moreover, treatment of TP$^{+/+}$ naïve T cells with TGFβ and IL-4 resulted in increased intracellular

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cAMP levels; however, this increase did not occur when TP$^{-/-}$ naïve T cells were treated with TGFβ and IL-4 (Figure 8B). Next, we examined the effect of cTXA$_2$ on phosphorylation of p38 MAPK. cTXA$_2$ enhanced p38 MAPK phosphorylation in naïve T cells treated with TGFβ and IL-4 (Figure 8C, Supplemental Figure 17). Consistent with this data, phosphorylation of p38 MAPK appeared reduced in TP$^{-/-}$ naïve T cells treated with TGFβ and IL-4 relative to TP$^{+/+}$ naïve T cells (Figure 8C, Supplemental Figure 17). The importance of cAMP/PKA and p38 MAPK in the cTXA$_2$-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 significantly alter the ability of cTXA$_2$ to inhibit Th9 cell differentiation (Figure 8D). We were unable to confirm the role of p38 MAPK in the cTXA$_2$ 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 TXA$_2$ effect.

TXA$_2$ 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, little is known about the role of PBX1, NFE2 or CREB in regulation of Th9 cell differentiation or function. Interestingly,

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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 naïve T cells in vitro (Figure 9B). Consistent with the role of IRF4 in Th9 cell differentiation, treatment of naïve T cells with TGFβ and IL-4 increased *Irf4* mRNA expression. cTXA$_2$ suppressed the induction of *Irf4* mRNA by approximately 50%. Notably, cTXA$_2$ suppression of *Irf4* mRNA was less pronounced than cTXA$_2$ suppression of *Il9* mRNA suggesting that cTXA$_2$ does not act solely through IRF4 to regulate Th9 cell differentiation. *Pbx1* and *Nfe2* mRNAs are both abundant in naïve T cells and treatment with TGFβ 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 cTXA$_2$ increased *Pbx1* expression and restored *Nfe2* expression close to that in naïve T cells. Thus, suppression of IL-9 by cTXA$_2$ may, at least in part, be due to restoration of the basal repression of the *Il9* promoter by PBX1 and NFE2. Treatment of naïve T cells with TGFβ and IL-4 to induce Th9 cell differentiation increased expression of *Creb* mRNA; however, cTXA$_2$ had no significant effect on *Creb* expression. Expression of *Pu.1* mRNA was low/undetectable in mouse naïve T cells and not changed during Th9 cell differentiation or by cTXA$_2$ treatment (data not shown).

Since cTXA$_2$ increased expression of *Pbx1* and *Nfe2* during Th9 cell differentiation, we examined whether it also influenced binding of these two transcription factors to the *Il9* promoter. We performed chromatin immunoprecipitation using PBX1

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or NFE2 specific antibodies followed by qPCR and direct sequencing of PCR products (Figure 9C, Supplemental Figure 18). $cTXA_2$ 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 TXA_2, we made a series of luciferase reporter constructs containing varying lengths of the mouse *Il9* promoter (Figure 10A, Supplemental Table 1). All of these constructs were transfected into 293T cells and luciferase activity was measured after correcting 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 $cTXA_2$. This indicates that the TXA_2 sensitive elements are contained within this V2 construct. We next used site-directed mutagenesis to disrupt the PBX1, NFE2 or CREB binding sites in the V2 promoter construct. Disruption of PBX1 or NFE2 or both binding sites increased promoter activity. This is consistent with the known repressor function of these two transcription factors. Importantly, disruption of NFE2 and PBX1 binding sites abolished the ability of the $cTXA_2$ to repress *Il9* promoter activity. Disruption of the CREB binding site reduced promoter activity suggesting that CREB is a transcriptional activator of the *Il9* promoter. This construct was not further inhibited by $cTXA_2$. Taken together, these data indicate that PBX1 and NFE2 likely mediate repression of the *Il9* promoter by TXA_2.

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Discussion

The well-described role of TXA$_2$ as a potent bronchoconstrictor suggested a novel therapeutic approach for allergic lung disease; however, TXAS 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: 1) cTXA$_2$ decreases lung inflammation, airway hyperresponsiveness and numbers of Th2, Th9 and Treg cells in the allergic mouse lung in vivo; 2) Allergic TP receptor knockout mice have increased numbers of Th9 cells in vivo; 3) cTXA$_2$ suppresses and TP receptor knockout enhances differentiation of naïve T cells to Th9 cells in vitro; 4) cTXA$_2$ is produced by myeloid cells and Th9 cells express the TP receptor; 5) cTXA$_2$ enhances and TP receptor knockout suppresses activation of p38 MAPK and cAMP/PKA signaling pathways; and 6) cTXA$_2$ induces NFE2 and PBX1 transcription factor binding to, and repression of, the *Il9* promotor. Thus, although anti-TXA$_2$ therapies attenuate bronchoconstriction, they may exacerbate the asthma phenotype by promoting Th9-mediated inflammation.

Our findings add to a growing body of evidence supporting the role of TXA$_2$ in regulation of immune cell function. Mouse thymus and spleen have the highest expression of TP receptors compared to other organs (55). Leung and Mihich first demonstrated that TXA$_2$ had immuno-regulatory; suppression of TXA$_2$ production inhibited splenocyte proliferation (56). Similarly, others have shown that TXAS inhibitors and TP receptor antagonists inhibit splenocyte proliferation but lack an additive or synergistic effect (57). Both mitogen- and antigen-induced spleenocyte proliferation responses are impaired in TP$^{-/-}$ spleen cells (58). More recent studies indicated that

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TXA$_2$-TP signaling dampens acquired immunity by suppressing the interactions between DCs and T cells (43). In our studies, cTXA$_2$ suppressed differentiation of purified naïve T cells to Th9 cells in the presence or absence of co-stimulatory DC cells suggesing that it directly suppresses Th9 cell differentiation and IL-9 production, with little or no effect on DCs.

TXA$_2$ 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 immune function (59). Our results showed that CD11c$^+$ and/or F4/80$^+$ myeloid cells express TXAS and produce the stable metabolite of TXA$_2$ (TXB$_2$) after stimulation with LPS. In contrast, the literature has conflicting evidence regarding TXAS expression and TXA$_2$ production by T cells. TXAS was reported to be absent in thymic lymphocytes (24); however, TXB$_2$ can be produced by some CD4$^+$ T cell subsets under certain conditions (43). Our results showed that naïve CD4$^+$ T cells do not produce significant amounts of TXB$_2$ even after LPS stimulation. In contrast, naïve T cells express the TP receptor and that expression is upregulated during Th9 differentiation. Thus, our data are consistent with a model wherein myeloid cells produce TXA$_2$ that binds to the TP receptor on T cells to inhibit Th9 cell differentiation.

TXA$_2$-TP signaling regulates cell function through multiple signaling pathways (60, 61). The precise signaling mechanisms though which TXA$_2$-TP receptor activation suppresses Th9 cell differentiation remain unknown. Our data suggest that TXA$_2$-TP receptor signaling can increase cAMP levels and activate p38 MAPK during Th9 cell differentiation. Interestingly, PKA inhibition modestly enhanced Th9 cell differentiation,

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but it did not significantly alter the ability of cTXA$_2$ 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 TXA$_2$ effect.

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), FOXO1 (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 naïve T cells, downregulated during Th9 cell differentiation and were known transcriptional repressors (67, 68). Interestingly, ChIP-qPCR analysis which showed that cTXA$_2$ 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 cTXA$_2$. Together, these data suggest that TXA$_2$ 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 TXA$_2$ 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

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example, PGE_2 can shift the balance of $CD4^+$ helper T 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 PGE_2 formation in vivo and increases Th2-mediated lung inflammation (71). PGD_2 has been reported to stimulate chemotaxis of Th2 cells (72). PGI_2 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 $cTXA_2$ 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, $cTXA_2$ 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 discrepencies 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 regards to the characteristics of the allergic response. Our data is consistent with others who find that LPS induces a lower level of lung inflammation than other aduvants (33). In our experiments, OVA/alum (Inflamation scores ~3, Figures 1D and 2C) induced more inflammation than OVA/LPS (Inflamation scores ~1, Supplemental Figures 7B and 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 to a pronounced eosoinophilic

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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 cTXA$_2$ 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 cTXA$_2$ 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 cTXA$_2$ 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 TXA$_2$ in human naïve T cells isolated from peripheral blood of healthy volunteers. Indeed, both cTXA$_2$ and the TP receptor agonist U46619 significantly attenuated Th9 cell differentiation of human naïve T cells ex vivo while the TP receptor antagonist ISAP was able to reverse the effects of cTXA$_2$. 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 possiblity that polymorphisms in the *TBXAS1* or *TBXA2R* genes may be associated with altered asthma risk in humans. Functional polymorphisms in these two 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 *TBXA2R 924C/T* polymorphism is associated with asthma risk and the *TBXA2R 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 catalytic activity and was protective in

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aspirin-intolerant asthma in a Korean population (77). Further work is necessary to determine if these or other polymorphisms in TXAS/TP receptor pathway genes are associated with allergic airway inflammation or asthma in other populations.

