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The Immunomodulatory Functions Of Diacylglycerol Kinase Zeta On Type 2 Immune Responses

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Abstract

Type 2 helper T cells (Th2) are beneficial for orchestrating protective immune responses against helminths but can also be pathogenic in settings of allergy and asthma. Weak TCR-mediated extracellular signal-regulated kinase (ERK) signals are thought to promote Th2 differentiation in vitro. However, it was unclear whether selective enhancement of specific TCR-mediated signal transduction pathways could suppress Th2 differentiation in vitro and block Th2 inflammation in vivo in a polyclonal setting. The lipid molecule diacylglycerol (DAG) is the main driver of TCR-mediated ERK activation. Here, we demonstrate that T cells lacking DAG kinase-ζ (DGKζ), a negative regulator of DAG, display impaired Th2 differentiation in vitro. Accordingly, mice lacking DGKζ exhibited decreased type 2 airway inflammation and were almost completely resistant to airway hyperresponsiveness (AHR) in vivo in an OVA-induced mouse model of allergic asthma. Surprisingly, we found that the mechanisms by which DGKζ protected against airway inflammation and AHR were separable. Conditional deletion of DGKζ in T cells led to decreased type 2 airway inflammation with no attenuation of AHR. In contrast, conditional deletion of DGKζ in airway smooth muscle cells led to diminished AHR with no attenuation of airway inflammation. Mechanistically, T-cell specific enhancement of ERK signaling was sufficient to diminish Th2 differentiation in vitro and attenuate type 2 airway inflammation with no changes in AHR in vivo. These data demonstrate that specific enhancement of DAG signaling downstream of the TCR is sufficient to attenuate Th2 differentiation in an ERK-dependent manner. Furthermore, our findings reveal that the inflammatory and AHR components of asthma are not as interdependent as generally believed.

Additionally, we also demonstrate a novel role for DGKζ in regulating protease allergen-mediated type 2 airway inflammation. We found that global but not hematopoietic-specific ablation of DGKζ was sufficient to protect from papain-induced airway inflammation. Further analysis revealed that protection from papain in the absence of DGKζ might be potentially due to an impairment in IL-33 production/release in response to papain. Collectively, this thesis highlights that DGKζ plays immunomodulatory roles during Th2 differentiation and in the non-hematopoietic compartments to regulate type 2 immune-mediated disease.

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THE IMMUNOMODULATORY FUNCTIONS OF DIACYLGLYCEROL KINASE ZETA ON TYPE 2

IMMUNE RESPONSES

DEDICATION

To Anu, who forever has my heart

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ABSTRACT

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Type 2 helper T cells (Th2) are beneficial for orchestrating protective immune responses against helminths but can also be pathogenic in settings of allergy and asthma. Weak TCR-mediated extracellular signal-regulated kinase (ERK) signals are thought to promote Th2 differentiation *in vitro*. However, it was unclear whether selective enhancement of specific TCR-mediated signal transduction pathways could suppress Th2 differentiation *in vitro* and block Th2 inflammation *in vivo* in a polyclonal setting. The lipid molecule diacylglycerol (DAG) is the main driver of TCR-mediated ERK activation. Here, we demonstrate that T cells lacking DAG kinase-ζ (DGKζ), a negative regulator of DAG, display impaired Th2 differentiation *in vitro*. Accordingly, mice lacking DGKζ exhibited decreased type 2 airway inflammation and were almost completely resistant to airway hyperresponsiveness (AHR) *in vivo* in an OVA-induced mouse model of allergic asthma. Surprisingly, we found that the mechanisms by which DGKζ protected against airway inflammation and AHR were separable. Conditional deletion of DGKζ in T cells led to decreased type 2 airway inflammation with no attenuation of AHR. In contrast, conditional deletion of DGKζ in airway smooth muscle cells led to diminished AHR with no attenuation of airway inflammation. Mechanistically, T-cell specific enhancement of ERK signaling was sufficient to diminish Th2 differentiation *in vitro* and attenuate type 2 airway inflammation with no changes in AHR *in vivo*. These data demonstrate that specific enhancement of DAG signaling downstream of the TCR is sufficient to attenuate Th2 differentiation in an ERK-dependent manner. Furthermore, our findings reveal that

the inflammatory and AHR components of asthma are not as interdependent as generally believed.

Additionally, we also demonstrate a novel role for DGKζ in regulating protease allergen-mediated type 2 airway inflammation. We found that global but not hematopoietic-specific ablation of DGKζ was sufficient to protect from papain-induced airway inflammation. Further analysis revealed that protection from papain in the absence of DGKζ might be potentially due to an impairment in IL-33 production/release in response to papain. Collectively, this thesis highlights that DGKζ plays immunomodulatory roles during Th2 differentiation and in the non-hematopoietic compartments to regulate type 2 immune-mediated disease.

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CHAPTER 1: INTRODUCTION

Introduction to Th responses

The immune system is critical for protection of the host from microbial exposure and environmental insults. $CD4^+$ helper T (Th) cells play a key role in orchestrating immune responses by providing help to B cells and $CD8^+$ cytotoxic T cells and activating cells of the innate immune system. Recognition of foreign-derived peptides presented on major histocompatibility complex class II (MHC II) molecules by T cell receptors (TCR) expressed on naïve, antigen-inexperienced Th cells lead to activation and proliferation of these cells. Additionally, engagement of the TCR in combination with costimulatory and cytokine signals initiate differentiation programs that result in the formation of highly specialized effector Th subsets that secrete distinct sets of cytokines to promote unique immune functions. These subsets include Th1 cells that produce IFNγ to promote immunity against viruses and intracellular bacteria, Th2 cells that produce IL-4, IL-5, and IL-13 to promote immunity against helminths, Th17 cells that produce IL-17A, IL-17F, and IL-22 that are important for protection against extracellular bacteria and fungi, Tfh cells that produce IL-21 to provide B cell help for the production of high-affinity antibodies, and iTregs that produce IL-10, IL-35, and TGFβ to limit excessive immune activation. In this manner, Th differentiation allows for the induction of an immune response that is tailored toward the nature of the encountered threat.

While decades of works have gone into understanding the signals that dictate differentiation of naïve $CD4^+$ T cells into specific Th lineages *in vivo*, major gaps still remain, particularly in the context of Th2 differentiation. The aim of this thesis is to interrogate the signal requirements for the differentiation of naïve $CD4^+$ T cells into the

Th2 lineage. This thesis will examine the roles of the lipid signaling molecule diacylglycerol (DAG) and the regulation of DAG metabolism by diacylglycerol kinase zeta (DGKζ) in modulating Th2 differentiation and Th2-mediated immune responses. Furthermore, this thesis will also discuss novel immunomodulatory roles of DGKζ in regulating non-immune responses that are critical for initiating and promoting the pathological aspects of aberrant type 2 immune responses**. Chapter 1** of this thesis will serve as an introduction into the key molecular signals that instruct Th2 differentiation and the various roles of DAG and DGKζ on immune cell signaling and function.

Signals regulating instruction of Th2 differentiation

Role of cytokine-driven signals in Th2 differentiation

IL-4

A key determinant of the Th differentiation decision is polarizing cues in the form of cytokines. Interleukin 4 (IL-4) is critical for instructing Th2 differentiation *in vitro (1).* IL-4 can bind and signal through the type I IL-4 receptor comprised of the IL-4Rα and IL-2Rγ subunits on T cells to induce activation of the Jak1/3-STAT6 pathway (*2*). Activation of the STAT6 pathway results in the induction of the transcription factor GATA3, which is the master regulator of Th2 cell identity, and increased accessibility of the *Il4* gene (*2*). Consequently, STAT6 KO and GATA3 KO T cells display complete abrogation of Th2 differentiation in the presence of exogenous IL-4 *in vitro (3-5)*. In addition to instructing the Th2 gene program, IL-4 also represses the induction of alternative gene programs through the induction of the transcriptional repressor Gfi-1 to suppress the induction of Th17 and iTreg lineage programs (*6*).

