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## Diacylglycerol kinase $\zeta$ promotes allergic airway inflammation and airway hyperresponsiveness through distinct mechanisms

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### Abstract

Asthma is a chronic allergic inflammatory airway disease caused by aberrant immune responses to inhaled allergens, which leads to airway hyperresponsiveness (AHR) to contractile stimuli and airway obstruction. Blocking T helper 2 (T<sub>H</sub>2) differentiation represents a viable therapeutic strategy for allergic asthma, and strong TCR-mediated ERK activation blocks T<sub>H</sub>2 differentiation. Here, we report that targeting diacylglycerol (DAG) kinase zeta (DGK $\zeta$ ), a negative regulator of DAG-mediated cell signaling, protected against allergic asthma by simultaneously reducing airway inflammation and AHR through independent mechanisms. Targeted deletion of DGK $\zeta$  in T cells decreased type 2 inflammation without reducing AHR. In contrast, loss of DGK $\zeta$  in airway smooth muscle cells decreased AHR but not airway inflammation. T cell-specific enhancement of

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SUPPLEMENTARY MATERIALS

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ERK signaling was only sufficient to limit type 2 airway inflammation, not AHR. Pharmacological inhibition of DGK diminished both airway inflammation and AHR in mice and also reduced bronchoconstriction of human airway samples in vitro. These data suggest that DGK is a previously unrecognized therapeutic target for asthma and reveal that the inflammatory and AHR components of asthma are not as interdependent as generally believed.

## INTRODUCTION

Asthma is a chronic allergic inflammatory airway disease that affects more than 300 million people worldwide, with an annual economic cost estimated to exceed \$56 billion in the United States alone (1). The pathogenesis associated with allergic asthma is characterized by airway inflammation that is mediated by aberrant immune responses to inhaled allergens at the mucosal surfaces of the lung and airflow obstruction driven in part by increased airway smooth muscle responses to contractile stimuli in a process known as airway hyperresponsiveness (AHR) (2–4). Current therapeutic approaches used to treat asthma involve combinatorial administration of bronchodilators and anticholinergic drugs to relax constricted airways and corticosteroids to inhibit airway inflammation (5). Although these treatments benefit many patients who have asthmatic disease, there is a substantial proportion of patients in whom these treatments never fully control asthma, particularly in those who have severe disease (3). Furthermore, cessation of these treatments often results in loss of asthma control and reoccurrence of asthma symptoms, suggesting that these treatments fail to reverse the underlying intrinsic changes in airway cells that mediate asthma pathology (6, 7). Therefore, there is an urgent unmet need for therapeutics that can offer better control and potentially mediate resolution of the disease.

Airway inflammation in allergic asthma is typically driven by type 2 immune responses in the lung, although other asthma endo-types driven by type 2-independent immune responses do exist (8, 9). Type 2 inflammation in the lung is mediated by T helper 2 (T<sub>H</sub>2) CD4<sup>+</sup> T cells and group 2 innate lymphoid cells (ILC2), which produce the type 2 cytokines, interleukin-4 (IL-4), IL-5, and IL-13, in response to antigen-dependent and antigen-independent activation (10–17). The production and release of these cytokines promote a variety of downstream responses, which include the recruitment and activation of eosinophils in the lung, immunoglobulin E (IgE) production by allergen-specific B cells to arm basophils and mast cells for degranulation, goblet cell-mediated mucus production, and excessive airway smooth muscle contraction, that ultimately result in the damage of the lung parenchyma and the impairment of lung function in asthma (2, 4, 13–16, 18–20). Furthermore, type 2 airway inflammation drives the nonimmune abnormalities, such as AHR, that are present in asthma.

Given the role of T<sub>H</sub>2 CD4<sup>+</sup> T cells in asthma pathogenesis, blocking T<sub>H</sub>2 differentiation of allergen-specific T cells represents a viable therapeutic strategy for the treatment of asthma. Cytokine [e.g., IL-4, TSLP (thymic stromal lymphopoietin), IL-25, and IL-33] and costimulatory (e.g., CD28, ICOS, OX40) signals are important for driving T<sub>H</sub>2 differentiation, but the strength and duration of T cell receptor (TCR) signaling can also contribute to the outcome of CD4<sup>+</sup> T cell differentiation. Strong and prolonged TCR-

mediated signals promote T<sub>H</sub>1 differentiation, whereas weak and transient signals skew differentiation toward T<sub>H</sub>2 (21–31). More specifically, TCR-mediated extracellular signal-regulated kinase (ERK) activation is a key determinant in driving CD4<sup>+</sup> T cell differentiation, and strong ERK signals block T<sub>H</sub>2 differentiation (28, 29). TCR-mediated ERK activation is largely dependent on diacylglycerol (DAG), which is a secondary lipid messenger that is generated after the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) by phospholipase C (PLC). ERK activation is promoted by DAG-mediated Ras-GRP1 activation, and nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation is promoted by DAG-mediated PKC activation (32). DAG activity is inhibited by its phosphorylation into phosphatidic acid by DAG kinase (DGK) enzymes. Among DGK family members, the  $\zeta$  isoform of DGK plays a predominant role in suppressing DAG-dependent ERK activation (33). Accordingly, T cells lacking DGK $\zeta$  accumulate DAG and display enhanced ERK activation (34).

The absence of DGK $\zeta$  enhances the magnitude of antitumor and antiviral immunity (34–37). However, in some instances (e.g., in allergic responses), DGK $\zeta$  knockout (KO) mast cells fail to degranulate, and DGK $\zeta$  KO mice are resistant to anaphylaxis (38). Thus, depending on the process, blocking DGK $\zeta$  could be either immunostimulatory or immunosuppressive (39). Given that ERK activation skews T cell responses away from T<sub>H</sub>2 differentiation, we hypothesized that the enhancement of DAG signaling by targeting DGK $\zeta$  would suppress rather than potentiate the development of allergic asthma. We observed that enhancement of DAG signaling by the loss of DGK $\zeta$  reduced T<sub>H</sub>2 differentiation, and this effect translated to protection from a mouse model of T<sub>H</sub>2-mediated allergic asthma. Unexpectedly, we found that the mechanisms by which DGK $\zeta$  promoted airway inflammation and AHR were separable. Conditional deletion of DGK $\zeta$  in T cells limited type 2 inflammation in an ERK-dependent manner with no effect on AHR. In contrast, targeted deletion of DGK $\zeta$  in smooth muscle cells impaired AHR without preventing airway inflammation. Furthermore, we found that pharmacological inhibition of DGK suppressed murine type 2 airway inflammation and AHR and inhibited carbachol-mediated bronchoconstriction of human airway slices. Thus, these data demonstrate that DGKs are previously unrecognized therapeutic targets for asthma and reveal that airway inflammation and AHR are not as interdependent as generally believed.

## RESULTS

### DGK $\zeta$ KO mice are protected from OVA-induced allergic airway inflammation and AHR

ERK activation drives T cells to differentiate into T<sub>H</sub>1 over T<sub>H</sub>2 phenotype (28, 29). Because ERK is hyperactivated in the absence of DGK $\zeta$  in T cells, we tested whether loss of DGK $\zeta$  in T cells impaired T<sub>H</sub>2 differentiation in vitro. When naïve CD4<sup>+</sup> T cells from DGK $\zeta$  KO mice were stimulated through their TCR and expanded in vitro under nonpolarizing conditions, we found that the proportion of T<sub>H</sub>1 cells was increased and the proportion of T<sub>H</sub>2 cells was decreased when compared with naïve CD4<sup>+</sup> T cells from wild-type (WT) mice (Fig. 1, A to C). Because DGK $\zeta$  KO T cells are hyperproliferative after TCR activation, it was possible that the decrease in the proportion of T<sub>H</sub>2 cells was due to selective outgrowth of T<sub>H</sub>1 cells among DGK $\zeta$  KO T cells. However, the total number of T<sub>H</sub>2 cells generated after TCR activation in the absence of DGK $\zeta$  significantly diminished

when compared with WT T cells (fig. S1). In addition, the differentiation of DGK $\zeta$  KO T cells into T<sub>H</sub>2 cells was reduced, whereas T<sub>H</sub>1 differentiation was increased at early time points after TCR activation. Thus, these data demonstrate that the loss of DGK $\zeta$  does not change the kinetics but rather the outcome of T<sub>H</sub> differentiation (fig. S2).

Conventional CD4<sup>+</sup> T cells can be an early source of IL-4 immediately after TCR activation that is sufficient to promote T<sub>H</sub>2 differentiation in the absence of exogenous cytokines (40–42). The generation of T<sub>H</sub>2 cells under nonpolarizing conditions was dependent on endogenous production of IL-4 from T cells (fig. S3A). To determine whether reduced T<sub>H</sub>2 differentiation in the DGK $\zeta$  KO T cells was due to impaired endogenous IL-4 production, naïve DGK $\zeta$  KO CD4<sup>+</sup> T cells were activated through their TCR in the presence of exogenous IL-4. We found that T<sub>H</sub>2 differentiation was completely restored in DGK $\zeta$  KO T cells treated with exogenous IL-4 (fig. S3B). Similarly, activation under T<sub>H</sub>2-polarizing conditions also restored T<sub>H</sub>2 differentiation in DGK $\zeta$  KO CD4<sup>+</sup> T cells (fig. S3C). These data revealed that the addition of exogenous IL-4 during TCR activation could bypass the impairment in T<sub>H</sub>2 differentiation in DGK $\zeta$  KO T cells, which indicated that the loss of DGK $\zeta$  might impair early TCR-mediated, T cell-intrinsic IL-4 production.