In summary, TXA_2/TP receptor signaling attenuates lung Th9 cell differentiation during OVA-induced allergic lung inflammation in vitro and in vivo. Within the immune synapse, TXA_2 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, TXA_2 induces p38 MAP kinase 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 spasmogenic effects, thromboxane exerts immunosuppressive effects which 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 for 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. Tissue culture media and supplements were from Gibco/Thermo Fisher. Male and female C57BL/6J mice (6-10-weeks of age) were purchased from Jackson Laboratories. Male $TP^{+/+}$ and $TP^{-/-}$ mice (6-10-week-old) on a pure C57BL/6 background (backcrossed >10 generations) were provided by Dr. Thomas Coffman (Duke University).

Ovalbumin-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) as an adjuvant by intraperitoneal injection on Days 0 and 1; 14–21 days later, mice were exposed to 1% OVA ($\geqslant$ 98% pure by agarose gel electrophoresis) in saline (or saline only) via inhalation for 30 minutes per day for four consecutive days. Vehicle (15% ethanol in PBS), $cTXA_2$, and/or Iodophenyl sulfonyl amino pinane TXA_2 (ISAP) (1 μmol/mouse/day) were delivered one week prior to airway OVA exposure via subcutaneously implanted osmotic minipumps (model 1007D; 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 O.C.T. compound (International Medical Equipment) at 25 cm $H_2O$and frozen on dry ice. Lung sections were fixed in methanol with 0.3% H_2O_2 for 10 min at 4° C and permeabilized with Triton x-100 (0.8%) for 10 min 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 hr at room temperature (Biolegend, Cat. Nos. 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 Axioplan 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 image J software (NIH).

In vitro differentiation of naïve T cells. Lung and spleen CD4$^+$ T cells were isolated by Magnetic-Activated Cell Sorting (MACS). Naïve CD4$^+$ T cells were isolated using the CD4$^+$CD62L$^+$ isolation kit (Miltenyi Biotec,; ~95% purity) or by fluorescent 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 302902; 1 µg/ml) and differentiated to Th1

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(with IL-12, 20 ng/ml), Th2 (IL-4, 20 ng/ml), Th9 (IL-4, 20 ng/ml and TGFβ, 2 ng/ml), Th17 (TGFβ, 10 ng/ml and IL-6, 30 ng/ml), and Tregs, (TGFβ, 10 ng/ml). In some experiments, cells were treated with $cTXA_2$ (7.5-300 nM), TXB_2 (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 incubating samples at 4°C for 20 min. 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 kit (BD Bioscience, San Jose, CA) using the PE-conjugated anti-IL-9 and APC-conjugated anti-IL-10 mAbs. Analysis of other T helper subsets were performed using anti-CD4 with IFNg (Th1; Biolegend 505807), IL-4 (Th2; Biolegend 50413) or FOXP3 (Treg; Biolegend 126403), or IL-17 (Th17; Biolegend 506915).   Samples were analyzed by FACS (BD LSRFortessa with HTS option) using *LSR II* and FlowJo software (Tree Star).

Naïve 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 min. 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 U/ml streptomycin, 100 mM sodium pyruvate, L-glutamine and nonessential amino acids (NEAAs). We used EasySep Mouse T cell Negative Selection kit or Mouse CD4⁺

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T cell enrichment kit as described in the manufacturer's instructions (STEMCELL Technologies). Naïve CD4$^+$ T cells were sorted based on staining by anti-mouse CD4-APC-Cy7 (Biolegend 100414) and anti-mouse CD62L-PE (Biolegend 161204). Sorted naïve CD4$^+$ T cell purities were >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). 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 the BD FACSAria II instrument.

Eicosanoid analysis of myeloid cells and naïve CD4$^+$ T cells. Purified myeloid cell subsets (CD11c$^+$, F4/80$^+$ and CD11c$^+$F4/80$^+$) and naïve CD4$^+$ T cells were either unstimulated or stimulated with LPS (1 µg/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 co-cultures. Lung DC-T cell co-cultures were performed in 24-well flat-bottom culture plates. Briefly, purified DCs (CD11c$^+$) from lung were treated with LPS (1 µg/ml) overnight, afterwhich the culture supernatants were removed, and the cells were extensively washed and resuspended in RPMI/10% FCS. Naïve CD4$^+$ T

cells (1×10^6/well) were then co-cultured with the DCs (1×10^5/well) in the presence of anti-CD3 (2 ug/ml), anti-CD28 (1 ug/ml), IL-4 (20 ng/ml) and TGFβ (2 ng/ml) for 5 days in 1ml 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 naïve T cells (2×10^6/ml) from C57BL/6, TP$^{+/+}$ and TP$^{-/-}$ mice were treated with vehicle, cTXA$_2$ and cytokines as described. cAMP levels were determined using a cAMP enzyme immunoassay kit following the manufacturer's instructions (Cayman Chemical, Cat No: 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* promoters 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) per Supplementary Table 1. Promoter fragments were sub-cloned into the promoterless pGL4-10 luciferase reporter vector (Promega E6651) and transfected into Jurkat T cells (Jurkat, Clone E6-1, ATCC® TIB-152). Twenty-four hours after transfection, cells were treated with 300 nM cTXA$_2$ or vehicle for 4 hours. Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP)-qPCR. Naïve CD4$^+$ T cells were either untreated or treated with TGFβ and IL-4 in the presence of vehicle or 300 nM cTXA$_2$. Chromatin immunoprecipitation (ChIP) assays were performed following the

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manufacturer's instructions (Agarose ChIP kit 26156; Pierce/Thermo Fischer). The fragmented DNA samples obtained from the ChIP with anti-NFE2 (Abcam ab140598), anti-PBX1 (ThermoFisher PA5-17223) and anti-IgG Abs (ThermoFisher 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 (AB System) were used to amplify *Il9*, *Il10*, *Irf4*, *PU.1* and *Gapdh* Taqman primers (*Il9*: Mm00434304_m1; *Il10*: Mm01288386_m1; *Irf4*: Mm00516431_m1 and *PU.1*[spic]: Mm00488428_m1; *Tbxa2r:* 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, 100 nM, Cayman) and p38 MAPK (SB203580, 1 μg/ml, Cayman) were used in Th9 cell differentiation studies in vitro*.*

Statistical analysis: Data are presented as means ± SEM. Statistical comparisons among treatment groups were performed by randomized-design, one-way ANOVA or multiple t-test comparisons with Holm-Sidak correction, or by unpaired Student's t-test for two groups using Prism 10.1 software (GraphPad Inc.), as indicated. Statistical significance was defined as a P value of less than 0.05.

Study approval: All animal experiments were performed according to National Institutes of Health guidelines and were approved by the National Institute of

Environmental Health Sciences Animal Care and Use Committee, RTP, North Carolina (95-18). Peripheral blood collected following written informed consent under a protocol that was approved by the National Institute of Environmental Health Sciences Institutional Review Board, RTP, North Carolina (10-E-0063).

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

Author Contributions:

H.L. (Hong Li) designed research studies, conducted experiments, analyzed data and wrote the manuscript.

J.A.B. performed data acquisition, data analysis and revised the manuscript.

M.L.E. performed data acquisition, data analysis and revised the manuscript.

A.G. designed research studies and generated reagents.

H.L. (Huiling Li) performed data acquisition and data analysis

J.P.G. performed data acquisition and data analysis.