In vivo, Th2 differentiation can occur through IL-4-dependent and IL-4-independent routes. For IL-4-dependent Th2 differentiation, one of the major questions in the field has been trying to identify the source of IL-4 for initiating Th2 differentiation. Many different cell types have been implicated as a relevant source of IL-4, including type 2 natural killer T cells (NKT2), granulocytes (basophils, eosinophils, and mast cells), and innate lymphoid cells (ILC2) (*7-13*). Additionally, conventional naïve $CD4^+$ T cells can be an early source of IL-4 immediately following TCR activation to initiate differentiation down the Th2 pathway through an autocrine/paracrine manner (*14-16*). Nevertheless, mice lacking IL-4, IL-4Rα, or STAT6 display relatively normal generation of Th2 cells in response to *Nippostrongylus brasiliensis* and *Trichuris muris*, suggesting that the IL-4-independent route for Th2 differentiation is relevant in certain infection models (*17*). However, the factors that regulate IL-4-independent Th2 differentiation are not completely understood.

IL-2

Interleukin 2 (IL-2) is essential for differentiation of naïve $CD4^+$ T cells into the Th2 lineage. Neutralization of IL-2 blocks Th2 differentiation without affecting T cell proliferation, suggesting that IL-2 promotes Th2 differentiation independently of the canonical role of IL-2 in T cell proliferation (*1, 3*). Indeed, IL-2 maintains chromatin accessibility of the DNase I hypersensitive sites II (HSII) and III (HSIII) in the second intron of the *Il4* locus through a STAT5-dependent manner (*3*). In accordance, IL-2 KO and STAT5a KO T cells exhibit impaired Th2 differentiation (*3, 18*). Furthermore, retroviral-mediated expression of a constitutively active STAT5 mutant is sufficient to drive Th2 differentiation independently of IL-4, IL-4Rα, and STAT6 but dependent on GATA3 by promoting *Il4* gene accessibility at non-overlapping regions within the locus

(HSII and HSIII regions by STAT5 and V_A and CNS1 regions by GATA3) (*19*). In addition to influencing the *Il4* locus, IL-2 enhances responsiveness to IL-4 by upregulating expression of IL-4Rα through STAT5-dependent binding to the GAS3 region in the *Il4ra* locus to promote Th2 differentiation (*20*).

Tissue-derived cytokines (IL-33, TSLP, and IL-25)

Tissue-derived cytokines (IL-33, TSLP, and IL-25) are produced by specialized, non-hematopoietic cells in mucosal barriers upon exposure to allergens, tissue damage, or helminth parasites. The production of these cytokines is critical for promoting type 2 effector responses from both the innate and adaptive arms of the immune system for helminth clearance or allergen-induced immunopathology. However, the production of these tissue-derived cytokines also directly regulates Th2 differentiation. Van Dyken et al. demonstrated that differentiation of naïve $CD4^+$ T cells into the Th2 lineage occurs in a two-step process in which the initiation of Th2 differentiation and the induction of IL-4 competence occurs during T cell priming in the draining lymph nodes while terminal Th2 differentiation and the acquisition of the ability to produce IL-5 and IL-13 occurs following T cell entry into the inflamed tissue (*21*). In accordance, blocking lymph node egress using the sphingosine-1-phosphate receptor antagonist, FTY20, failed to increase the appearance of $IL\text{-}5^+$ and $IL\text{-}13^+$ Th2 cells in the draining lymph nodes following infection with *Nippostrongylus brasiliensis* despite no alterations in the frequency of IL-4-competent Th cells, thus demonstrating that the signals for instructing full Th2 differentiation but not IL-4-competence is absent in the lymph nodes (*21*). Rather, the induction of the terminal effector programs in Th2 cells was instructed by the tissue-derived signals elicited by IL-33, IL-25, and TSLP because T cells doubly-deficient in ST2 and TSLPR displayed impaired ability to produce IL-5 and IL-13 following

Nippostrongylus brasiliensis infection, which was further enhanced in the absence of IL-25 (*21*).

In addition to promoting terminal Th2 differentiation, TSLP can instruct the initiation of Th2 differentiation in a STAT5-dependent manner that can occur though IL-4-dependent and IL-4-independent routes (*22, 23*). Moreover, TSLP can repress the induction of Bcl-6 in a STAT5-dependent manner to selectively promote Th2 differentiation rather than differentiation into the follicular helper T (Tfh) cell lineage (*23*). Furthermore, TSLP can induce a pathogenic Th2 program, which is accompanied by low expression of IL-4 and Bcl-6 and high expression of IL-5, IL-9, IL-13, and GATA3, to drive the generation of IL-4^{neg} Th2 effector cells *in vivo (23, 24)*.

Role of costimulatory-driven signals in Th2 differentiation

CD28

CD28 is a costimulatory molecule expressed on T cells and binds the B7-1 (CD80) and B7-2 (CD86) molecules expressed on many different cell types, including professional antigen-presenting cells (APC). Engagement of CD28 is critical for full activation of T cells following TCR activation and preventing anergy induction. In addition to regulating T cell activation, CD28 costimulatory signals can also influence Th2 differentiation. Seder et al. demonstrated that ligation of CD28 is required for the induction of Th2 differentiation following activation of $CD4^+$ TCR transgenic T cells in the presence of APCs lacking B7 molecules (*25*). Furthermore, blockade of B7-CD28 interactions using CTLA4-Ig impaired Th2 differentiation by T-cell depleted APCs (*25*). Mechanistically, impairment of Th2 differentiation in the absence of CD28-mediated costimulatory signals could be overcome by the addition of exogenous IL-2, suggesting that CD28 promotes

Th2 differentiation by augmenting IL-2 production (*25*). In contrast, other groups have suggested that the effects of CD28 costimulation on promoting Th2 differentiation occurs through an IL-2-independent but IL-4-dependent mechanism (*26, 27*). Accordingly, Kubo et al. demonstrated that engagement of CD28 enhances the sensitivity and responsiveness of the IL-4 receptor to promote IL-4-mediated Th2 differentiation (*28*). Overall, these data suggest that CD28 is critical for the induction of Th2 differentiation potentially through multiple mechanisms.

OX40

OX40 is a costimulatory molecule from the tumor necrosis factor receptor (TNFR) superfamily. While not expressed in naïve T cells, OX40 expression is transiently induced 12 hours following TCR activation (*29*). Expression of OX40 remains elevated until 4-5 days after TCR activation, and can be rapidly induced upon TCR restimulation (*29*). While engagement of OX40 does not significantly influence initial $CD4^+$ T cell activation and proliferation, OX40 activation helps sustain T cell proliferation, cytokine production, and survival at the effector stage (*29, 30*).