To test whether this reduction in T<sub>H</sub>2 differentiation correlated with protection against asthma in DGK $\zeta$  KO mice, WT and DGK $\zeta$  KO mice were subjected to an ovalbumin (OVA)-induced allergic asthma mouse model. In line with our in vitro data, total inflammatory cell and eosinophil numbers in the bronchoalveolar lavage (BAL) fluid, the amount of T<sub>H</sub>2 cytokines in BAL, and OVA-specific IgG1 serum antibody were significantly reduced in OVA-challenged DGK $\zeta$  KO mice compared with OVA-challenged WT controls (Fig. 1, D to F). This correlated with decreased inflammatory infiltrates in the lungs of OVA-challenged DGK $\zeta$  KO mice (Fig. 1G). AHR was almost completely abolished in OVA-challenged DGK $\zeta$  KO compared with WT mice (Fig. 1H). In contrast to our in vitro data, T<sub>H</sub>1 responses were not increased in OVA-challenged DGK $\zeta$  KO mice, which had no difference in the amount of BAL interferon- $\gamma$  (IFN- $\gamma$ ) or OVA-specific IgG2a serum antibody compared with WT controls (Fig. 1, E and F). To test whether the loss of DGK $\zeta$  could protect from other models of allergic asthma, WT and DGK $\zeta$  KO mice were subjected to chronic house dust mite (HDM) exposure. Airway eosinophilia and the amounts of T<sub>H</sub>2 cytokines in the BAL were significantly diminished in HDM-challenged DGK $\zeta$  KO mice compared with HDM-challenged WT controls (Fig. 1, I and J).

Although DGK $\zeta$  is the predominant isoform that limits DAG-mediated signaling in T cells, another DGK isoform known as DGK $\alpha$  also contributes to this process (33). In DGK $\alpha$  KO T cells, T<sub>H</sub>1 differentiation was enhanced and T<sub>H</sub>2 differentiation was reduced after TCR stimulation (fig. S4, A and B). In accordance, AHR and airway inflammation were partially but significantly reduced in OVA-induced DGK $\alpha$  KO mice when compared with WT controls (fig. S4, C to F). Thus, the manipulation of DAG signaling by targeting DGK enzymes attenuates OVA-induced allergic asthma.

### Protection from OVA-induced airway inflammation and AHR in the absence of DGK $\zeta$ is independently mediated by separate compartments

The near-complete abolition of AHR in DGK $\zeta$  KO mice despite a statistically significant but only partial reduction in airway inflammation prompted us to test whether DGK $\zeta$  deficiency in nonhematopoietic cells also contributed to protection against OVA-induced allergic asthma. The hematopoietic compartment of lethally irradiated WT and DGK $\zeta$  KO mice was reconstituted with bone marrow (BM) cells from either WT or DGK $\zeta$  KO mice. Similar to DGK $\zeta$  KO mice, eosinophilic inflammation, the amount of T<sub>H</sub>2 cytokines in the BAL fluid, and AHR responses were reduced in DGK $\zeta$  KO  $\rightarrow$  DGK $\zeta$  KO BM chimeric mice when compared with WT  $\rightarrow$  WT BM chimeric mice (Fig. 2, A to C). Unexpectedly, however, we found that the reduction in inflammation and AHR was mediated by two separate cell compartments. Although WT mice reconstituted with DGK $\zeta$  KO BM cells had reduced eosinophilic inflammation and less T<sub>H</sub>2 cytokines in the BAL fluid, they were not protected against OVA-induced AHR (Fig. 2, A to C). In contrast, DGK $\zeta$  KO mice reconstituted with WT BM cells were completely protected from OVA-induced AHR, despite the presence of eosinophilic inflammation and T<sub>H</sub>2 cytokines in the BAL fluid (Fig. 2, A to C). These data suggested that DGK $\zeta$  deficiency in hematopoietic cells contributes to reduced airway inflammation, whereas loss of DGK $\zeta$  in nonhematopoietic cells leads to protection against AHR.

### DGK $\zeta$ deficiency in T cells protects from OVA-induced airway eosinophilia and partially attenuates OVA-induced T<sub>H</sub>2 differentiation

To more precisely interrogate the impact of DGK $\zeta$  deficiency in hematopoietic cells in OVA-induced airway inflammation, DGK $\zeta$  was conditionally deleted in hematopoietic cells or T cells using a Vav-inducible Cre (Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> mice) or CD4-inducible Cre (CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice), respectively. In Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> mice OVA-induced eosinophil accumulation and the amount of IL-4 in the BAL fluid were significantly reduced, whereas the OVA-induced AHR response was completely intact (Fig. 2, D to F). Similarly, in CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice, OVA-induced eosinophil accumulation and the amount of IL-4 in the BAL fluid were also reduced (Fig. 2, G and H).

Whereas IL-4 abundance in the BAL was diminished in Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> and CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice after OVA challenge, the IL-5 and IL-13 amounts in the BAL were relatively intact in these mice (Fig. 2, F and H). To test whether the loss of DGK $\zeta$  in T cells selectively attenuated the ability of T cells to produce IL-4 after T<sub>H</sub>2 differentiation in vivo, we adoptively transferred a mixture of CD45.1<sup>+</sup>CD45.2<sup>+</sup> WT and CD45.2<sup>+</sup> DGK $\zeta$  KO OT-II CD4<sup>+</sup> T cells into naïve CD45.1<sup>+</sup> WT mice followed by OVA immunization. OT-II T cells express a transgenic TCR specific for the OVA<sub>323–339</sub> peptide presented on the MHC (major histocompatibility complex) class II I-A<sup>b</sup> molecule. In accordance with the Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> and CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> data, we found that the proportion of DGK $\zeta$  KO OT-II T cells expressing IL-4 was significantly diminished, whereas the proportions of DGK $\zeta$  KO OT-II T cells expressing IL-5 and IL-13 were relatively unaltered compared with WT OT-II T cells after OVA sensitization (Fig. 2I and fig. S5). These data suggest that the loss of DGK $\zeta$  selectively impaired the ability of T cells to produce IL-4 in a T cell–intrinsic manner during T<sub>H</sub>2 differentiation in vivo. In addition, we observed that the frequency of DGK $\zeta$  KO

OT-II T cells expressing IFN- $\gamma$  was significantly increased compared with WT OT-II T cells after OVA sensitization, suggesting that T<sub>H</sub>1 differentiation was enhanced in the absence of DGK $\zeta$  in vivo (Fig. 2I and fig. S5). Overall, these data demonstrate that DGK $\zeta$  deficiency inhibits airway inflammation in a T cell–intrinsic manner by partially attenuating T<sub>H</sub>2 differentiation in vivo.

### DGK $\zeta$ deficiency in airway smooth muscle cells protects from OVA-induced AHR

AHR is increased indirectly in part by the production of contractile mediators from sensory neurons that innervate the lungs, and directly by contraction of airway smooth muscle cells driven by the activation of receptors, such as muscarinic type 3 (M3) receptors, that bind to these mediators (43–45). Furthermore, DGK $\zeta$  is expressed in sensory neurons arising from the dorsal root ganglion and in smooth muscle cells (46–50). To identify the nonhematopoietic cell type that was responsible for protection against OVA-induced AHR in the absence of DGK $\zeta$ , DGK $\zeta$  was conditionally deleted in sensory neurons (Pirt-Cre DGK $\zeta^{fl/fl}$ ) and smooth muscle cells (Myh11-Cre DGK $\zeta^{fl/fl}$ ). Pirt-Cre DGK $\zeta^{fl/fl}$  mice exhibited similar OVA-induced AHR compared with control mice (Fig. 3A). However, Myh11-Cre DGK $\zeta^{fl/fl}$  mice were protected from OVA-induced AHR despite unaltered airway inflammation (Fig. 3, B to D). Moreover, methacholine-induced contractile force was reduced in tracheal rings isolated from either DGK $\zeta$  KO or Myh11-Cre DGK $\zeta^{fl/fl}$  mice when compared with WT or Myh11-Cre controls (Fig. 3, E and F, and fig. S6). These data demonstrate that DGK $\zeta$  promotes airway smooth muscle cell contraction and allergen-induced AHR independently of inflammation in a cell-intrinsic manner.