L.M.D. acquired data and conducted experiments.

F.B.L. performed data acquisition and data analysis.

C.F. performed data acquisition and data analysis.

E.R.W. performed data acquisition and data analysis.

C.D.B. performed data acquisition and data analysis.

S.J.L. performed data acquisition and data analysis.

M.A.S. acquired data and conducted experiments.

T.M.C. provided reagents.

D.C.Z. designed research studies, analyzed data and revised the manuscript.

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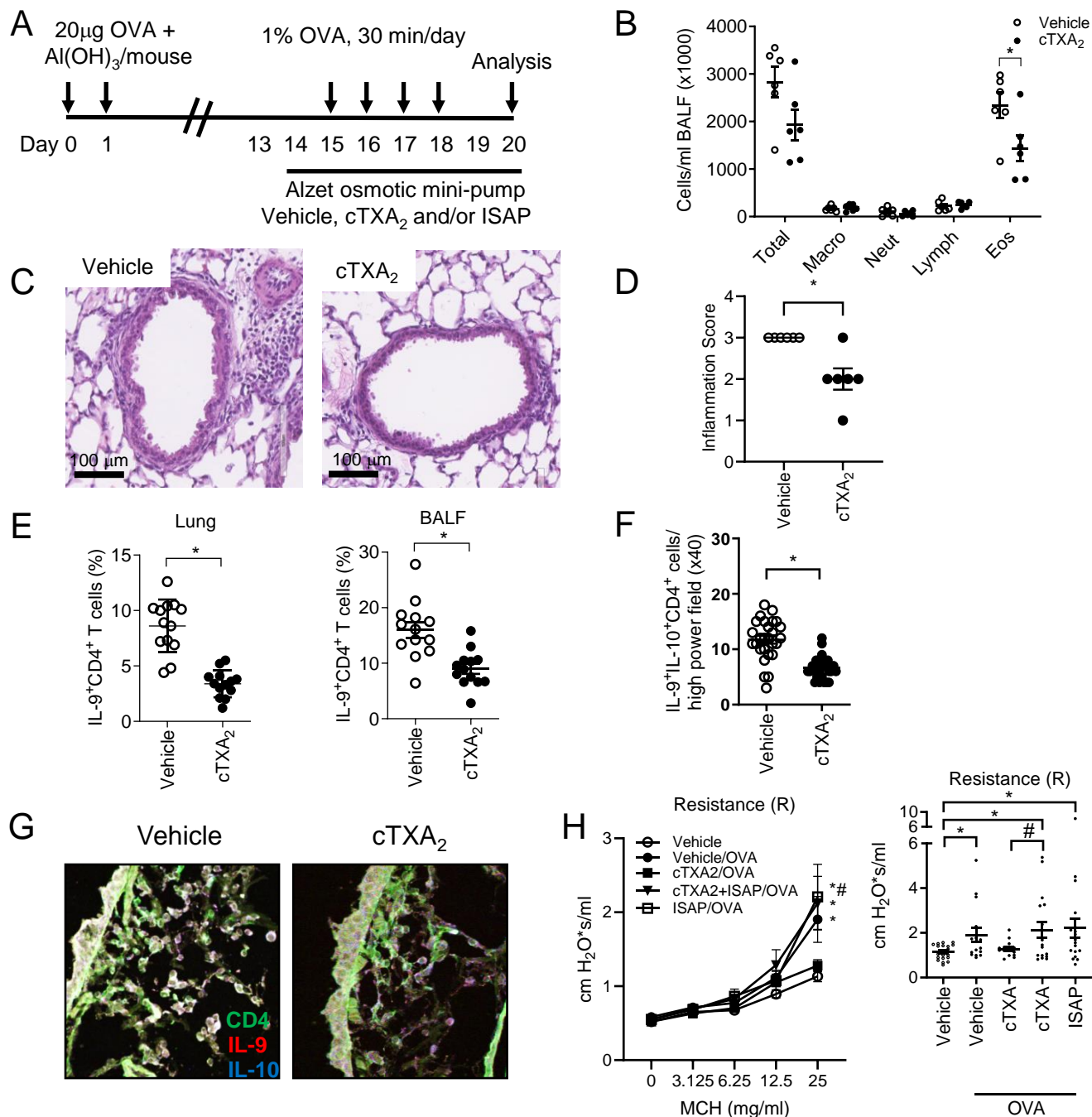
Figure 1. cTXA$_2$ attenuates Th9 cell responses to allergen exposure in vivo. A) Mice were sensitized with OVA/alum and exposed OVA in the presence of vehicle or cTXA$_2$ and/or ISAP (delivered by osmotic minipumps) as indicated. **B)** Total cell number and cell differentials in BALF were analyzed 48 hours after the last airway OVA exposure (N=6/group, *p<0.05). **C)** H&E-stained lung sections from vehicle- and cTXA$_2$-treated mice after OVA sensitization/exposure. **D)** Scoring of lung sections revealed decreased inflammation in lungs from cTXA$_2$-treated mice compared to vehicle-treated controls (N=6/group, *p<0.05; note that the lack of an error bar in the vehicle group is because all vehicle-treated lungs receiving a score of 3, "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). Th9 cells in mouse lung tissue sections were visualized by immunofluorescent staining using anti-IL-9, anti-IL-10 and anti-CD4 antibodies. All images are shown at original magnification X 40. Quantitation **(F)** of the number of IL-9$^+$IL-10$^+$CD4$^+$ T cells per high power field and representative 40X images **(G)** are shown; N=7 lungs per group, 5 HPF/lung, *p<0.05. **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, cTXA$_2$, cTXA$_2$+ISAP or ISAP alone. N=15-20/group, *p<0.05. vs. non-allergic (vehicle), # p<0.05 vs. OVA sensitized/exposed cTXA$_2$-treated mice. Significance for panels B was evaluated using multiple t-tests, panels D through F were evaluated by t-test and panel H was evaluated by one-way ANOVA.
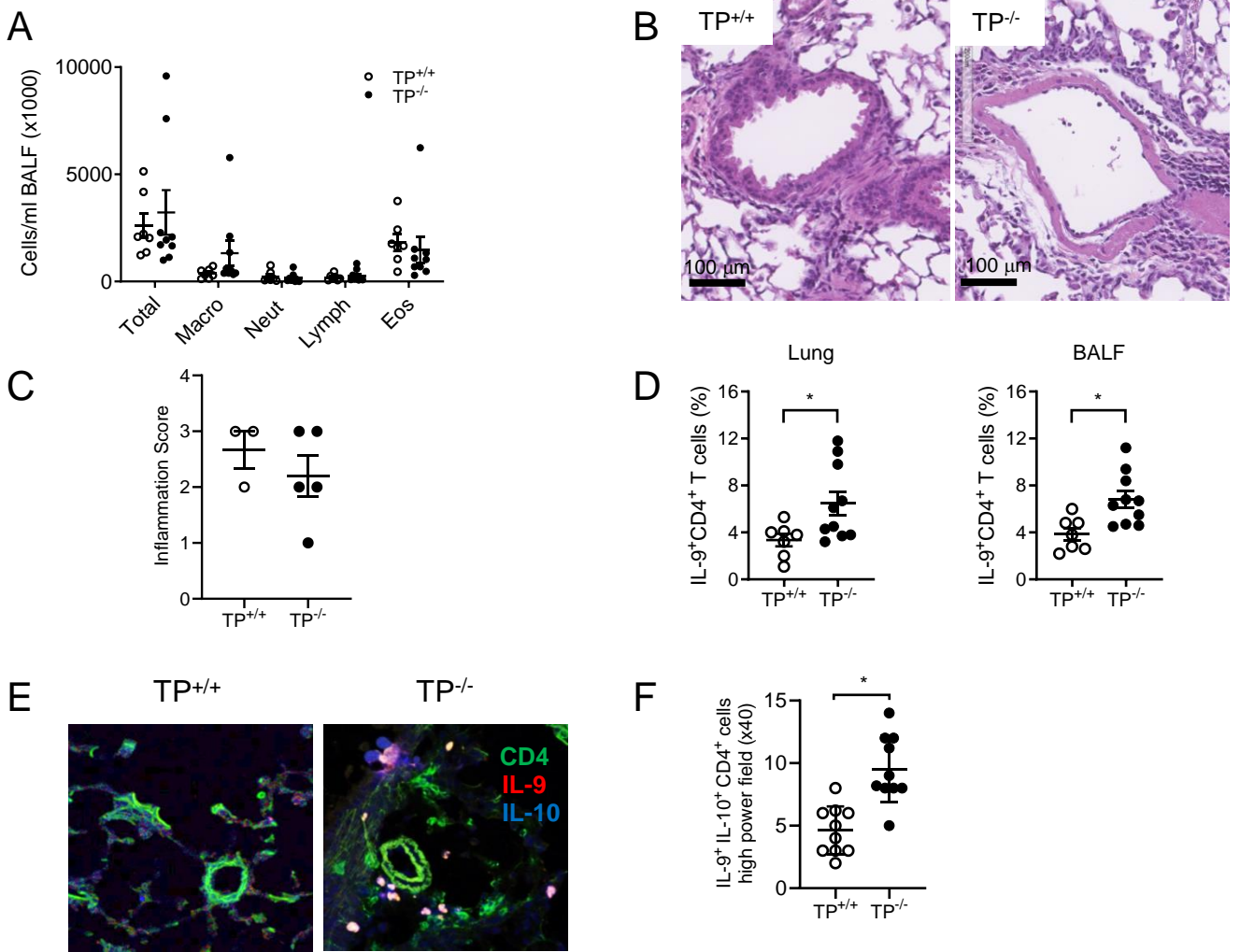
Figure 2. TP⁻ᐟ⁻ mice have increased Th9 cell responses to allergen exposure in vivo. TP⁺ᐟ⁺ and TP⁻ᐟ⁻ mice were sensitized to OVA/alum and exposed to OVA via the airway as described in Methods. **A)** Total cell number and cell differentials from BALF were analyzed 48 hours after the last OVA exposure (N=10/group). **B)** H&E-stained lung sections from TP⁺ᐟ⁺ and TP⁻ᐟ⁻ mice after OVA sensitization/exposure. **C)** Scoring of lung sections revealed no differences in overall inflammation between allergic TP⁺ᐟ⁺ and TP⁻ᐟ⁻ lungs (N=8/group). **D)** Percent IL-9⁺CD4⁺ T cells in the lung and BALF after OVA-induced allergic lung inflammation. N=7-10 mice per group, *p<0.05. **E)** Th9 cells in mouse lung tissue sections were visualized by immunofluorescent staining using anti-IL-9, anti-IL-10 and anti-CD4 antibodies. All images are shown at original magnification X 40. F) Quantitation of the number of IL-9⁺IL-10⁺CD4⁺ T cells in lung; N=5 lungs per group, 2 HPF/lung, *p<0.05. Significance for panel A was evaluated by multiple t-test and panels C, D and F were evaluated by t-test.
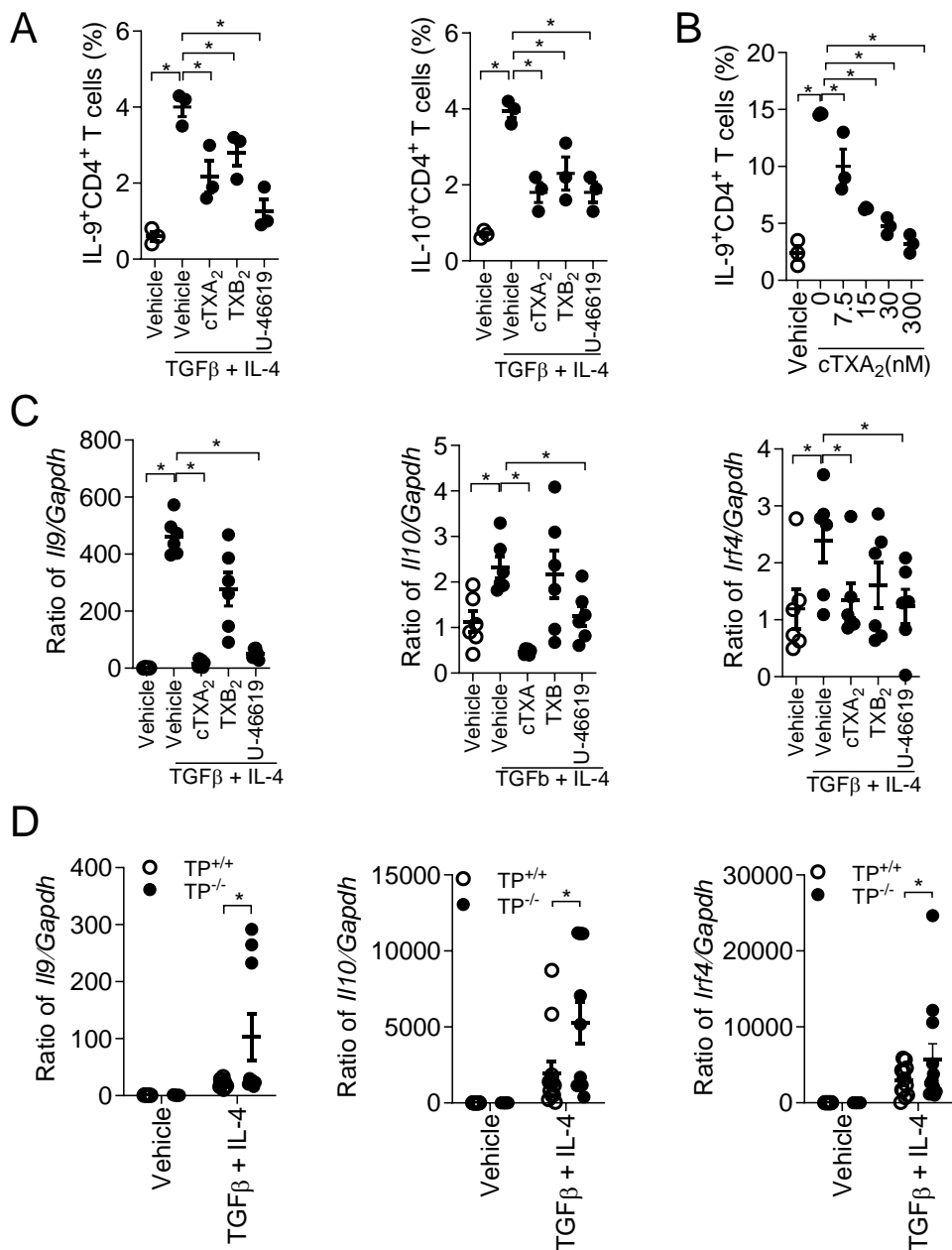