OX40 costimulation can also instruct Th2 differentiation by regulating early IL-4 production in $CD4^+$ T cells following TCR activation (*31-33*). So et al. demonstrated that the absence of OX40-OX40L interactions, either due to genetic deletion of OX40 or the addition of a blocking antibody against OX40L, could attenuate Th2 differentiation following activation of $CD4^+$ TCR transgenic T cells in the presence of APC and cognate peptide *in vitro (33)*. Mechanistically, OX40 costimulation promoted early IL-4 transcription by enhancing nuclear translocation of NFATc1 in an IL-4R-independent but calcineurin and PI3K-dependent manner (*33*). Consistent with this notion, Th2

generation has been shown to be diminished in OX40 KO mice subjected to an OVA-induced model of asthma and following infections with *Leishmania major* and *Heligmosomoides polygyrus in vivo (34-36)*.

ICOS

ICOS is a member of the CD28 superfamily of receptors and binds to ICOSL that is expressed primarily on professional APCs, such as B cells, dendritic cells (DC), and macrophages, as well as on endothelial and alveolar type II cells (*37*). Similar to OX40, ICOS is not expressed in naïve T cells but is upregulated after TCR activation (*38, 39*). Triggering of ICOS promotes T cell proliferation and enhances the secretion of various cytokines, such as IL-4, IL-10, IFNγ, but not secretion of IL-2 following TCR activation (*38, 39*).

ICOS expression is high in Th2 cells and costimulatory signals delivered by ICOS can promote Th2 responses because blocking ICOS interactions in mice impairs Th2 generation following OVA-induced asthma induction or infection with *Nippostrongylus brasiliensis (40, 41)*. However, the mechanism by which ICOS regulates the differentiation of naïve $CD4^+$ T cells into the Th2 lineage is not entirely clear. Nurieva et al. reported that ICOS activation promotes Th2 generation by directly enhancing IL-4R-independent early IL-4 transcription in T cells following TCR activation to instruct Th2 differentiation (*42*). Specifically, ICOS promoted the upregulation and nuclear translocation of NFATc1 and was potentially important for the induction of c-Maf to potentiate early IL-4 transcription (*42*). In contrast, Watanabe et al. demonstrated that the ligation of ICOS did not impact early IL-4 transcription or NFATc1 nuclear localization

in T cells (*43*). Instead, activation of ICOS promoted Th2 differentiation by enhancing IL-4R signaling through an unknown mechanism (*43*).

Notch

Notch receptors are an evolutionarily conserved family of receptors that are important for regulation of a variety of cell processes, including cell proliferation, differentiation, survival, and death (*44*). Notch receptors can bind to two classes of membrane-bound Notch ligands, which are the Jagged ligand family (Jagged1 and Jagged 2) or the Delta-like ligand family (DLL1, DDL3, and DDL4) (*44*). For T cells, the Notch signaling pathway is critical for T cell development by inducing commitment of multipotent hematopoietic progenitor cells to the T lineage decision and maintenance of the T lineage program as early thymic progenitors (ETP) progress through $\alpha\beta$ T cell development in the thymus (*45-47*).

In addition to T cell development, engagement of the Notch signaling pathway is important for Th2 differentiation. Early reports demonstrated the activation of the Notch signaling pathway was critical for Th2 differentiation because loss of Notch signaling by either deletion of RBPJκ, the major transcriptional effector for Notch signaling, or expression of a dominant negative MAML protein, a scaffold protein important for Notch transcriptional activation complex, attenuated Th2 differentiation *in vitro* and impaired the generation of protective Th2 responses against *Trichuris muris in vivo (48, 49)*. Mechanistically, *Gata3* and *Il4* were direct transcriptional targets of Notch and engagement of Notch signaling regulated transcription of both genes in IL-4/STAT6-independent manner to promote optimal Th2 differentiation (*48, 50*). However, it is now appreciated that Notch acts as a broad regulator of Th differentiation by promoting

multiple Th lineage programs through direct transcriptional activation of Th2-specific (*Il4 and Gata3*) and non-Th2 targets (*Tbx21, Ifng, Il17a, Rorc*) rather than instructing specific helper T cell lineage choice (*51-54*).

Role of TCR-mediated signal strength in Th2 differentiation

While cytokine and costimulatory signals are important for instructing Th2 differentiation, the strength and duration of TCR signaling can also contribute to the outcome of Th differentiation. Early reports demonstrated that activation of $CD4^+$ TCR transgenic T cells in the presence of APCs and high doses of cognate peptide, which are conditions that elicit strong and prolonged TCR signaling, resulted in preferential differentiation toward the Th1 lineage (*55, 56*). In contrast, T cell activation by APCs given low doses of cognate peptide, which are conditions that elicit weak and transient TCR signaling, potentiated Th2 differentiation (*55, 56*). Accordingly, activation of $CD4^+$ TCR transgenic T cells with altered peptide ligands (APL), which are cognate peptides with signal amino acid substitutions that result in lower affinity TCR interactions, promoted Th2 differentiation as compared to activation with WT peptides (*57, 58*). Similarly, lowering TCR affinity for cognate peptide-MHC complexes by the introduction of a point mutation (L51 → S51) in the CDR2 region of the TCRα chain of the D10 $CD4^+$ TCR transgenes to generate L51S $CD4^+$ TCR transgenic T cells altered Th differentiation toward Th2 and away from Th1 following TCR activation with high doses of cognate peptide (*59*). Overall, these results demonstrate that weak TCR signaling promotes differentiation of naïve $CD4^+$ T cells into the Th2 lineage.

The strength of TCR signaling instructs Th differentiation by regulating early T-cell-intrinsic IL-4 transcription through a TCR-dependent but IL-4R-independent

mechanism. Weak TCR signaling induces the early upregulation of GATA3 in an IL-2-independent and IL-4-independent manner (*60*). TCR-mediated early GATA3 induction works in conjunction with IL-2-mediated STAT5 signaling to promote accessibility of *Il4* locus to drive early IL-4 transcription in the first 14-24 hours following TCR activation independently of IL-4 signaling (*3, 60*). Subsequently, secretion of IL-4 protein then further reinforces GATA3 induction during the polarization phase of Th differentiation through an IL-4R/STAT6-dependent mechanism to potentiate Th2 differentiation (*61*).

Augmenting TCR signaling can block this process of early IL-4 transcription through multiple mechanisms. Similar to previous reports, Yamane et al. demonstrated that activation of $CD4^+$ TCR transgenic T cells with APCs and high doses of cognate peptide, which mimics strong TCR signaling, blocked Th2 differentiation by dampening early GATA3 induction and IL-4 transcription (*60*). Importantly, they found that TCR-mediated ERK signaling was a key determinant in regulating Th differentiation because inhibition of ERK signaling using the MEK inhibitor, U0126, could restore Th2 differentiation at these high peptide concentration conditions through upregulation of early GATA3 expression and IL-4 transcription (*60*). Additionally, strong TCR-mediated ERK signaling blocked IL-2R-mediated STAT5 activation independently of the effects of ERK signaling on GATA3 to attenuate Th2 differentiation (*60*). However, despite the importance of strong TCR-mediated ERK signaling in blocking early GATA3 induction and promoting IL-2R desensitization to suppress Th2 differentiation, the molecular mechanisms through which ERK mediates these effects are not entirely understood. Interestingly, Jorritsma et al. suggested that weak TCR-mediated ERK signaling either by activation with APLs or through inhibition of ERK signaling can promote Th2 differentiation through an alternative mechanism by altering the composition of TCR-

mediated activation of AP-1 complexes from Fos-JunB heterodimers to more JunB-JunB homodimers (*58*). However, evidence that alterations in AP-1 complex formation by changes in TCR-signal strength directly instructs Th differentiation is lacking. Overall, the data supports that TCR signal strength regulates Th2 differentiation through the regulation of early T cell-intrinsic IL-4 production.