### Enhancement of ERK signaling in T cells is sufficient to protect from OVA-induced allergic airway inflammation but insufficient to protect from OVA-induced AHR

We tested whether increased ERK signaling was responsible for the effect of DGK $\zeta$  deficiency on T<sub>H</sub>2 differentiation. To this end, we assessed T<sub>H</sub> differentiation of naïve DGK $\zeta$  KO CD4<sup>+</sup> T cells activated through their TCR in the presence of the U0126, a pharmacological inhibitor of mitogen-activated protein kinase kinase 1/2 (MEK1/2), the kinase that phosphorylates and activates ERK. We found that inhibition of ERK signaling was sufficient to restore T<sub>H</sub>2 differentiation in DGK $\zeta$  KO T cells (Fig. 4A and fig. S7). In addition, although treatment with U0126 did not alter the frequency of IL-4– and IL-13–producing WT T cells, inhibition of ERK signaling increased the frequency of WT T cells producing IL-5 (Fig. 4A and fig. S7). In contrast, T<sub>H</sub>1 differentiation in WT and DGK $\zeta$  KO T cells was reduced in the presence of U0126. Together, these data establish that enhanced TCR-mediated DAG signaling impairs T<sub>H</sub>2 differentiation and promotes T<sub>H</sub>1 differentiation in an ERK-dependent manner (Fig. 4A and fig. S7).

We took a gain-of-function approach to test whether the enhancement of ERK signaling in T cells was sufficient to limit OVA-induced airway inflammation. Sevenmaker (ERK<sup>SEM</sup>) transgenic mice express a transgene that encodes a gain-of-function mutant of Erk2 driven from the human CD2 promoter and locus control region, which results in selective enhancement of the ERK signaling pathway specifically in T cells (51). Similar to DGK $\zeta$  KO T cells, T<sub>H</sub>1 differentiation was increased and T<sub>H</sub>2 differentiation was reduced after TCR stimulation of ERK<sup>SEM</sup> T cells in vitro (Fig. 4, B to D). Furthermore, ERK<sup>SEM</sup> mice

had less eosinophilia and  $T_H2$  cytokines in the airways after OVA challenge compared with WT controls. Thus, enhancement of ERK signaling was sufficient to inhibit  $T_H2$  differentiation and protect from OVA-induced airway inflammation *in vivo* (Fig. 4, E and F). Similar to the Vav-Cre  $DGK\zeta^{fl/fl}$  mice, OVA-induced AHR in  $ERK^{SEM}$  mice was similar to WT controls, despite suppressed type 2 airway inflammation (Fig. 4G). Overall, these data demonstrate that  $DGK\zeta$  controls  $T_H2$  differentiation in an ERK-dependent manner in T cells to promote OVA-induced airway inflammation independently of AHR.

### Pharmacological inhibition of DGK is sufficient to protect from OVA-induced asthma

We tested whether DGK could represent a previously unrecognized target for the prevention and treatment of asthma. Although there are no selective inhibitors of  $DGK\zeta$ , a pan-DGK inhibitor, R59949, that is relatively selective for  $DGK\alpha$  is commercially available (52, 53).  $DGK\alpha$  and  $DGK\zeta$  are expressed in both T cells and smooth muscle cells (33, 34, 48). Because  $DGK\alpha$  KO mice have a partial but statistically significant reduction in OVA-induced airway inflammation and AHR, we tested whether pharmacological inhibition of  $DGK\alpha$  kinase activity with R59949 could block OVA-induced type 2 airway inflammation and AHR. Mice were treated with R59949 during the late sensitization and airway challenge phases of the murine model of OVA-induced asthma. Compared with vehicle, treatment with R59949 significantly inhibited AHR and reduced eosinophilia and  $T_H2$  cytokines in the BAL fluid (Fig. 5, A to C). To test whether  $DGK\alpha$  inhibition could block type 2 airway inflammation and AHR after allergen-specific T cell responses were established, we treated OVA-sensitized mice with R59949 only during the airway challenge phase of the OVA-induced asthma model. Compared with vehicle, treatment with R59949 again exhibited significantly reduced OVA-induced AHR (Fig. 5D). In contrast, treatment with R59949 during the airway challenge phase failed to alter type 2 airway inflammation after OVA challenge (Fig. 5, E and F). Together, these data suggest that DGK can be pharmacologically targeted to reduce AHR and airway inflammation. However, whereas the acute administration of a DGK inhibitor is sufficient to attenuate OVA-induced AHR, the DGK inhibitor must be administered during the sensitization stage to inhibit the type 2 airway inflammation.

To further examine the therapeutic potential of targeting DGK, we tested whether inhibition of  $DGK\alpha$  by R59949 affects human airway smooth muscle (HASM) contraction. Human airway smooth muscle cells were pretreated with or without R59949 and stimulated with the nonselective M3 receptor agonist, carbachol. The phosphorylation of myosin light chain (MLC), a critical step in smooth muscle cell contraction, was reduced by R59949 (Fig. 5G). To test whether this effect translated to the attenuated contraction of human airways, precision-cut lung slices (PCLS) were obtained from lung transplant donors and treated with R59949. After overnight incubation with R59949, carbachol-induced bronchoconstriction was significantly decreased (Fig. 5H). Overall, these data highlight that acute inhibition of DGK is sufficient to protect from the development of AHR in human samples. These data suggest that DGK is a potential therapeutic target for the prevention and treatment of asthma.



## DISCUSSION

Our findings demonstrated that augmenting DAG signaling by limiting the activity of DGK $\zeta$  and DGK $\alpha$  reduced disease in a mouse model of allergen-induced asthma. Genetic ablation of DGK $\zeta$  or DGK $\alpha$  protected from OVA-induced airway inflammation and AHR. Using BM chimeras, we demonstrated that inhibition of AHR was due to the loss of DGK $\zeta$  in the radioresistant compartment, whereas reduced airway inflammation was due to the loss of DGK $\zeta$  in the radio-sensitive compartment. Thus, DGK $\zeta$  limits these processes in separate compartments and independently of each other. Generation of cell type-specific conditional DGK $\zeta$  KO mice revealed that the protection from OVA-induced airway inflammation was mediated by the loss of DGK $\zeta$  in T cells, whereas the attenuation of OVA-induced AHR was facilitated by the absence of DGK $\zeta$  in airway smooth muscle cells. Last, acute loss of DGK activity through pharmacological blockade with a DGK $\alpha$  inhibitor was sufficient to protect mice from allergen-induced asthma, limit allergen-induced AHR in mice with established allergen-induced immune responses, and reduce carbachol-induced bronchoconstriction of human airways. These data suggest that modulating DGK activity could represent a new therapeutic strategy for the treatment of asthma.

Given that DAG promotes signal transduction downstream of activating receptors, one might predict that the inhibition of DGK would always enhance immune responses. Our findings highlight the idea that augmenting DAG-mediated signaling does not necessarily increase activation. In settings of T<sub>H</sub>1 and cell-mediated immune responses, increased DAG signaling caused by the loss of DGK $\zeta$  augments the function of T cells and NK cells (34–37). In contrast, mast cell degranulation during allergic responses is inhibited in the absence of DGK $\zeta$  (38). Thus, targeting DGK $\zeta$  is immunomodulatory, i.e., it is immunostimulatory or immunosuppressive depending on the context. Our data presented here reinforce this notion because the loss of DGK $\zeta$  inhibits T<sub>H</sub>2-mediated inflammation.

We demonstrated that the loss of DGK $\zeta$  diminished T<sub>H</sub>2 differentiation in vitro and suppressed eosinophilic inflammation and T<sub>H</sub>2 cytokine release in the BAL fluid of asthmatic mice in vivo. Although all major T<sub>H</sub>2 cytokines (IL-4, IL-5, and IL-13) were reduced in the BAL of DGK $\zeta$  KO mice, only IL-4, but not IL-5 or IL-13, was reduced in the airways of Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> and CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice. In accordance, DGK $\zeta$  KO OT-II cells displayed a selective impairment in their potential to produce IL-4, but not IL-5 or IL-13, compared with cotransferred WT OT-II cells after OVA sensitization in vivo. These results suggest that the loss of DGK $\zeta$  selectively impairs the ability of T cells to produce IL-4 in a T cell-intrinsic manner during T<sub>H</sub>2 differentiation in vivo. This could potentially explain the selective reduction in BAL IL-4 amounts seen in the hematopoietic-specific and T cell-specific DGK $\zeta$  KO mice after OVA challenge in vivo. It is still unclear why all three T<sub>H</sub>2 cytokines were reduced in WT mice reconstituted with DGK $\zeta$  KO BM cells. The BM reconstitution studies may need to be interpreted with caution given the unknown effects of irradiation, reconstitution efficiency, and increased age of BM-transplanted mice on asthma.

The signaling mechanism by which DGK deficiency protects against asthma is not entirely clear. We started our studies by hypothesizing that DGK deficiency would limit T<sub>H</sub>2 differentiation by enhancing ERK phosphorylation. However, the role of ERK in T<sub>H</sub>2

differentiation and allergic asthma is controversial. Although ERK activation downstream of strong TCR stimulation negatively correlates with T<sub>H</sub>2 differentiation (22, 29), very strong TCR-mediated signaling can also induce T<sub>H</sub>2 differentiation (23). Similarly, in ERK1 KO mice, allergic airway inflammation and AHR were decreased due to reduction in T<sub>H</sub>2 differentiation (54), yet others report that T<sub>H</sub>2 differentiation is intact in ERK1 KO mice (55). In these studies, the defects in OVA-induced allergic asthma in ERK1 KO mice might be secondary to enhanced IL-12 production by ERK1 KO dendritic cells (56), which stimulates a T<sub>H</sub>1 response in a T cell–extrinsic manner. T cells expressing a dominant negative Ras transgene (dnRas Tg mice) exhibit reduced T<sub>H</sub>2 differentiation in vitro and in vivo (OVA-induced asthma), which further suggests that ERK activation promotes T<sub>H</sub>2 differentiation (57, 58). However, TCR-activated dnRas Tg T cells also display impaired IL-4R signaling as demonstrated by decreased pSTAT6, pJak1, pJak3, and pIL-4R $\alpha$  after IL-4 stimulation (57). As a result, the defect in T<sub>H</sub>2 differentiation seen in dnRas Tg T cells might reflect impaired IL-4–mediated T<sub>H</sub>2 polarization. As opposed to DGK $\zeta$  KO T cells, exogenous IL-4 was not sufficient to restore T<sub>H</sub>2 differentiation in dnRas Tg T cells.