Figure 3. TXA₂ inhibits Th9 cell differentiation in vitro. A) Naïve CD4⁺CD62L⁺ T cells were purified by FACS and treated with TGFβ and IL-4 in presence of anti-CD28 and anti-CD3 to induce Th9 cell differentiation in vitro. During differentiation, cells were treated with either vehicle, 300 nM cTXA₂, 300 nM U-46619 (TP receptor agonist) or 500 nM TXB₂ (stable TXA₂ metabolite). Compared to vehicle-treated controls, treatment with cTXA₂, TXB₂ or U-46619 significantly attenuated differentiation to IL-9⁺CD4⁺ and IL-10⁺CD4⁺ T cells (N=3/group, *p<0.05). **B)** Inhibition of Th9 differentiation by cTXA₂ was dose-dependent with significant effects at 7.5 nM (N=3/group, *p<0.05). **C)** cTXA₂ and U-46619 also decreased *Il9*, *Il10* and *Irf4* mRNA levels during Th9 differentiation (N=6/group, *p<0.05). **D)** Naïve CD4⁺CD62L⁺ T cells from either TP⁺/⁺ or TP⁻/⁻ mice were treated with vehicle or TGFβ and IL-4 to induce Th9 cell differentiation. Th9 cell differentiation was enhanced in TP⁻/⁻ T cells compared to TP⁺/⁺ T cells as demonstrated by increased *Il9, Il10* and *Irf4* mRNA levels. N=5-6 per group, *p<0.05. Significance for panels A-C were evaluated by one-way ANOVA. Panel D was evaluated by t-test.
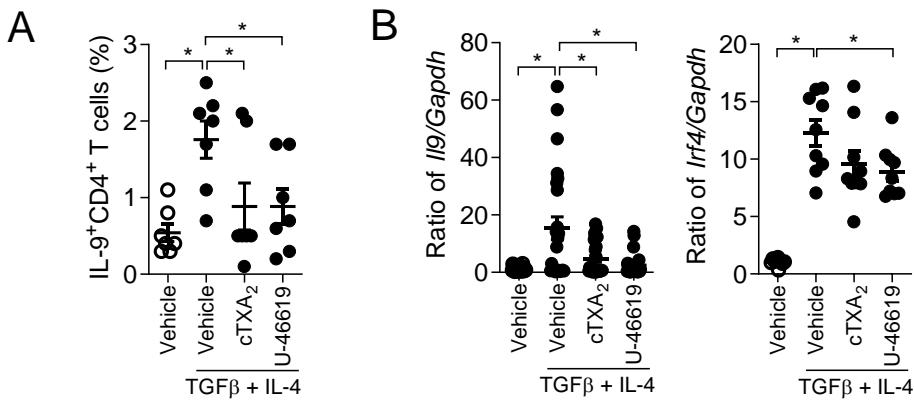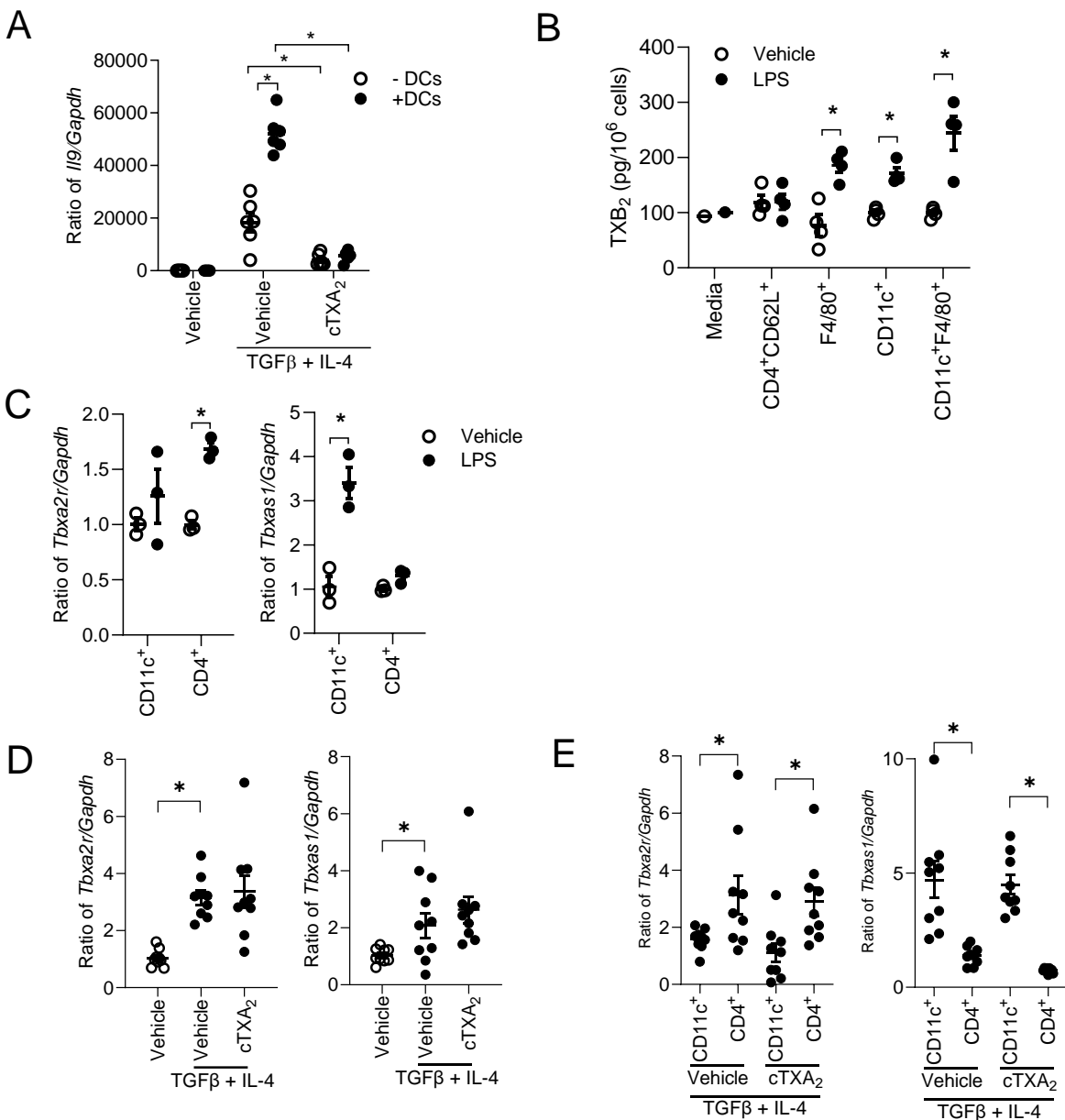
Figure 4. TXA$_2$ inhibits human Th9 cell differentiation in vitro. Naïve CD4^{+} T cells were purified from peripheral blood of healthy subjects and differentiated to Th9 cells with TGFβ and IL-4. cTXA$_2$ and U-46619 significantly attenuated human Th9 cell differentiation as determined by (**A**) percentages of IL-9^{+}CD4^{+} cells by FACS analysis, and (**B**) *Il9*, *Il10* and *Irf4* mRNA levels. N=5-12 per group, *p<0.05. Significance for all panels were evaluated by one-way ANOVA.