Given that the lipid molecule diacylglycerol (DAG) is the main driver of TCR-mediated ERK activation, the following sections will review the regulation of DAG by diacylglycerol kinases (DGK) and the various roles that DGKs play in regulating immune cell function.

Regulation of DAG by diacylglycerol kinases (DGK)

Diacylglycerol (DAG) is a key secondary lipid messenger for transducing signals downstream of many receptors expressed by hematopoietic cells. DAG has shown to be important in driving the activation, proliferation, migration and effector function of adaptive and innate immune cells. The generation of DAG can be accomplished by the activation of various cell-surface receptors, including G_q-mediated G-protein coupled receptors (GPCR)s (e.g., muscarinic and histamine receptors) and immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors (e.g., T cell receptor (TCR), FcεRI) (*62-65*). The activation of these receptors results in the formation of proximal signaling complexes that are critical for the activation of phospholipase C (PLC). PLC activation leads to enzymatic cleavage of phosphoinositol 4,5-bisphosphate (PIP_2) into DAG and inositol 1,4,5-triphosphate (IP_3) (*66*). While IP_3 mobilizes Ca^{2+}, DAG activates the NF-κB and extracellular regulated kinase (ERK) pathways through protein kinase C (PKC) and RasGRP, respectively, to promote cell function (*67-72*). Consequently, the levels of DAG

must be tightly regulated to control the magnitude and duration of the responses generated.

Diacylglycerol kinases (DGK) are negative regulators of DAG-mediated signaling. DGKs regulate DAG signaling by phosphorylating DAG and converting it into phosphatidic acid (PA) (*64, 73*). The loss of DGKs increases DAG levels and the duration of DAG-mediated signaling. One might expect that elevated DAG levels would lead to general enhancement of effector responses. However, the enhancement of DAG signaling through the loss of DGKs can lead to either hyperactivation or hyporesponsiveness depending on the cell type and the type of response. There are 10 different isoforms comprising 5 different classes of DGKs, each of which control different cellular functions based on their distinct structural motifs and subcellular localization (*64, 73-76*). The three major isoforms that are abundantly expressed in lymphoid tissues are DGKα, DGKδ, and DGKζ (*77*). In particular, mice that lack the zeta (ζ) isoform of DGK, which is highly expressed in hematopoietic cells, display profound effects on the functional behavior of various cell types. In following sections, we will focus on how DGKζ plays both negative and positive roles in immune responses mounted by different cell types.

Negative regulation of effector responses by DGKζ

$CD4^+$ and $CD8^+$ conventional T cells

DGKζ serves as a critical negative regulator of DAG signaling downstream of the TCR and can modulate the strength of TCR signaling. Early experiments using the immortalized Jurkat T cell line showed that overexpression of DGKζ inhibits TCR signaling by reducing the levels of active GTP-bound Ras and, consequently, diminishing ERK activation (*78*). Furthermore, the overexpression of DGKζ was associated with decreased AP-1

transcription factor activity and CD69 expression (an early T cell activation marker) following TCR stimulation, both of which are regulated by the Ras-ERK pathway. Importantly, Ca^{2+} flux was normal regardless of DGKζ overexpression, suggesting that DGKζ selectively regulated DAG-mediated signaling pathways downstream of the TCR. Further biochemical analysis through the use of a kinase dead DGKζ mutant revealed that the enzymatic activity of DGKζ was critical for its inhibitory effects on TCR signaling.

To test the physiological role of DGKζ in T cells, Zhong et al. generated DGKζ knockout mice (*79*). Initial phenotypic analysis showed that DGKζ KO mice contained similar frequencies and numbers of $CD4^+$ and $CD8^+$ T cells in secondary lymphoid organs and displayed no obvious defects in lymphoid architecture or cellularity. Furthermore, thymic development as analyzed by the number and frequency of CD4 single-positive (SP), CD8 SP, double-positive (DP), and double-negative (DN) thymocytes in DGKζ-deficient mice was similar to wild-type (WT) mice. However, upon TCR stimulation, naïve DGKζ KO $CD4^+$ and $CD8^+$ T cells displayed enhanced upregulation of activation markers CD25 and CD69 and increased proliferation compared to WT T cells. The increased expression of activation markers was associated with enhanced phosphorylation of ERK but normal induction of non-DAG mediated pathways including Ca^{2+} flux and JNK activation. Importantly, bypassing TCR activation with a DAG analogue, phorbol-12-myristate-13-acetate (PMA), abolished differences in activation between DGKζ KO and WT T cells, suggesting that the hyperactivation of DGKζ KO T cells was secondary to defective regulation of DAG.

In agreement with enhanced TCR signaling, DGKζ KO T cells also display improved anti-viral responses (*79*). DGKζ KO mice infected with LCMV Armstrong showed enhanced viral-specific T cell responses as evidenced by decreased viral titers at day 7

following infection. This effect correlated with an increased number of total and $CD44^{hi}CD62L^{lo}$ effector $CD8^+$ T cells in the spleen. Furthermore, LCMV-infected DGKζ KO mice exhibited increased number of IFNγ-producing $CD8^+$ and $CD4^+$ T cells when restimulated with LCMV-specific peptides, suggesting that DGKζ KO T cells displayed enhanced effector function following LCMV infection.

Similarly, DGKζ-deficient mice also exhibit enhanced anti-tumor responses. DGKζ KO mice subcutaneously injected with OVA-expressing EL4 T cell lymphoma, had significantly reduced tumor mass compared to their WT counterparts (*80*). This effect was accompanied by an increased number of total and antigen-specific tumor-infiltrating $CD44^{hi}CD8^+$ T cells. To show that T cells were responsible for the enhanced anti-tumor effect by DGKζ deficiency, DGKζ KO and WT OVA-specific OT-I T cells were adoptively transferred into naïve recipient WT mice. Mice receiving DGKζ KO compared to WT OVA-specific OT-I T cells also exhibited lower tumor mass upon challenge with OVA-expressing EL4 cells. Isolation of tumor-infiltrating OT-I cells revealed that the loss of DGKζ increased the frequency of $CD44^{hi}$ and IL-2-producing OT-I cells in a cell-intrinsic manner. In addition to preventing tumor engraftment, DGKζ deficiency also improves tumor rejection of established tumors, as the adoptive transfer of DGKζ KO but not WT OT-I effector T cells into tumor-bearing mice significantly reduced tumor burden (*81*). Thus, DGKζ could represent a novel target for enhancing anti-tumor responses in adoptive immunotherapy. This could also be applied to engineered T cells that express chimeric antigen receptors (CAR) directed against the tumor, as DGKζ deficiency was also shown to promote CAR T cell-mediated anti-tumor responses (*81*). How DGKζ deficiency augments anti-tumor responses is unclear. Although DGKζ KO $CD8^+$ T cells display increased cytokine production and increased proliferation, their cytotoxic function is comparable to WT $CD8^+$

T cells (*80*). Nevertheless, these studies demonstrate that DGKζ serves to constrain T cell activation and anti-viral and anti-tumor T cell responses. Thus, inhibition of DGKζ might provide a therapeutic opportunity to enhance immune-mediated viral and tumor clearance.