Our findings demonstrated that partial attenuation of ERK signaling by U0126 restored T<sub>H</sub>2 differentiation in DGK $\zeta$  KO T cells. Furthermore, T cells from ERK<sup>SEM</sup> mice showed increased T<sub>H</sub>1 and decreased T<sub>H</sub>2 differentiation in vitro, which correlated with protection from OVA-induced type 2 airway inflammation in vivo. Thus, the effect of DGK $\zeta$  deficiency on the inhibition of T<sub>H</sub>2 differentiation was dependent on increased ERK activation. However, NF- $\kappa$ B activity is also increased in DGK $\zeta$  KO T cells after TCR activation (59). Although, whether enhancement of NF- $\kappa$ B signaling also contributes to the attenuation of T<sub>H</sub>2 differentiation in DGK $\zeta$  KO T cells remains unclear. The NF- $\kappa$ B p50 subunit is required for GATA3 expression at late time points after TCR activation under T<sub>H</sub>2 polarization conditions (60). Yet, the requirement for the NF- $\kappa$ B p50 subunit and other members of the NF- $\kappa$ B family in the instruction of T<sub>H</sub>2 differentiation by weak TCR-mediated signaling is unknown.

The mechanism by which DGK affects acetylcholine-induced smooth muscle cell contraction is still unclear. Akin to what we observed with airway smooth muscle cells, inhibition of DGK $\alpha$  activity also reduces vascular smooth muscle contraction through an unknown mechanism (49, 50). Similar to TCR signaling, activation of muscarinic type 3 receptors, which are G protein–coupled receptors (GPCRs) that signal through G $\alpha_q$  proteins, promotes PLC-dependent DAG generation and ERK activation (61, 62). Thus, it is possible that the attenuation of smooth muscle contraction in DGK $\zeta$  KO airway smooth muscle cells is also ERK dependent. However, ERK signaling promotes rather than suppresses carbachol-induced contraction in ileal smooth muscle cells and  $\alpha_1$ -adrenergic receptor–induced contraction, which is another G $\alpha_q$ -coupled GPCR, in vascular smooth muscle cells (63, 64). In addition, PLC activation increases intracellular Ca<sup>2+</sup> necessary for the cross-bridge cycling of actin and myosin to drive smooth muscle contraction, and DAG-dependent PKC activation maintains contraction by preventing activation of an inhibitor of the cross-bridge cycling (65–67). Consequently, one might expect that the lack of DGK $\zeta$ , which would lead to enhanced DAG-dependent PKC activation after muscarinic type 3 receptor activation, would exacerbate bronchoconstriction. However, the diminution of smooth muscle contraction by the loss of DGK $\zeta$  suggests that other biological mechanisms are involved. It

is possible that enhanced DAG-mediated PKC activation results in feedback inhibition of PLC activity, which would lead to diminished  $Ca^{2+}$  responses and attenuated smooth muscle contraction. This feedback inhibition of PLC activity is seen after a short term treatment with DAG analogs, such as phorbol esters (68, 69). Similarly, DGK $\zeta$  KO mast cells display impaired Fc $\epsilon$ RI-mediated degranulation due to impaired PLC $\gamma$  and  $Ca^{2+}$  responses after Fc $\epsilon$ RI activation (38).

Our study demonstrated that AHR and eosinophilic airway inflammation were separate and distinct processes that mediated the development of asthma independently of each other. In recent clinical trials, although inhibition of type 2 cytokine signaling reduces eosinophilia and decreases the frequency of asthma exacerbations in asthmatic patients, these approaches failed to improve baseline lung function or prevent histamine-induced airway responses (70, 71). Thus, suppressing inflammation is not sufficient to reverse AHR and airway smooth muscle dysfunction in asthma. Our findings formally demonstrated that airway eosinophilic inflammation and AHR can be regulated independently of each other and revealed that DGK $\zeta$  plays a central role in the induction of these processes during the development of asthma. We envision that therapeutic targeting of DGK $\zeta$  may prevent or resolve asthma by suppressing both the immune and nonimmune responses that drive the disease.

## MATERIALS AND METHODS

### Mice

C57BL/6, B6.SJL-*Ptprca*<sup>a</sup>*Peppc*<sup>b</sup>/BoyCrCrl (CD45.1<sup>+</sup>), and C57BL/6-Tg(TeraTcrb)425Cbn/Crl (CD45.2<sup>+</sup> WT OT-II) mice were purchased from the Jackson laboratory or Charles River Laboratories. Generation of DGK $\zeta$  KO, DGK $\zeta$  floxed (DGK $\zeta$ <sup>fl/fl</sup>), and DGK $\alpha$  KO mice were described previously (33, 59, 72). CD45.1<sup>+</sup>CD45.2<sup>+</sup> WT OT-II mice were generated by crossing CD45.2<sup>+</sup> WT OT-II to CD45.1<sup>+</sup> WT mice. CD45.2<sup>+</sup> DGK $\zeta$  KO mice were crossed to CD45.2<sup>+</sup> WT OT-II mice to generate CD45.2<sup>+</sup> DGK $\zeta$  KO OT-II mice. C57BL/6-*Il4*<sup>tm1Nnt</sup>/J (IL-4 KO) mice were purchased from the Jackson laboratory. B6.Cg-*Commd10*<sup>Tg(Vav1-cre)</sup>A2Kio/J (Vav-Cre) and Tg(Cd4-cre)1Cwi/BfluJ (CD4-Cre) mice were purchased from the Jackson laboratory and crossed to DGK $\zeta$ <sup>fl/fl</sup> mice to generate Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> and CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice, respectively. B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J (Myh11-Cre) mice were purchased from the Jackson laboratory and crossed to DGK $\zeta$ <sup>fl/fl</sup> mice to generate Myh11-Cre DGK $\zeta$ <sup>fl/fl</sup> mice. Pirt-Cre mice were crossed to DGK $\zeta$ <sup>fl/fl</sup> mice to generate Pirt-Cre DGK $\zeta$ <sup>fl/fl</sup> mice (73). Sevenmaker (ERK<sup>SEM</sup>) mice were provided by L. Samuelson from the National Institutes of Health and were originally developed by S. Hedrick from the University of California, San Diego (51). Unless otherwise specified, all mice were 7 to 12 weeks old at the time of use, were housed in pathogen-free conditions, and were treated in strict compliance with the Institutional Animal Care and Use Committee regulations at the University of Pennsylvania.

### Flow cytometry, cell sorting, and data analysis

For flow cytometric analyses, cells were stained with antibodies against cell surface antigens at 4°C for 15 min in phosphate-buffered saline (PBS). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was used to exclude nonviable cells. Intracellular cytokine staining was performed

with the BD Cytotfix/Cytoperm Kit according to the manufacturer's protocol. Flow cytometry was performed with an LSR II or FACSCanto flow cytometer (BD Biosciences). For cell sorting, freshly isolated splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5, eBioscience) and anti-FITC magnetic-activated cell sorting (MACS) beads (Miltenyi Biotec) and, subsequently, passed through MACS columns (Miltenyi Biotec) according to the manufacturer's protocol to enrich for CD4<sup>+</sup> T cells. MACS-enriched CD4<sup>+</sup> T cells were subjected to cell surface staining before cell sorting. FACS (fluorescence-activated cell sorting) was performed with a FACS Aria cell sorter (BD Biosciences). Data were analyzed and plotted with FlowJo software (TreeStar). All fluorochrome-conjugated antibodies are listed in table S1.

### CD4<sup>+</sup> T cell differentiation assays

MACS-enriched CD4<sup>+</sup> T cells were sorted for naïve T cells (CD4<sup>+</sup>CD45RB<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup>). Sorted naïve CD4<sup>+</sup> T cells were plated (30,000 cells per well) in tissue culture media [MEM $\alpha$  (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2 mM L-glutamine, 12.5 mM HEPES (Life Technologies), and 22.9  $\mu$ M  $\beta$ -mercaptoethanol (Bio-Rad)] and activated with soluble anti-CD3 (10  $\mu$ g/ml; 145-2C11, BD Pharmingen) and soluble anti-CD28 (3  $\mu$ g/ml; 37.51, BD Pharmingen) in the presence of irradiated (50 Gy) CD4-depleted splenocytes (150,000 cells per well). In some experiments, sorted naïve CD4<sup>+</sup> T cells were activated in the presence of either media alone or exogenous IL-4 (10 ng/ml; Peprotech) before TCR activation. In other experiments, sorted naïve CD4<sup>+</sup> T cells were activated under T<sub>H</sub>2 conditions [anti-IL-12 (10  $\mu$ g/ml; C15.6, BioLegend), anti-IFN- $\gamma$  (10  $\mu$ g/ml; XMG1.2, BioLegend), IL-4 (10 ng/ml; Peprotech)]. In other experiments, sorted naïve CD4<sup>+</sup> T cells were pretreated with either dimethyl sulfoxide (DMSO) or the MEK1/2 inhibitor U0126 (0.4  $\mu$ M; Cell Signaling Technology) for 30 min before TCR activation. On day 5 after activation, T cells were restimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma-Aldrich) and ionomycin (1  $\mu$ g/ml; Sigma-Aldrich) in the presence of brefeldin A (5  $\mu$ g/ml; Cell Signaling Technology) for 5 hours. In other experiments, on day 3 or day 4 after activation, T cells were restimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. After restimulation, activated T cells were washed twice with PBS and prepared for cell surface and intracellular cytokine staining.