Figure 5. TXA₂ inhibits promotion of Th9 cell differentiation by DCs in vitro. A) Co-culture of purified naïve CD4⁺CD62L⁺ T cells with CD11c⁺ DCs (from lung) enhanced Th9 cell differentiation compared to naïve T cells alone. Treatment with 300 nM cTXA₂ significantly impairs Th9 cell differentiation of naïve T cells, whether cultured alone or in the presence of DCs. N=10 per group, *p<0.05. **B)** Purified naïve 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 naïve 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. Mixed cultures of CD11c⁺ and CD4⁺ T cells **(D)** or transwell-separated CD11c⁺ and CD4⁺ T cells **(E)** were incubated with vehicle, cTXA₂, and TGFβ + IL-4 as indicated and assayed for *Tbxa2r* and *Tbxas1* mRNA levels. N=9, *p<0.05. Significance for panels A and D were evaluated by one-way ANOVA. Panels B, C and E were evaluated by multiple t-tests.
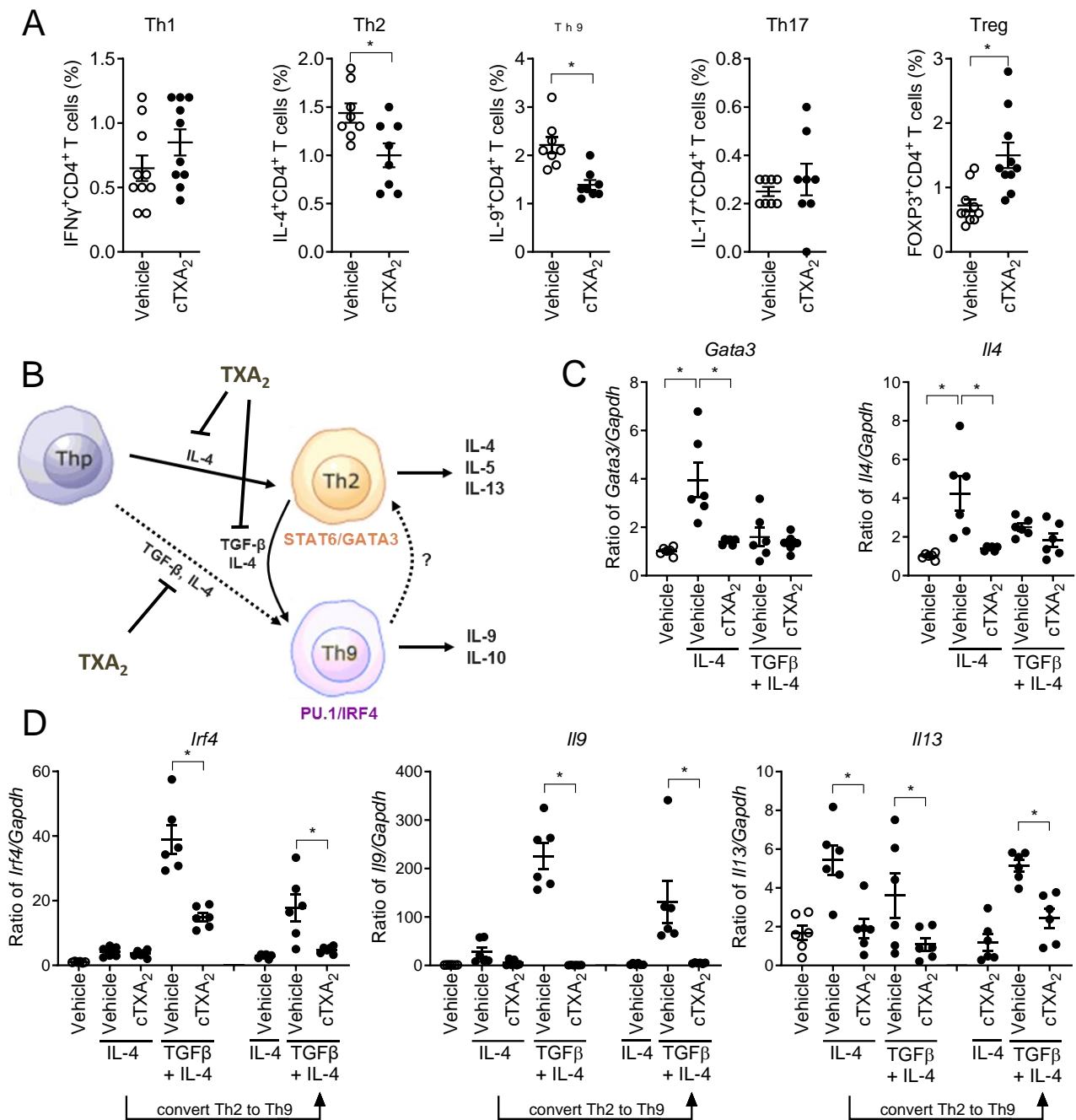
Figure 6. TXA₂ inhibits Th2 cell differentiation in vivo and in vitro. Mice were sensitized with OVA/alum and exposed to OVA via the airway as depicted in Figure 1A. **A)** Forty-eight hours after the last OVA exposure, the percentages of Th1, Th2, Th9, Th17 and Treg cells were determined by FACS analysis. N=8, *p<0.05. **B)** Th9 cells can differentiate directly from naïve T cells or from Th2 cells. **C)** IL-4 alone, but not TGFβ and IL-4, induced Th2 cell differentiation from naïve T cells as determined by mRNA levels of Th2 markers *Gata3* and *Il4*. cTXA₂ treatment attenuated Th2 cell differentiation from naïve T cells. **D)** TGFβ and IL-4, but not IL-4 alone, induced Th9 cell differentiation from naïve T cells as determined by mRNA levels of Th9 markers *Irf4, Il9 and Il13.* cTXA₂ attenuated Th9 cell differentiation directly from naïve T cells. Naïve T cells were treated with IL-4 alone to generate Th2 cells, and then the Th2 cells were treated with TGFβ 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 using t-tests for panel A and one-way ANOVA for panels C and D.
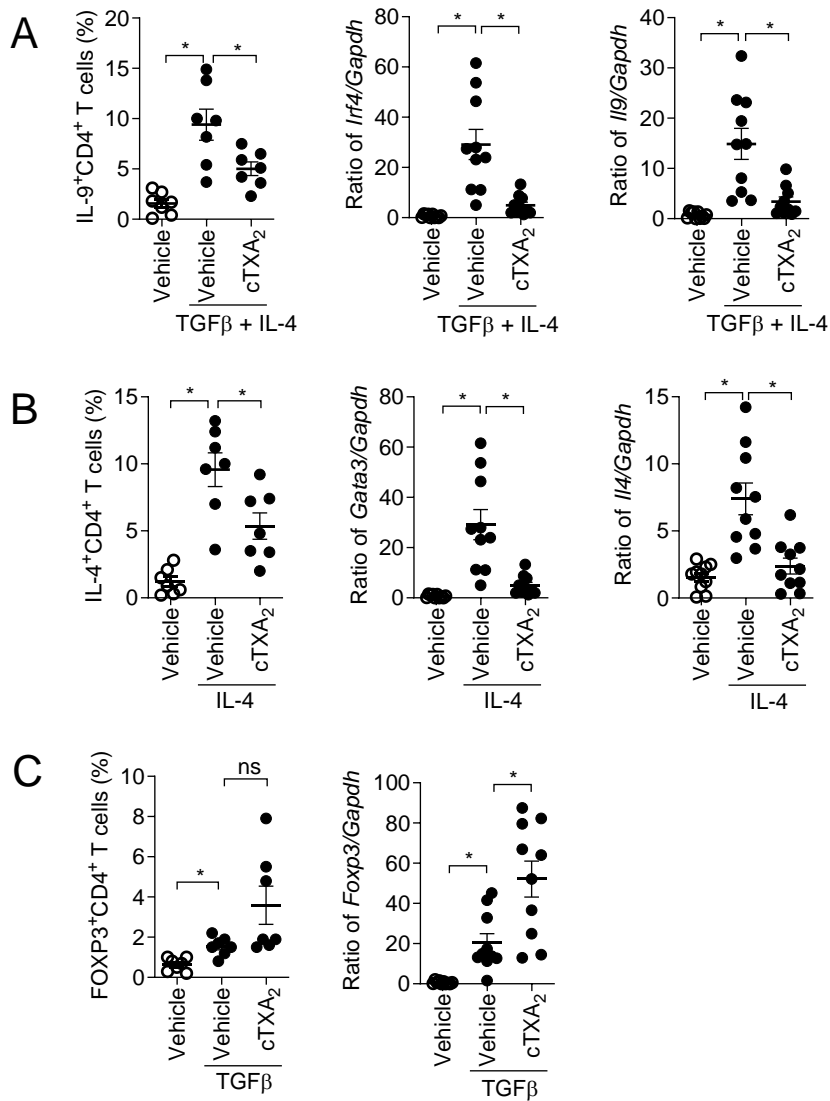
Figure 7. cTXA$_2$ decreases human Th2 and Th9 cell differentiation and increases Treg cell differentiation in vitro. Naïve CD4$^+$ T cells were purified from peripheral blood of healthy subjects and differentiated to Th2 cells with IL-4, to Th9 cells with TGFβ + IL-4 or to Treg cells with TGFβ. **A)** cTXA$_2$ significantly attenuated human Th9 differentiation as determined by percentages of IL-9$^+$CD4$^+$ cells and expression of Irf4 and Il9 mRNA. **B)** cTXA$_2$ significantly attenuated human Th2 cell differentiation as determined by the percentages of IL-4$^+$CD4$^+$ cells and expression of Gata3 and Il4 mRNAs. **C)** cTXA$_2$ treatment during TGFβ-induced Treg differentiation revealed a non-significant increase in FOXP3$^+$CD4$^+$ cells, and a similar but significant increase in *Foxp3* mRNA levels. N=8 per group, *p<0.05. Significance was determined by one-way ANOVA for all panels.