It is possible that DGKζ is physiologically important for limiting over-activation and inducing anergy in inappropriately activated T cells. The expression level of DGKζ can be controlled depending on the type of stimulation the T cell receives. T cells that are stimulated through their TCR and co-stimulatory molecules downregulate DGKζ transcript levels, thereby allowing appropriately activated T cells to become fully activated (*82-84*). In contrast, T cells that receive TCR stimulation alone in the absence of co-stimulation do not downregulate DGKζ levels, potentially leading to attenuated DAG-mediated signaling and decreased activation. Consistent with this notion, DGKζ KO T cells resist anergy induction when activated by TCR alone in the absence of co-stimulatory signals (*83*). In addition to TCR-mediated regulation, DGKζ might also be regulated by environmental cytokines. In particular, IL-33 has been shown to up-regulate DGKζ in cardiomyocytes following stimulation (*85*). Although it is unknown if IL-33 can upregulate DGKζ in immune cells, it is tantalizing to speculate that cytokine signaling can affect the TCR responsiveness of T cells by regulating DGKζ levels.

NK cells

NK cells are cytotoxic members of the innate lymphoid cell (ILC) family and play an important role in protection against viral infection and clearance of tumors (*86*). Unlike their adaptive counterparts ($CD8^+$ T cells), they do not possess a somatically-rearranged antigen receptor but rather express a variety of activating receptors specific for ligands displayed on virally-infected, stressed, or transformed cells (*87*). NK cell activating

receptors can be categorized into three main families based on the signaling adaptors used to relay downstream activation signals. These families include SAP-dependent (e.g., 2B4), ITAM-dependent (e.g., CD16), or DAP10-dependent (e.g., NKG2D) receptors (*87-90*). The activation of any of these three families of receptors relies on proximal signaling complexes involving SLP-76, which subsequently leads to the activation of PLCγ and the production of DAG (*91-93*). In addition to these activating receptors, NK cells express an assortment of inhibitory receptors, many of which bind to MHC class I alleles and negatively regulate activating receptor signaling by the recruitment of phosphatases such as SHP-1 and SHIP (*87, 94*).

NK cell activation is determined by the net balance of the activating and inhibitory inputs that the NK cell receives through its receptors. For example, NK cells are activated when neoplastic cells upregulate ligands such as RAE-1 or MICA, which are recognized by the activating receptor NKG2D (*95*). Likewise, NK cells are activated through disinhibition when tumor cells lose MHC class I, a process known as missing self-recognition (*96*). Since SHP-1 and SHIP negatively regulate activating receptor signaling, one might predict that the loss of these molecules would boost the effector function of NK cells. Surprisingly, however, SHP-1 and SHIP deficiency in NK cells renders them less functional than their WT counterparts (*97-99*). One explanation of this seemingly paradoxical finding is that NK cells continuously adjust their responsiveness to activating stimuli in their local environment, a phenomenon known as tuning (*100*). Thus, NK cells that chronically lack inhibitory signals, such as in SHP-1 or SHIP deficiency, require more stimulation to achieve their threshold of activation (*97-99*). While NK cell tuning may protect the host from NK cell-mediated immunopathology, this process can hamper important effector responses against chronic viral infections or tumors.

Although the molecular mechanism of NK cell tuning is unknown, stimulation of NK cells with PMA and a calcium ionophore, ionomycin, can bypass the hyporesponsiveness of SHP-1 and SHIP KO NK cells (*97, 99*). These data suggest that the tuning process is proximal to PLCγ-mediated production of DAG. Thus, we speculated that NK cells may not be able to tune their responsiveness in response to enhanced DAG-mediated signaling by DGKζ deficiency. Indeed, we recently demonstrated that DGKζ KO NK cells are hyperfunctional compared to WT NK cells (*101*). DGKζ KO NK cells displayed increased cytokine production and cytotoxicity following stimulation through ITAM, SAP, and DAP10-dependent activating receptors. In contrast, IFNγ production by DGKζ KO and WT NK cells was similar following stimulation with IL-12 and IL-18, which utilize a DAG-independent signaling pathway, suggesting that the loss of DGKζ selectively augmented NK cell responsiveness to DAG-dependent stimuli. Like T cells, the hyperfunctionality of DGKζ KO NK cells was dependent on enhanced ERK signaling. Importantly, DGKζ KO mice cleared the NK cell-sensitive RMA-S tumor more efficiently than WT mice. Thus, the inactivation of negative regulators distal to PLCγ such as DGKζ might prove therapeutically useful in enhancing NK cell function.

B cells

B cells comprise the second arm of the adaptive immune system and are critical for the generation of protective antibody responses during infection. The induction of antibody production results from the stimulation of the somatically rearranged B cell receptor (BCR) by cognate antigen (*102, 103*). Similar to the TCR, activation of the BCR leads to downstream biochemical cascades that ultimately result in the generation of DAG through PLCγ and, subsequently, the activation of ERK (*104, 105*). ERK has been shown to play multiple roles during B cell responses, including the promotion of B cell survival,

proliferation, and differentiation into antibody-secreting plasma cells (*106-108*). Furthermore, attenuation of ERK activation has been shown to important during B cell development, since ERK signals decrease as B cells progress from the immature transitional stage to mature follicular B cells (*109-111*).

Given the role of ERK in these B cell processes, controlling the levels of BCR-induced DAG through DGKζ might be important in regulating B cell development, activation, and antibody secretion capabilities. For example, mRNA transcripts of DGKζ are upregulated as B cells progress from early transitional to the mature follicular stage, which is associated with decreased ERK activation (*112*). Accordingly, the loss of DGKζ only affected ERK activation and IκBα degradation in the follicular but not early immature transitional B cell pool in response to BCR stimulation. Importantly, the augmentation of BCR-induced activation in DGKζ KO follicular B cells was seen even under less optimal BCR activation conditions, suggesting that DGKζ might control the BCR activation threshold in these cells.

The effects of DGKζ on B cell signaling threshold translate to functional consequences on B cell effector responses. BCR stimulation of purified DGKζ KO splenic B cells in vitro led to increased expression of CD69 and enhanced proliferation compared to WT B cells. DGKζ KO mice displayed enhanced antibody responses to T-independent and T-dependent antigens (*112*). The heightened antibody response by DGKζ-deficiency was accompanied by increased antigen-specific expansion of both germinal center (GC) B cells and plasma cells. These results demonstrate that regulation of DAG-dependent ERK activation by DGKζ is critical for selectively controlling the activation threshold of mature B cells to limit their activation.

The immunomodulatory role of DGKζ

We have so far described how the loss or inhibition of DGKζ can lead to increased immune responses against viruses or cancer. As DGKζ is a negative regulator of DAG-mediated signaling, it is conceivable that immune responses would be enhanced in the absence of DGKζ. However, DGKζ deficiency may also lead to dampening or regulation of immune responses. In the sections below, we will discuss how the absence of DGKζ can direct and indirectly suppress or modulate rather than enhance immune responses.