### OVA-induced allergic asthma model

Mice were sensitized with an intraperitoneal injection of 10  $\mu$ g of OVA (Sigma-Aldrich) and 2.25 mg of Imject Alum (Thermo Fisher Scientific), which contains aluminum hydroxide and magnesium hydroxide with inactive stabilizers, in 200  $\mu$ l of PBS on day 0 and day 14. After sensitization, mice were intranasally challenged with 10  $\mu$ g of OVA in 20  $\mu$ l of PBS on days 28 to 30. Airway responses to methacholine were measured about 16 hours after the last intranasal challenge. Mice were anesthetized with an intraperitoneal injection of ketamine (87.5 mg/kg; Hospira Inc.) and xylazine (12.5 mg/kg; Akorn Inc.), cannulated through the trachea, and attached to a lung mechanics analyzer (FlexiVent, SCIREQ Inc.). Airway responses were measured after the administration of increasing doses of methacholine through the use of a nebulizer as we previously described (74). Briefly, mice were ventilated with a tidal volume of 8 ml/kg at a rate of 150 breaths/min and a positive

end-expiratory pressure of 3 cm H<sub>2</sub>O by using a computerized flexiVent system. Using the low-frequency forced oscillation technique, respiratory mechanical input impedance ( $Z_{rs}$ ) was derived from the displacement of the ventilator's piston and the pressure in its cylinder. By fitting  $Z_{rs}$  to the constant phase model, the flexiVent software calculated the airway resistance (cmH<sub>2</sub>O.s.ml<sup>-1</sup>), which was normalized to body weight. Baseline measurements and responses to aerosolized saline were followed by measurements of responses to increasing doses of aerosolized methacholine (Sigma-Aldrich, St. Louis, MO). Recorded values were averaged for each dose and used to obtain dose-response curves for each mouse.

After measurement of airway mechanics, the lung was flushed three times with 0.7 ml of MACS buffer [PBS with 5% bovine serum albumin (Fisher Bioreagents) and 2 mM EDTA (Invitrogen)] containing complete Mini, EDTA-free protease inhibitor cocktail (Roche) to collect BAL fluid. The BAL fluid was centrifuged at 6797g at 4°C for 2 min in a microcentrifuge. BAL supernatant was collected and stored at -80°C before analysis. For measurement of cytokine concentrations in cell-free BAL fluid, mouse-specific IL-4 (BD Biosciences), IL-5 (BioLegend), and IL-13 (eBioscience) ELISA (enzyme-linked immunosorbent assay) kits were used according to the manufacturer's protocol. The remaining BAL cell pellet was treated with erythrocyte lysis buffer (ELB), pelleted, and resuspended in PBS for analysis by cytospin or flow cytometry. For cytospin analysis, BAL cells were spun down onto cytospin slides using a cytocentrifuge. Slides were air dried and stained with Shandon Kwik-Diff (Thermo Fisher Scientific) according to the manufacturer's protocol. Differential BAL cell counts were performed manually with at least 200 total cells counted for each slide as previously described (75–77). For analysis by flow cytometry, BAL cells were stained with antibodies against cell surface markers and identified by the following gating schemes: eosinophils (Ly6G<sup>-</sup>CD11c<sup>-</sup>Siglec F<sup>+</sup>), neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>), macrophages (Ly6G<sup>-</sup>F4/80<sup>+</sup>), and lymphocytes (CD3<sup>+</sup> or CD19<sup>+</sup> or B220<sup>+</sup>). All cells were pregated on CD45<sup>+</sup> live cells.

After BAL fluid collection, murine lungs were injected with 10% buffered formalin (Fisher Healthcare) through the trachea, harvested, and incubated in 10% buffered formalin overnight to allow for fixation. Next, lung tissue was dehydrated, paraffin embedded, and sliced into sections. Tissue sections were stained with hematoxylin and eosin (H&E) to determine cellular infiltrates in the lung (78, 79). Inflammation was scored by two blinded investigators on a scale of 0 (no tissue inflammation) to 5 (severe inflammation with involvement of the peribronchial, perivascular, and parenchymal regions).

### HDM-induced allergic asthma model

Mice were intranasally challenged with 25 µg of HDM extract (*Dermatophagoides pteronyssinus*, Greer Laboratories) in 20 µl of PBS for 5 days a week for three consecutive weeks (intranasal HDM challenge on days 0 to 4, days 7 to 11, and days 14 to 18). Mice were taken down 16 hours after the last intranasal challenge, and the lung was flushed once with 0.7 ml of MACS buffer [PBS with 5% bovine serum albumin (Fisher Bioreagents) and 2 mM EDTA (Invitrogen)] containing complete Mini, EDTA-free protease inhibitor cocktail (Roche) to collect BAL fluid. The BAL fluid was processed as described earlier to measure immune cell recruitment and cytokine release in the airways.

### Adoptive transfer of WT and DGK $\zeta$ KO OT-II

MACS-enriched CD4<sup>+</sup> T cells isolated from spleens and lymph nodes of CD45.1<sup>+</sup>CD45.2<sup>+</sup> WT OT-II TCR transgenic mice and CD45.2<sup>+</sup>DGK $\zeta$  KO OT-II TCR transgenic mice were sorted for naïve T cells (CD4<sup>+</sup>CD45RB<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup>). Sorted naïve CD45.1<sup>+</sup>CD45.2<sup>+</sup>WT OT-II and CD45.2<sup>+</sup> DGK $\zeta$  KO OT-II cells were transferred intravenously into CD45.1<sup>+</sup> WT hosts at a 1:1 ratio (400,000 WT OT-II:400,000 DGK $\zeta$  OT-II). One day after transfer, mice were subjected to OVA sensitization. Five days after the second intraperitoneal OVA/Alum immunization, splenic CD4<sup>+</sup> T cells were isolated by MACS enrichment and restimulated with PMA (100 ng/ml; Sigma-Aldrich) and ionomycin (1  $\mu$ g/ml; Sigma-Aldrich) in the presence of brefeldin A (5  $\mu$ g/ml; Cell Signaling Technology) for 5 hours. After restimulation, activated CD4<sup>+</sup> T cells were washed twice with PBS and prepared for cell surface and intracellular cytokine staining.

### Generation of BM chimeric mice

Donor BM cells from CD45.1<sup>+</sup> WT or CD45.2<sup>+</sup> DGK $\zeta$  KO donor mice were transferred ( $3 \times 10^6$  to  $4 \times 10^6$  BM cells) intravenously into lethally irradiated (11 Gy) CD45.1<sup>+</sup> WT or CD45.2<sup>+</sup> DGK $\zeta$  KO hosts. Ten weeks after BM transfer, mice were subjected to OVA sensitization and challenge.

### Murine tracheal ring contraction

Airway contractility in isolated murine tracheal rings was determined using multiwire myograph (ADInstruments) as described previously (80). Tracheal rings were mounted on the myograph, bathed in Krebs-Henseleit (K-H) buffer (pH 7.4, 37°C, and 95% O<sub>2</sub>/5% CO<sub>2</sub>), and baseline tension was set at ~2.5 mN. After the rings attained a stable baseline tension, airways were challenged with increasing concentrations of methacholine and the change in tension was recorded. At the end of the last dose of methacholine, the airways were washed with K-H buffer. Data acquisition and analysis were performed using the Chart 7 software.

### Airway contraction measurements in human PCLS

Human PCLS preparation and bronchoconstriction experiments were performed as previously described (81). Briefly, healthy whole lungs from nonasthmatic donors were received from the National Disease Research Interchange (Philadelphia, PA). Lungs were inflated with 2% low-melting temperature agarose, sectioned, cored (8-mm diameter), and sliced at a thickness of 350  $\mu$ m (Precisionary Instruments VF300 Vibratome). Lung slices were rested in Ham's F12 medium in 12-well tissue culture plates at 37°C for 2 days after isolation and washed three times with fresh media to rid airways of agarose on days 1 and 2 during the resting period. Next, slices were incubated overnight (18 hours) with DMSO or DGK inhibitor R59949 (Sigma-Aldrich). After overnight incubation, slices were challenged with increasing doses of carbachol (Sigma-Aldrich), and live video images of the airways were taken after each dose using a microscope (Nikon Eclipse TE2000-U,  $\times 40$  magnification) connected to a live video feed (Evolution QEi 32-0074A-130). Suitable airways on slices were chosen on the basis of the following criteria: presence of a full smooth muscle wall, intact beating cilia, and absence of shared muscle walls at airway

branch points to exclude possible counteracting contractile forces. Changes in airway luminal area were measured using Image-Pro Plus software (version 6.0, Media Cybernetics) as previously described (81). Area under curve was calculated from the dose-response curves generated.