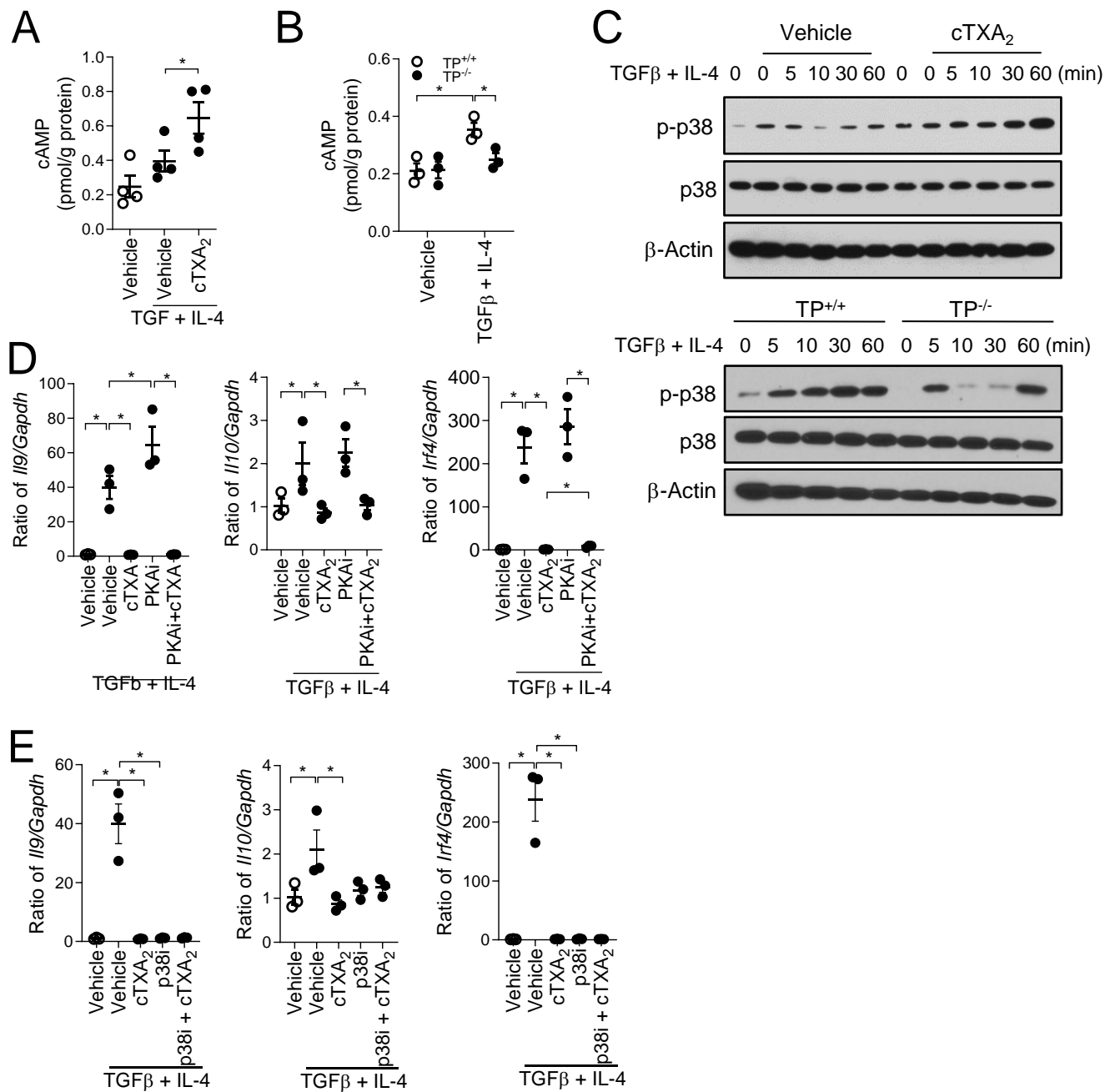
Figure 8. Role of cAMP/PKA and p38 MAPK signaling in Th9 cell differentiation. A) Naïve T cells were incubated with TGFβ and IL-4 to induce Th9 cell differentiation in the presence/absence of cTXA$_2$ and intracellular cAMP levels were measured by ELISA. cAMP levels were increased by cTXA$_2$ during Th9 cell differentiation. N=5 per group, *p<0.05. **B)** Naïve T cells from TP$^{+/+}$ and TP$^{-/-}$ mice were incubated with vehicle or TGFβ and IL-4 to induce Th9 cell differentiation. cAMP levels were increased during Th9 cell differentiation in TP$^{+/+}$ cells (white bars), but not in TP$^{-/-}$ cells (black bars); N=5 per group; *p<0.05. **C)** Treatment with cTXA$_2$ enhanced phosphorylation of p38 MAPK in naïve T cells incubated with TGFβ and IL-4. Incubation of naïve T cells from TP$^{+/+}$ mice with TGFβ and IL-4 increased phosphorylation of p38 MAPK (representative of 3 experiments), an effect that was attenuated in naïve T cells isolated from TP$^{-/-}$ mice (single experiment). **D)** Naïve T cells were incubated with TGFβ and IL-4 in the presence or absence of cTXA$_2$ and a PKA inhibitor (PKAi). Although Th9 cell differentiation (as measured by expression of *Il9*, *Il10* and *Irf4* expression) was modestly enhanced by PKAi, it did not significantly alter the ability of cTXA$_2$ to inhibit Th9 cell differentiation. N=5, *p<0.05. **E)** Naïve T cells were incubated with TGFβ and IL-4 in the presence or absence of cTXA$_2$ and a p38 MAPK inhibitor (SB203580, p38i). p38i abolished Th9 cell differentiation (as measured by expression of *Il9*, *Il10* and *Irf4* expression). N=5, *p<0.05. Significance was determined by one-way ANOVA for all panels.
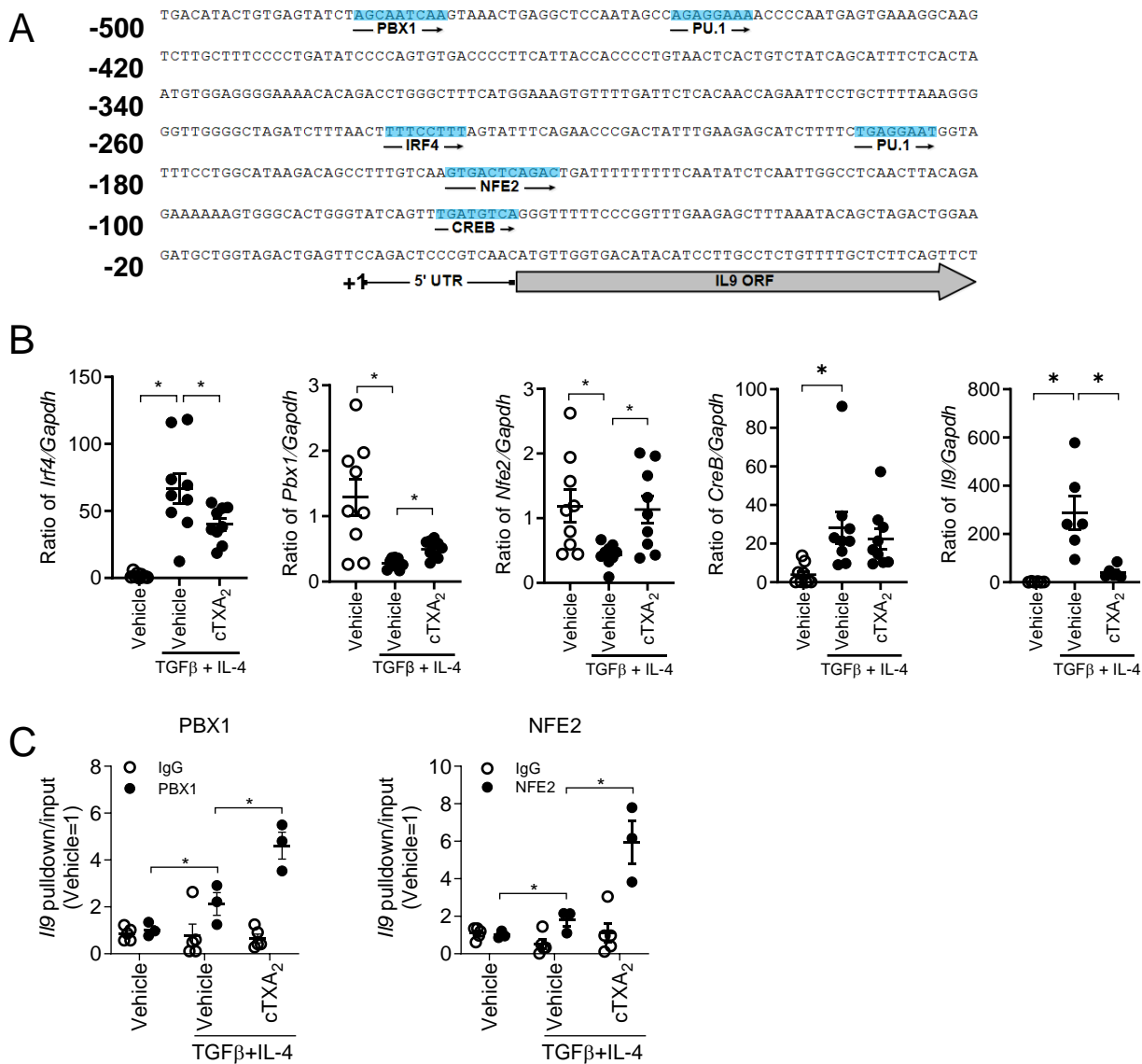
Figure 9. TXA₂ alters expression/binding of PBX1 and NFE2 to the Il9 promoter. **A)** The locations of transcription factor binding sites in the mouse proximal *Il9* promoter. Conservation analysis and motif prediction were used to identify unique binding sites for NFE2 (-150 bp), PBX1 (-481 bp), CREB (-101 bp), PU.1 (-450 bp, -192 bp) and IRF4 (-237 bp) transcription factors relative to the transcription start site (TSS). **(B)** Naïve T cells were treated with TGFβ and IL-4 to induced Th9 cell differentiation in the presence/absence