Regulatory T Cells

Regulatory T cells (Tregs) are a key subset of T cells that display suppressive function and are important for the regulation of adaptive immune responses. Tregs are governed by the master transcription factor, forkhead box P3 (Foxp3), and exert their immunosuppressive function via the production of immunoregulatory cytokines and through cell contact dependent mechanisms (*113*). Loss of function mutations in the *Foxp3* gene, as seen in Scurfy mice and humans with immune dysregulation, polyendocrinopathy, and X-linked lymphoproliferative disease (IPEX), leads to lethal systemic autoimmunity early in life, highlighting the importance of Tregs in inducing immunotolerance against self-antigens (*114-117*)

T cells that strongly recognize self-antigens are deleted during thymic development in a process known as negative selection. Specifically, T cells that receive strong TCR signals in the thymus, implying overt self-reactivity, undergo apoptosis. As an alternative fate, strong TCR stimulation in developing thymocytes can also lead to Treg differentiation (*113*). Thus, we hypothesized that enhancement of TCR-mediated DAG signaling by DGKζ deficiency in developing thymocytes may increase Treg generation. Indeed, the

loss of DGKζ resulted in a significant increase in Treg development in the thymus in a cell-intrinsic manner (*118*). DAG-mediated signaling leads to the activation of the NF-κB (through activation of PKC) and ERK pathways. One NF-κB family member, c-Rel, was previously shown to be important for inducing Foxp3 expression in thymocytes (*119, 120*). Although Treg generation in DGKζ KO mice was reduced in the absence of c-Rel, there was still residual Tregs in the thymus, suggesting that c-Rel was only partially responsible for the increased generation of Tregs in DGKζ KO mice (*118*). In fact, ERK activation appeared to be more important in the enhancement of Treg generation in DGKζ KO mice. Using an *in vitro* Treg development assay, we found that the inhibition of ERK phosphorylation by a MEK inhibitor led to decreased Treg generation in a dose-dependent manner, whereby the level of phosphorylated ERK (pERK) directly correlated to the magnitude of Treg generation. Importantly, Treg generation was also increased in sevenmaker mice (*121*), which express a gain of function ERK mutation that leads to increased resistance to dephosphorylation of active pERK, suggesting that the selective enhancement of the ERK pathway alone is sufficient to increase Treg generation.

In addition to Treg generation in the thymus, TCR signaling plays an important role in the function of Tregs. Although some Treg function may be preserved in the absence of TCR signaling, we demonstrated that Tregs lacking SLP-76 cannot suppress TCR-driven proliferation of conventional T cells (*122*). Furthermore, Tregs with a Y→F mutation at tyrosine 145 (Y145F) of SLP-76, which leads to defective PLCγ activation, also display attenuated suppressive function, suggesting that PLCγ is important for Treg function. Consistent with this notion, Tregs lacking DGKζ display significantly increased suppression of TCR-driven conventional T cell proliferation compared to WT Tregs. Together, these data demonstrate that DGKζ limit Treg generation and function. Thus,

DGKζ deficiency may indirectly lead to the suppression of immune responses through Tregs.

Mast Cells

Mast cells are critical mediators in type 2 immune responses involved in protection against helminthes and in pathologic responses in asthma and allergy (*123, 124*). A key feature of mast cell function is the immediate release of pre-formed inflammatory mediators such as histamine, cytokines, and proteases in a process called degranulation. In addition, mast cells produce arachidonic acid metabolites and cytokines in a protracted manner (*124*). One major stimulus for the release of these inflammatory mediators is crosslinking of FcεRI, the high affinity receptor for the Fc region of immunoglobulin E (*125*).

The interaction of allergens with IgE-FcεRI complexes results in formation of signaling complexes that converge on the activation of PLCγ (*126, 127*). PLCγ and subsequent PKC activation have been shown to be critical in controlling mast cell degranulation, suggesting that controlling the levels of DAG might be important for regulating this process (*128-131*). Indeed, the loss of DGKζ in FcεRI-stimulated mast cells leads to increased DAG levels, along with enhancement of downstream DAG-dependent signals, including RasGTP and ERK (*132*). Accordingly, DGKζ deficiency leads to enhanced mast cell production of IL-6 following FcεRI stimulation.

Intriguingly, however, FcεRI-stimulated DGKζ KO mast cells display impaired degranulation and are resistant to local skin anaphylaxis (*132*). The differential effect of DGKζ deficiency on mast function (the hypersecretion of IL-6 vs. decreased degranulation) may be explained by the negative feedback of DAG on PLCγ activation in mast cells. The elevation of DAG by DGKζ deficiency appears to negatively regulate the

phosphorylation and subsequent activity of PLCγ. Thus, although DAG accumulates, the production of IP_3, and hence Ca^{2+} flux is attenuated in DGKζ KO mast cells. As degranulation responses are highly dependent on elevation of intracellular Ca^{2+} levels, this may cause a differential effect on degranulation and cytokine production by mast cells (*132, 133*). Thus, as opposed to T cells and NK cells, DGKζ exerts both activating and inhibitory effects on mast cell functional responses.

Macrophages and Dendritic Cells

Macrophages and dendritic cells (DC) play a key role in bridging the adaptive and innate immune responses (*134-136*). Toll-like receptors (TLR) serve as an important mechanism for equipping macrophages and DCs with the ability to recognize the presence of pathogenic infection and, subsequently, instruct adaptive immune cells on the type of response needed to effectively clear the infection. TLRs can signal through either MyD88 and/or TRIF to induce activation of the NF-κB and ERK pathways (*134*). While TLR activation does not generally lead to PLCγ activation, DAG has been shown to be induced in macrophages following stimulation with LPS (TLR4 agonist) and lipopeptide (TLR2 agonist) (*137-139*). Furthermore, inhibition of PLC or PLD reduced cytokine production and nitric oxide formation by macrophages following TLR stimulation, suggesting that control of DAG levels through DGK might be important in regulating TLR-mediated responses.

Interestingly, modulation of DAG levels by the loss of DGKζ resulted in impairment rather than enhancement of cytokine production by macrophages and DCs in response to TLR stimulation. Specifically, in a developmentally independent manner, bone marrow derived macrophages (BMMΦ) and splenic DCs produced substantially less IL-12p40 and

TNFα following in vitro stimulation through a variety of TLR agonists (*140*). This paradoxical finding may be explained by the role of DGK in converting DAG into PA. Biochemical analysis revealed that the loss of DGKζ resulted in selective elevation of the PI3K-Akt pathway but no difference in activation of the ERK or NK-κB pathways following TLR stimulation. Activation of the PI3K pathway has been shown to negatively regulate TLR stimulation (*141-143*) and chemical inhibition of the PI3K restored LPS-induced IL-12p40 production from DGKζ KO BMMΦs (*140*). Intriguingly, the addition of PA also restored LPS-induced IL-12p40 production, suggesting that the cytokine production defect in DGKζ KO DCs and macrophages may be due to reduced PA rather than elevated DAG levels. Exactly how PA rescues TLR-induced cytokine production is unknown, but one possible mechanism is through the recruitment of SHP-1 to negatively regulate PI3K activation (*144-146*).

Defective cytokine production was also observed in vivo following intraperitoneal injection of TLR agonists, which correlated with enhanced survival of DGKζ KO mice after LPS-induced septic shock (*140*). However, while DGKζ KO mice were protected from TLR-mediated pathology, the loss of DGKζ conferred susceptibility to Toxoplasma gondii. DGKζ KO mice infected with Toxoplasma gondii displayed decreased serum IL-12p40 and IFNγ levels compared to WT mice. Furthermore, IFNγ production by DGKζ KO splenocytes isolated at day 15 and 30 post-infection was significantly attenuated following restimulation with T. gondii antigen STAg. Intriguingly, total $CD4^+$ and $CD8^+$ T cell numbers were similar between WT and DGKζ KO mice following infection with the frequency of $CD44^+CD62^{lo}$ effector T cells higher in infected DGKζ KO mice. As TLR-induced IL-12p40 production and the subsequent induction of a Th1 response are critical for protection against Toxoplasma gondii infection, the impairment of immune responses against T. gondii by

DGKζ KO mice could be secondary to a defect in macrophage and DC-derived cytokines that drive Th1 responses.