### **HASM isolation, culture, and immunoblotting**

Primary HASM were generated from nondiseased tracheas received from the National Disease Research Interchange (Philadelphia, PA). HASM culture was performed as previously described (82). HASM cells were cultured in Ham's F12 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (2.5 mg/ml) for up to one to five passages before use.

For immunoblotting analysis, HASM cells were grown to confluence in 12-well tissue culture plates. Cells were serum starved for 24 hours before stimulation. Cells were incubated overnight (18 hours) with DMSO or R59949. After overnight incubation, cells were stimulated with 10  $\mu$ M carbachol for 10 min. After stimulation, cells were fixed with perchloric acid, scraped, pelleted, and lysed in RIPA (radioimmunoprecipitation assay) buffer. Lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes. Immunoblotting for phosphorylated MLC (pMLC), phospho-Akt (pAkt), and phosphorylated MLC phosphatase (pMYPT1) was performed using tubulin as a loading control. The antibodies used for immunoblotting are listed in table S2.

### **In vivo systemic administration of DGK inhibitor**

For pharmacological inhibition of DGK activity in vivo, mice were intraperitoneally injected once daily with either DMSO or the DGK inhibitor R59949 (10 mg/kg; Sigma-Aldrich) in 50% polyethylene glycol (PEG) 400 (Sigma-Aldrich) solution beginning immediately after the second intraperitoneal OVA/Alum immunization and ending before the last intranasal OVA challenge (late sensitization and challenge phases) or beginning immediately before the first intranasal OVA challenge and ending before the last intranasal OVA challenge (challenge phase only).

### **Statistical analysis**

Statistical analysis of data was performed with the Prism 6 software (GraphPad).

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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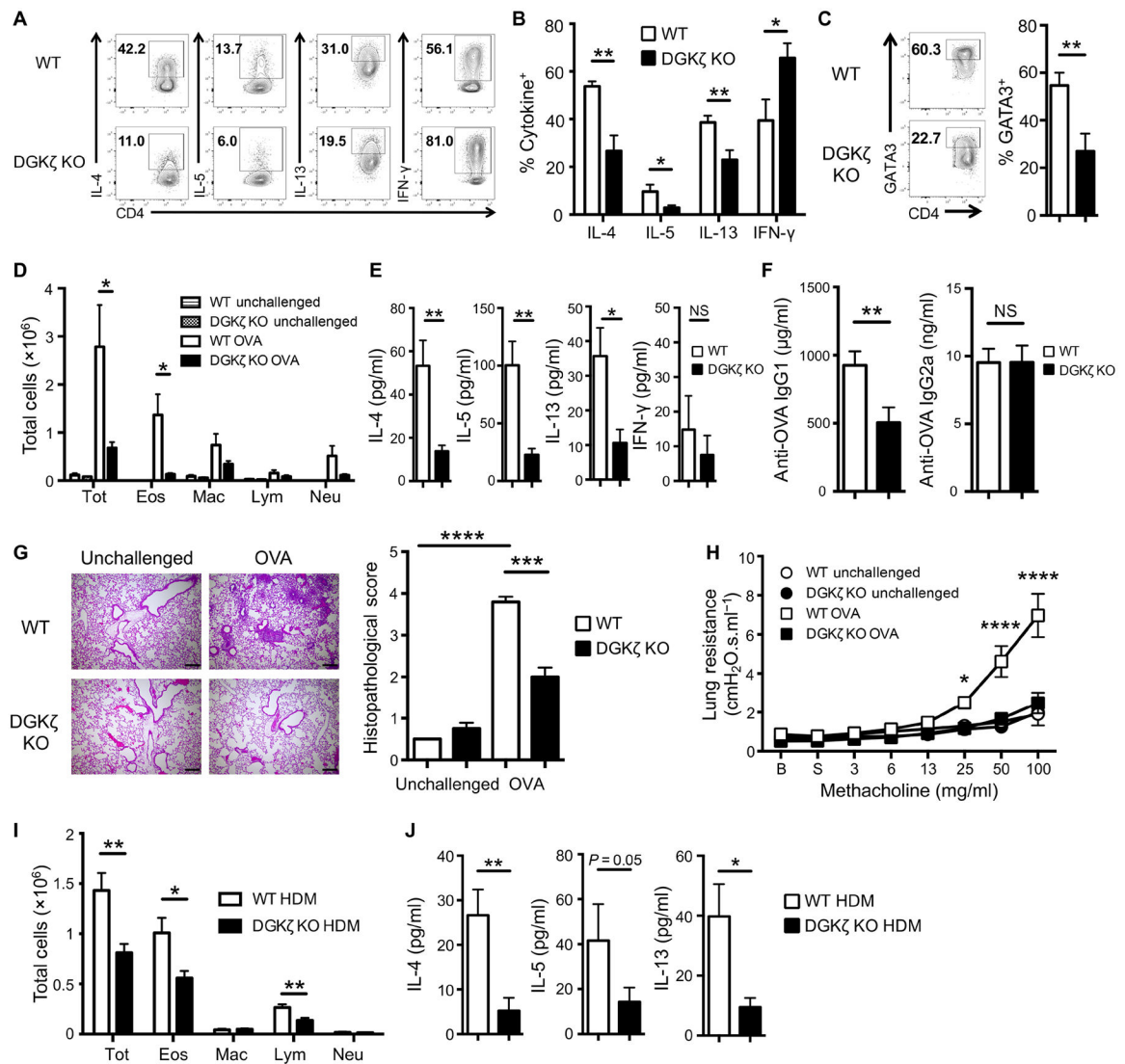