of cTXA₂ and expression of *Irf4*, *Pbx1*, *Nfe2*, *Creb* and *Il9* mRNAs was determined by qPCR; N=9, *p<0.05. **C)** Chip-PCR assays of PBX1 and NFE2 binding to genomic DNA from naïve T cells during Th9 cell differentiation with/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 pulldown 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 one-way ANOVA for all panels. Significance was determined by one-way ANOVA for panel B and multiple t-tests for panel C.
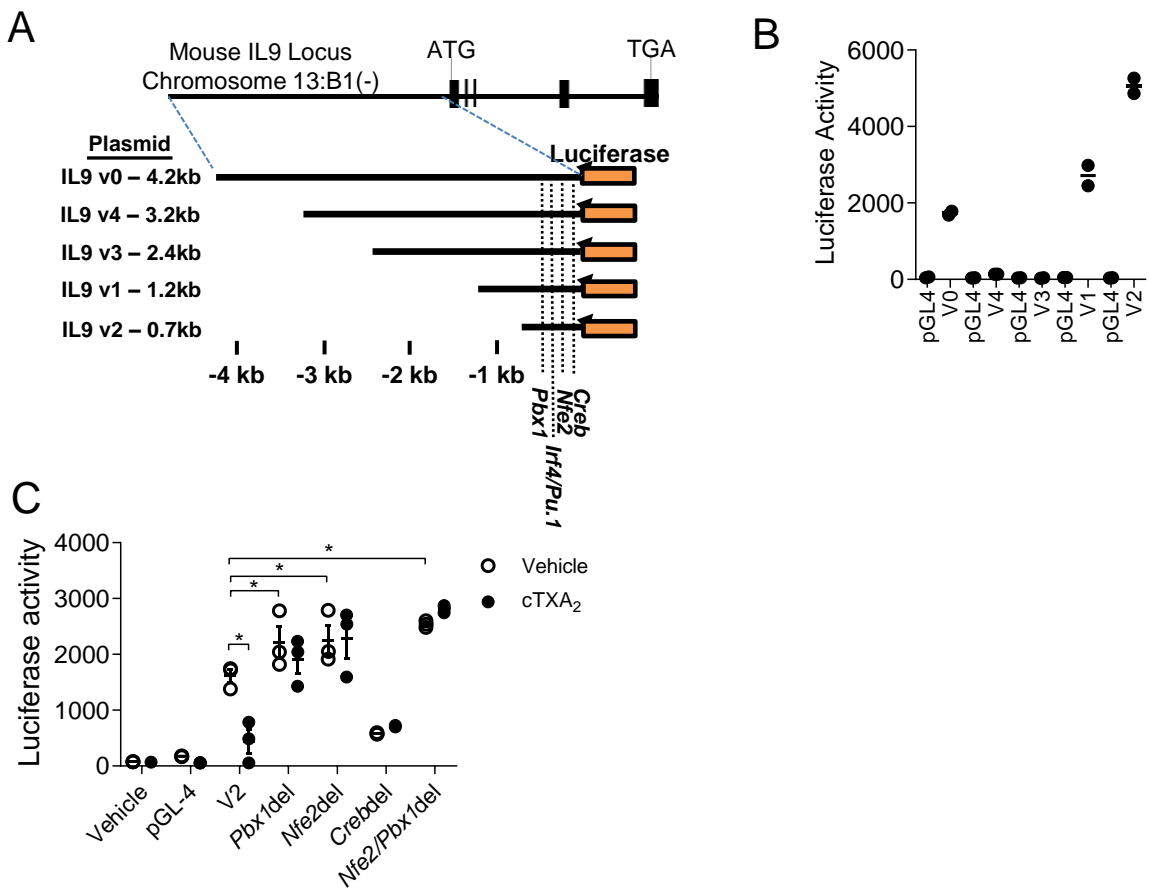
Figure 10. Role of PBX1 and NFE2 in *Il9* promoter activity and effect of TXA$_2$. **A)** Luciferase reporter constructs of different *Il9* promoter region fragments. Dashed lines denote locations of *Nfe2*, *Pbx1*, *Creb*, *Pu.1* and *Irf4* response elements. **B)** Luciferase activity of empty vector (pGL4) or truncated *Il9* promoter region fragments. The V2 construct, which contains a 700 bp promoter sequence containing consensus binding sites for the PBX1, NFE2, CREB, PU.1 and IRF4 transcription factors, showed strong luciferase activity when transfected into 293T cells. **C)** The V2 construct was modified to delete nucleotides in the consensus *Nfe2, Pbx1* and *Creb* binding sites. Compared to the parent *Il9* V2 construct, 293T cells transfected with the *Pbx1*del, *Nfe2*del and *Nfe2/Pbx1*del constructs had increased luciferase activity and cells transfected with the *Crebdel* construct had decreased luciferase activity. Luciferase activity from the parent V2 construct was inhibited by cTXA$_2$. In contrast, luciferase activity in *Pbx1*del, *Nfe2*del, and *Nfe2/Pbx1*del transfected cells was not inhibited by cTXA$_2$. Significance was determined by one-way ANOVA for panel B and multiple t-tests for panel C.
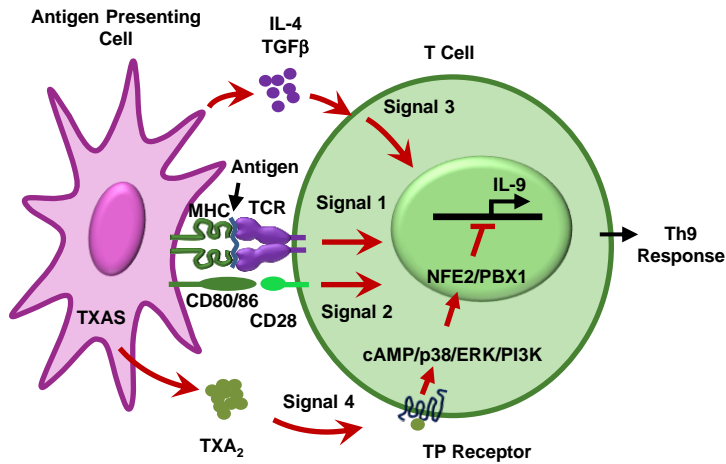
Figure 11. Model for TXA$_2$ regulation of Th9 cell differentiation. The interaction of T cells with antigen presenting cells involves four signals. Signal 1 involves the interaction between MHC molecules containing peptide fragments on the antigen presenting cell and the TCR on the T cell. Signal 2 involves the interaction between co-stimulatory molecules (such as CD80/86) on the surface of the antigen presenting cell with ligands (such as CD28) on the T-cell surface. Signal 3 involves the secretion of bioactive cytokines (IL-4 and TGFβ) by the antigen presenting cell. Signal 4 involves secretion of TXA$_2$ by antigen presenting cells that binds to the TP receptor on T cells to limit Th9 cell differentiation via a mechanism that involves the transcription factors NFE2 and PBX1 (adapted from Li et al, *World J Stem Cells*, 2014).