The role of other DGK isoforms on DGKζ-regulated immune function

So far, we have discussed isoform-specific regulation of immune function by DGKζ, however it is possible that the loss of DGKζ has other functional consequences that might be masked by redundant functions of other DGK isoforms. Indeed, DGKα has been shown to display some redundant function with DGKζ during conventional T and invariant NKT cell (iNKT) development. While singly-deficient DGKα KO and DGKζ KO mice display no gross defects in thymic T cell development, mice deficient in both DGKα and DGKζ (DGKαζ DKO) have significant reductions in CD4 and CD8 SP populations in the thymus due to a cell-intrinsic block in positive selection from the DP to SP stage (*147*). Interestingly, the addition of PA to fetal thymic organ cultures could partially restore T cell maturation defect in DGKαζ DKO thymocytes, suggesting that DGKα and DGKζ regulate T cell development partly through redundant production of PA.

Similarly, the development of iNKT cells is intact in mice singly-deficient for either DGKα or DGKζ (*148*). However, the loss of both DGKα and DGKζ results in a complete impairment of iNKT cell maturation in the thymus, spleen, and liver at both early and terminal stages in a cell-intrinsic manner. Selective enhancement of the ERK pathway through the expression of a constitutively active K-ras resulted in a significant reduction in mature iNKT cells due a block in Stage II to Stage III maturation of iNKT precursors. Furthermore, augmented activation of the NF-κB pathway through the expression of a constitutively active IKKβ also resulted in an impairment in iNKT maturation at both early and terminal stages of development. These results suggest that DGKα and DGKζ play

redundant roles in the regulation of iNKT maturation by controlling DAG-mediated activation of the ERK and NF-κB pathways.

In addition to controlling innate and conventional T cell development, DGKα has also been shown to promote T cell anergy in conjunction with DGKζ. Overexpression of either DGKα or DGKζ in Jurkat T cells induces an anergic-like state that is highlighted by reduced DAG-dependent TCR signals without the impairment of calcium flux (*83*). Similar to DGKζ KO T cells, T cells deficient in DGKα resist anergy induction when activated through their TCR in the absence of costimulation and during superantigen-induced activation. Furthermore, pharmacological inhibition of DGKα in DGKζ-deficient T cells can further enhance proliferation and IL-2 production in response to anergy-inducing conditions, suggesting that both DGKα and DGKζ contribute to anergy induction in inappropriately activated T cells through the synergistic regulation of TCR-induced DAG-mediated signaling.

While DGKα and DGKζ can share similar functions, DGKα does not simply compensate for all DGKζ-regulated functions. For example, unlike DGKζ KO mice, DGKα-deficient mice do not display an enhancement in Treg generation in the thymus or hyperfunctional NK cell responses, thus emphasizing that the regulation of these processes by DGKs is isoform-specific and unique to DGKζ (*101, 149*). The independent and redundant roles of DGKα and other DGK isoforms on DGKζ-regulated functions in other immune cells remain unexplored.

Structure of the thesis

While factors that regulate Th2 differentiation has been extensively studied since the discovery of Th subsets by Tim Mosmann and Robert Coffman in the late 1980's,

major gaps still remain regarding the key determinants for dictating the Th2 fate decision of naïve $CD4^+$ T cells following T cell activation. While TCR-driven signaling is thought to be highly important in this process, it is still not well understood how alteration of specific TCR signal transduction pathways influence the development of this lineage. The aim of this thesis is to investigate the role of DGKζ in modulating selective TCR-signal transduction pathways to regulate Th2 differentiation and control type 2 immune responses *in vivo* (Fig. 1.1). In addition, this thesis will also examine the novel immunomodulatory roles of DGKζ in regulating non-immune responses that are critical for initiating and promoting the pathological aspects of aberrant type 2 immune responses. **Chapter 2** will examine the cellular and molecular mechanisms by which DGKζ modulates Th2 differentiation and controls T-cell mediated airway inflammation in a mouse model of allergic asthma. Additionally, this chapter will interrogate the relationship between inflammation and airway hyperresponsiveness and how DGKζ influences both processes through independent mechanisms. **Chapter 3** will explore how DGKζ influences innate immune type 2 responses in the airways in response to protease allergens and discuss a novel role for DGKζ in regulating protease allergen sensing by the non-immune compartment to control airway inflammation. Lastly, **Chapter 4** will discuss the implications of these findings in context of what is known about the initiation, propagation, and maintenance of type 2 immune responses. Furthermore, this chapter will discuss the future directions for this work and the broader implications of these pathways in sites outside of the lung.

Figures

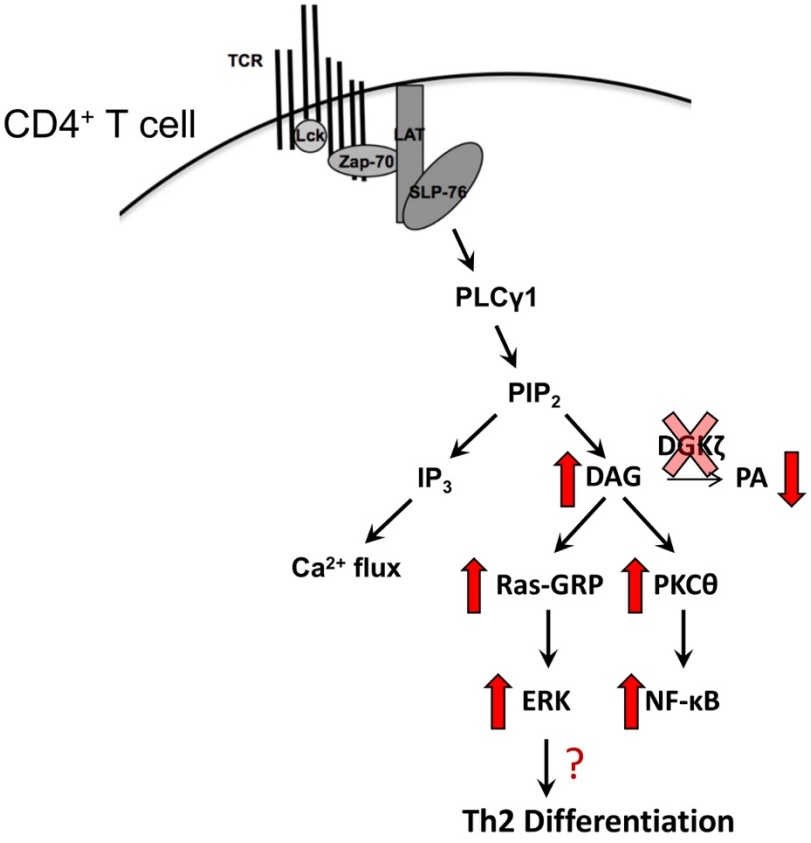

Figure 1.1. Enhanced TCR-mediated ERK activation in the absence of diacylglycerol kinase zeta (DGKζ). In the absence of DGKζ, T cells display accumulation of diacylglycerol (DAG) and, consequently, enhancement of the NF-κB and ERK pathways following TCR ligation. Additionally, T cell lacking DGKζ also display reduction in phosphatidic acid (PA) signaling following triggering of the TCR.