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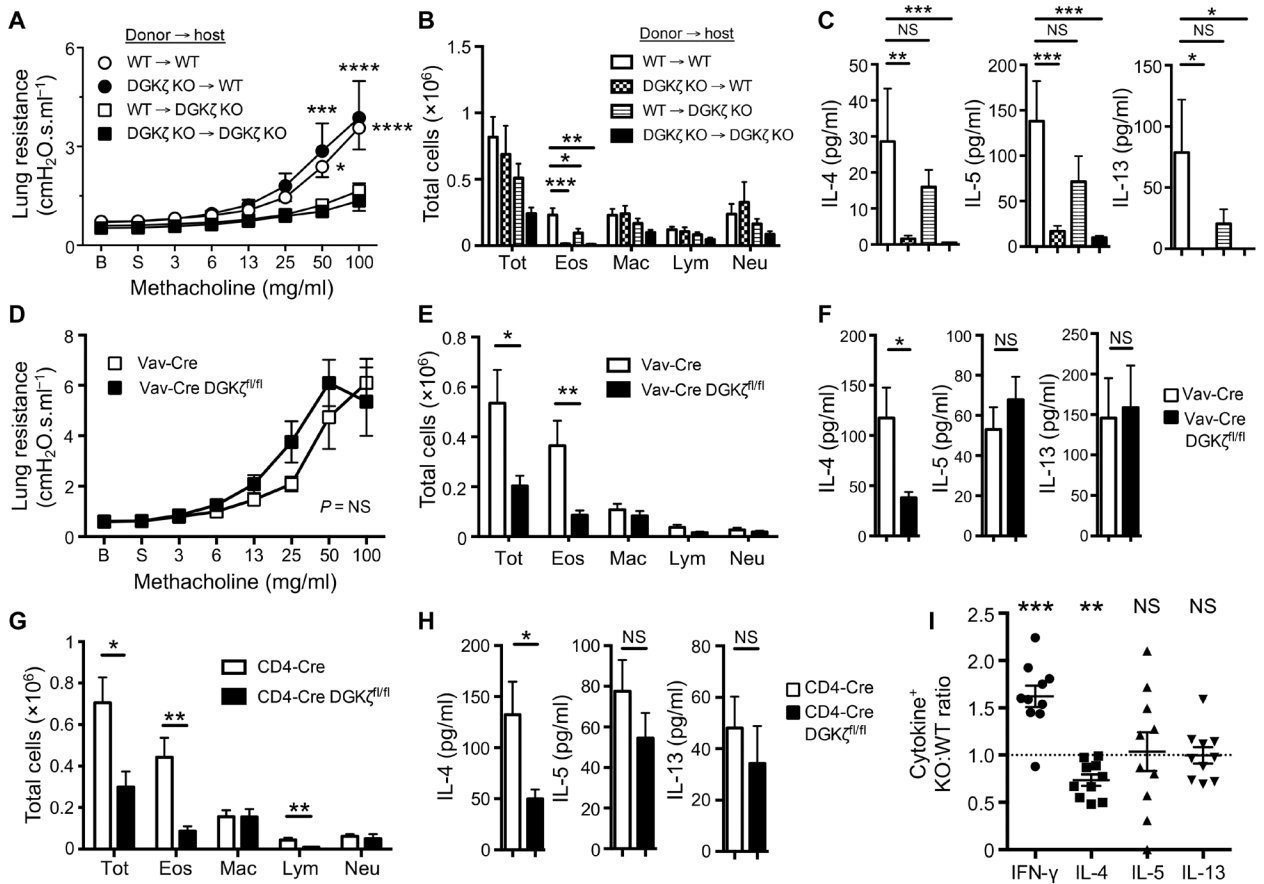
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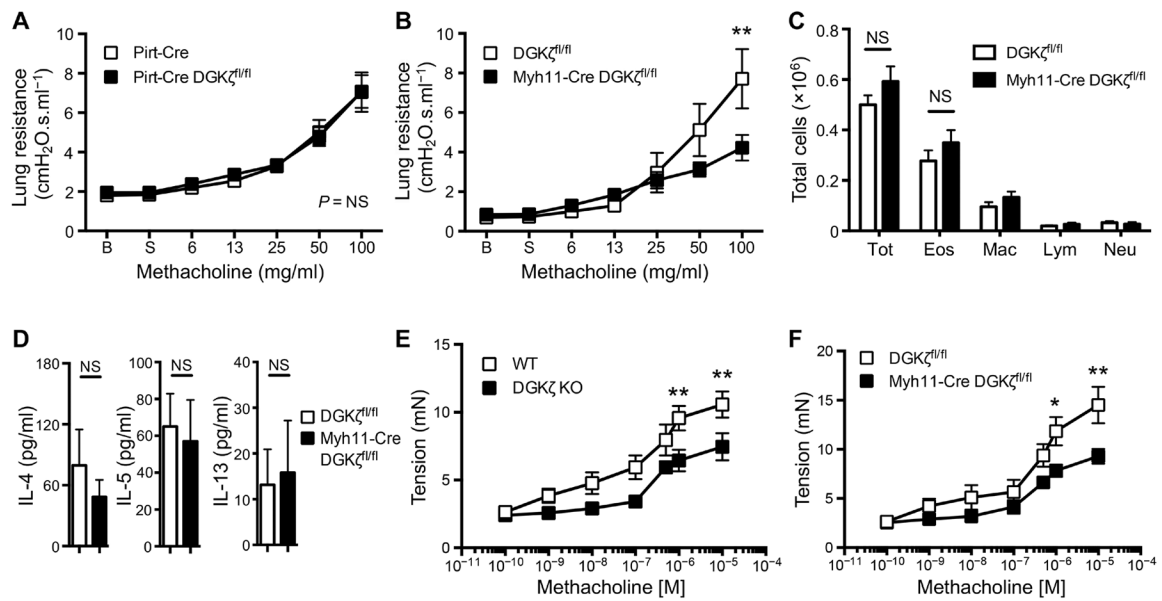
**Fig. 1. DGK $\zeta$  KO mice are protected from OVA-induced allergic airway inflammation and AHR.** (A to C) Flow cytometry analysis of cytokine production and GATA3 abundance in WT and DGK $\zeta$  KO CD4<sup>+</sup> T cells activated in vitro with antibodies against CD3 and CD28 for 5 days. Dot plots (A and C) are representative of five independent experiments. The frequency of cytokine-producing cells (B) and the frequency of GATA3<sup>+</sup> cells (C) are means  $\pm$  SEM of 10 mice per group pooled from all experiments. (D) Cytospin analysis of total number of eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu) in BAL fluid from unchallenged or OVA-challenged WT and DGK $\zeta$  KO mice, as indicated. Data are means  $\pm$  SEM of 6 mice per group (unchallenged) and 19 to 22 mice per group (OVA-challenged) from three independent experiments. (E and F) ELISA analysis of the amount of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines in BAL fluid (E) and OVA-specific IgG1 and IgG2a antibody in the serum (F) from OVA-challenged WT and DGK $\zeta$  KO mice. Data are means  $\pm$  SEM of 14 to 16 mice per group from two independent experiments. (G) H&E staining of lung tissue (left) and compiled histopathological scores (right) from unchallenged and OVA-challenged WT and DGK $\zeta$  KO mice. Images (left) are representative of two independent experiments. Scale

bars, 200  $\mu\text{m}$ . Quantified scores (right) are means  $\pm$  SEM of four to five mice per group pooled from all experiments. **(H)** FlexiVent analysis of airway resistance after methacholine treatment of unchallenged or OVA-challenged WT and DGK $\zeta$  KO mice, as indicated. Data are means  $\pm$  SEM of six to nine mice per group from two independent experiments. **(I and J)** Flow cytometry analysis of total immune cells (I) and ELISA for cytokine amounts (J) in BAL fluid from HDM-challenged WT and DGK $\zeta$  KO mice. Data are means  $\pm$  SEM of nine mice per group from two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ; NS, not significant by two-sided unpaired Student's  $t$  test (B to G and I), two-way analysis of variance (ANOVA) with Bonferroni's posttest (H), or Mann-Whitney  $U$  test (J).



**Fig. 2. Hematopoietic and nonhematopoietic cells differentially contribute to OVA-induced airway inflammation and AHR in the absence of DGK $\zeta$ .**

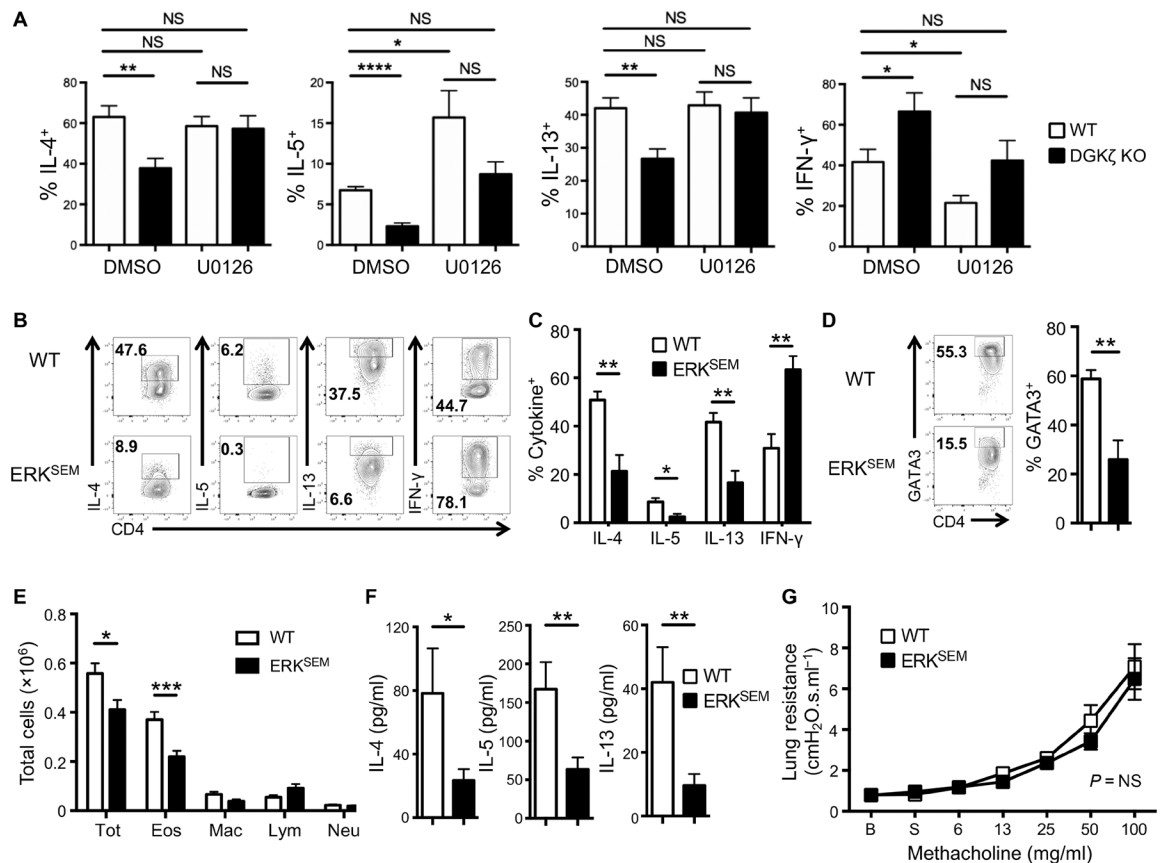
(A to C) FlexiVent analysis of airway resistance after methacholine treatment (A), cytospin analysis of total immune cells in BAL fluid (B), and ELISA for cytokine abundance in BAL fluid (C) from OVA-challenged WT and DGK $\zeta$  KO BM chimeras. Data are means  $\pm$  SEM of seven to nine mice per group from three independent experiments. (D to F) FlexiVent analysis of airway resistance after methacholine treatment (D), cytospin analysis of total immune cells in BAL fluid (E), and ELISA for cytokine abundance in BAL fluid (F) from OVA-challenged Vav-Cre DGK $\zeta$ <sup>fl/fl</sup> mice and Vav-Cre controls. Lung resistance values (D) are means  $\pm$  SEM of seven mice per group from two independent experiments. BAL cell numbers (E) and cytokine abundance (F) are means  $\pm$  SEM of 13 to 14 mice per group from four independent experiments. (G and H) Cytospin analysis of total immune cells (G) and ELISA for cytokine amounts (H) in BAL fluid from OVA-challenged CD4-Cre DGK $\zeta$ <sup>fl/fl</sup> mice and CD4-Cre controls. Data are means  $\pm$  SEM of 12 to 17 mice per group from at least two independent experiments. (I) Flow cytometry analysis of the ratio of adoptively transferred cytokine-producing WT OT-II and DGK $\zeta$  KO OT-II CD4<sup>+</sup> T cells in the spleen of OVA-sensitized congenic WT hosts after ex vivo restimulation with PMA/ionomycin. Data are means  $\pm$  SEM of 10 mice per group from two independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001; NS, not significant by two-way ANOVA with Bonferroni's posttest (A and D), two-sided unpaired Student's  $t$  test (B and E to H), Mann-Whitney  $U$  test (C), or one-sided Student's  $t$  test (I).



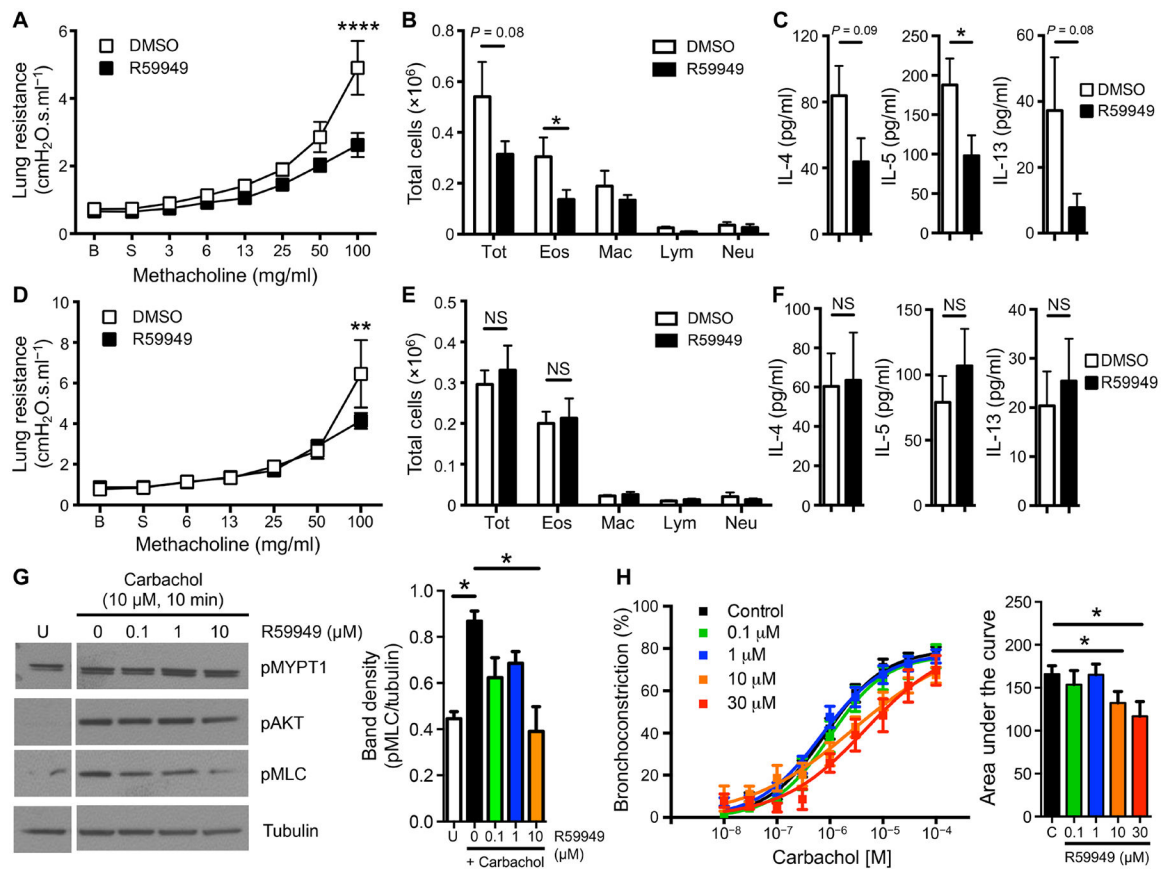
**Fig. 3. The loss of DGK $\zeta$  in airway smooth muscle cells protects against AHR.**

(A) FlexiVent analysis of airway resistance after methacholine treatment from OVA-challenged Pirt-Cre controls and Pirt-Cre DGK $\zeta^{fl/fl}$  mice. Data are means  $\pm$  SEM of 8 to 11 mice per group from two independent experiments. (B to D) FlexiVent analysis of airway resistance after methacholine treatment (A), flow cytometry analysis of total immune cells in BAL fluid (B), and ELISA for cytokine abundance in BAL fluid (C) from OVA-challenged DGK $\zeta^{fl/fl}$  controls and Myh11-Cre DGK $\zeta^{fl/fl}$  mice. Lung resistance values (B) are means  $\pm$  SEM of 9 to 14 mice per group from four independent experiments. BAL cell numbers (C) and cytokine abundance (D) are means  $\pm$  SEM of 18 to 21 mice per group from six independent experiments. (E) Myograph analysis of the contractile forces generated after methacholine treatment ex vivo of tracheal rings isolated from WT and DGK $\zeta$  KO mice. Data are means  $\pm$  SEM of 8 to 11 mice per group from three independent experiments. (F) Myograph analysis of the contractile forces generated after methacholine treatment ex vivo of tracheal rings isolated from DGK $\zeta^{fl/fl}$  controls and Myh11-Cre DGK $\zeta^{fl/fl}$  mice. Data are means  $\pm$  SEM of eight mice per group from two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ ; NS, not significant by two-way ANOVA with Bonferroni's posttest (A, B, E, and F) or two-sided unpaired Student's  $t$  test (C and D).





**Fig. 4. Enhancement of ERK signaling in T cells is sufficient to protect from OVA-induced allergic airway inflammation but insufficient to protect from OVA-induced AHR.** (A) The frequency of cytokine-producing WT and DGK $\zeta$  KO CD4<sup>+</sup> T cells after pretreatment with either vehicle control or U0126 followed by in vitro activation with antibodies against CD3 and CD28 for 5 days. Data are means  $\pm$  SEM of five mice per group from two independent experiments. (B to D) Flow cytometry analysis of cytokine production and GATA3 abundance in WT and ERK<sup>SEM</sup> CD4<sup>+</sup> T cells after in vitro activation with antibodies against CD3 and CD28 for 5 days. Dot plots (B and D) are representative of three independent experiments. The frequency of cytokine-producing cells (C) and the frequency of GATA3-expressing cells (D) are means  $\pm$  SEM of six mice per group from three independent experiments. (E to G) Flow cytometry analysis of total immune cells in BAL fluid (E), ELISA for cytokine abundance in BAL fluid (F), and flexiVent analysis of airway resistance after methacholine treatment (G) from OVA-challenged WT and ERK<sup>SEM</sup> mice. BAL cell numbers (E) and cytokine abundance (F) are means  $\pm$  SEM of 21 to 26 mice per group from five independent experiments. Lung resistance values (G) are means  $\pm$  SEM of eight to nine mice per group from two independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001; NS, not significant by two-sided unpaired Student's  $t$  test (A to F) or two-way ANOVA with Bonferroni's posttest (G).



**Fig. 5. Pharmacological inhibition of DGK protects against OVA-induced allergic airway inflammation and AHR.**

(A to C) FlexiVent analysis of airway resistance after methacholine treatment (A), flow cytometry analysis of total immune cells in BAL fluid (B), and ELISA for cytokine abundance in BAL fluid (C) from OVA-challenged WT mice treated with either vehicle control or R59949 during the late sensitization and airway challenge phases of the OVA-induced asthma model. Lung resistance values (A) are means  $\pm$  SEM of seven to eight mice per group from three independent experiments. BAL cell numbers (B) are means  $\pm$  SEM of 15 to 17 mice per group from four independent experiments. Cytokine amounts (C) are means  $\pm$  SEM of 10 to 11 mice per group from two independent experiments. (D to F) FlexiVent analysis of airway resistance after methacholine treatment (D), flow cytometry analysis of total immune cells in BAL fluid (E), and ELISA for cytokine abundance in BAL fluid (F) from OVA-challenged WT mice treated with either vehicle control or R59949 only during the airway challenge phase of the OVA-induced asthma model. Data are means  $\pm$  SEM of 9 to 10 mice per group from two independent experiments. (G) Western blot for pMLC, pAkt, and pMYPT1 in lysates of carbachol-stimulated HASM cells pretreated with vehicle versus R59949. Blots (left) are representative of three independent experiments. Normalized band densities of pMLC (right) are means  $\pm$  SEM of three donors per condition pooled from all experiments. (H) Analysis of bronchoconstriction (left) and area under curve values (right) after carbachol treatment of human PCLS pretreated with vehicle versus R59949. Data are means  $\pm$  SEM of 9 to 23 slices per condition from three to seven donors

per condition. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ ; NS, not significant by two-way ANOVA with Bonferroni's posttest (A and D) or two-sided unpaired Student's  $t$  test (B, C, and E to H).