CHAPTER 2: DIACYLGLYCEROL KINASE ZETA CONTROLS ALLERGIC AIRWAY INFLAMMATION AND AIRWAY HYPERRESPONSIVENESS THROUGH DISTINCT MECHANISMS

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 (*150*). 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) (*151-153*). Current therapeutic approaches used to treat asthma involve combinatorial administration of bronchodilators and anti-cholinergic drugs to relax constricted airways and corticosteroids to inhibit airway inflammation (*154*). While these treatments benefit many patients who have asthmatic disease, there is a significant proportion of patients in whom these treatments never fully control asthma, particularly in those who have severe disease (*152*). Furthermore, cessation of these treatments often results in reoccurrence of asthma symptoms and loss of asthma control, suggesting that these treatments fail to reverse the underlying intrinsic changes in airway cells that mediate asthma pathology (*155, 156*). Therefore, there is an urgent unmet need for therapeutics that can offer better control and potentially mediate resolution of the disease.

Airway inflammation present in allergic asthma is typically driven by type 2 immune responses in the lung, although other asthma endotypes driven by type 2-independent immune responses do exist (*157, 158*). Type 2 inflammation in the lung is mediated by Th2 $CD4^+$ T cells and group 2 innate lymphoid cells (ILC2), which produce

the type 2 cytokines, IL-4, IL-5, and IL-13, in response to antigen-dependent and antigen-independent activation (*159-166*). The production and release of these cytokines promote a variety of downstream responses, which include the recruitment and activation of eosinophils in the lung, 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 (*151, 153, 162-165, 167-169*). Furthermore, it is generally thought that type 2 airway inflammation drives the non-immune abnormalities, such as AHR, that are present in asthma.

Given the role of Th2 $CD4^+$ T cells in asthma pathogenesis, blocking Th2 differentiation of allergen-specific T cells represents a viable therapeutic strategy for the treatment of asthma. While cytokine (e.g. IL-4, TSLP, IL-25, and IL-33) and costimulatory (e.g. CD28, ICOS, OX40) signals are known to be important for driving Th2 differentiation, the strength and duration of TCR signaling can also contribute to the outcome of $CD4^+$ T cell differentiation, in which strong and prolonged TCR-mediated signals promote Th1 differentiation while weak and transient signals skew differentiation toward Th2 (*1, 21, 23, 26, 27, 34, 41, 55, 56, 58, 60*). More specifically, TCR-mediated ERK activation is a key determinant in driving $CD4^+$ T cell differentiation, in which strong ERK signals block Th2 differentiation (*58, 60*). TCR-mediated ERK activation is largely dependent on DAG, which is a secondary lipid messenger that is generated upon the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). DAG is negatively regulated by its phosphorylation into phosphatidic acid by diacylglycerol kinase (DGK) enzymes. Among DGK family members, the ζ isoform of DGK plays a predominant role in suppressing DAG-dependent ERK activation (*170*).

Accordingly, T cells lacking DGKζ accumulate DAG and display enhanced ERK activation (*171*).

Being a negative regulator of DAG-mediated signaling, the absence of DGKζ would be predicted to enhance the magnitude of immune responses in general. Indeed, this is the case in anti-tumor and anti-viral immunity, whereby DGKζ KO T cells and NK cells display enhanced activation (*171-174*). However, in some instances (e.g., in allergic responses), DGKζ KO mast cells fail to degranulate and DGKζ KO mice are resistant to anaphylaxis (*175*). Thus, depending on the process, blocking DGKζ could be either immunostimulatory or immunosuppressive (*176*). Given that ERK activation skews T cell responses away from Th2 differentiation, we hypothesized that the enhancement of DAG signaling by targeting DGKζ would suppress rather than potentiate the development of allergic asthma. Indeed, we show that enhancement of DAG signaling by the inhibition of diacylglycerol kinases attenuates Th2 differentiation and this effect translated to protection from a mouse model of Th2-mediated allergic asthma. Surprisingly, we found that the mechanisms by which DGKζ mediated airway inflammation and AHR were separable. Conditional deletion of DGKζ in T cells led to impairment of type 2 inflammation in an ERK-dependent manner with no attenuation of AHR. In contrast, targeted deletion of DGKζ in smooth muscle cells led to impairment of AHR with no attenuation of airway inflammation. Furthermore, we demonstrate that pharmacological inhibition of DGK suppresses murine type 2 airway inflammation and AHR and inhibits carbachol-mediated bronchoconstriction of human airways. Thus, these data demonstrate that DGKs are novel therapeutic targets for asthma and reveal that airway inflammation and AHR are not as interdependent as generally believed.

Results

Conventional CD4$^+$ T cells produce IL-4 following TCR activation to drive Th2 differentiation under nonpolarizing conditions in vitro

While IL-4 is critical for instructing Th2 differentiation, the relevant sources of IL-4 for initiating differentiation of naïve CD4$^+$ T cells into the Th2 lineage is controversial. Many different cell types, such as non-conventional T cells (NKT2), granulocytes (eosinophils, basophils, and mast cells), innate lymphoid cells (ILC2), have been implicated as a relevant source of IL-4 for driving Th2 differentiation *in vivo* (*7-13*). However, previous work has shown that conventional CD4$^+$ T cells can be an early source of IL-4 immediately following TCR activation that is sufficient to promote Th2 differentiation in the absence of exogenous cytokines (*14-16*). We found that activation of naïve CD4$^+$ T cells under nonpolarizing conditions with anti-CD3 and anti-CD28 in the presence of irradiated CD4-depleted splenocytes in the absence of exogenous cytokines resulted in robust generation of Th2 cells that expressed high levels of GATA3 and Th2 cytokines, IL-4, IL-5, and IL-13 (Fig 2.1, A). Strong Th2 development under these conditions was dependent on STAT6 signaling because Th2 differentiation was completely blocked in STAT6 KO T cells (Fig. 2.1, B and C). Activation of T cells in the presence of neutralizing antibodies to IL-4 completely diminished Th2 generation following TCR activation, thus revealing that the induction of Th2 cells was dependent on IL-4 signaling (Fig. 2.1, D). To determine the source of IL-4 for instructing Th2 differentiation in this system, we activated WT and IL-4 KO T cells with either WT or IL-4 KO irradiated CD4-depleted splenocytes. To our surprise, Th2 differentiation was completely intact in T cells activated with IL-4 KO irradiated feeders (Fig. 2.1, E). However, if T cells were deficient in IL-4, Th2 differentiation was completely lost

following TCR activation (Fig. 2.1, E). Overall, these data demonstrated that conventional $CD4^+$ T cells produce relevant amounts of IL-4 immediately following TCR activation to instruct Th2 differentiation in the absence of exogenous sources of IL-4 *in vitro*.

DGKζ KO T cells display impaired Th2 differentiation in vitro

ERK activation has been shown to drive T cells to differentiate into Th1 over Th2 phenotype (*58, 60*). Since ERK is hyperactivated in the absence of DGKζ in T cells, we first tested if DGKζ knockout (KO) T cells display impaired Th2 differentiation *in vitro*. When naïve $CD4^+$ T cells from DGKζ KO mice were stimulated through their TCR and expanded *in vitro* under nonpolarizing conditions, the proportion of Th1 cells was increased while the proportion of Th2 cells was decreased compared to naïve $CD4^+$ T cells from WT mice (Fig. 2.2, A and B). To determine if the attenuation of Th2 differentiation in the DGKζ KO T cells was due to impaired endogenous IL-4 production, naïve DGKζ KO $CD4^+$ T cells were activated through their TCR in the presence of exogenous IL-4. Indeed, Th2 differentiation was completely restored in DGKζ KO T cells treated with exogenous IL-4 (Fig. 2.2, C and D). Induction of early IL-4 transcripts is detectable within ~1 hour following TCR activation while production of IL-4 protein is detectable ~48 hours after TCR activation in an IL-4R-independent manner, suggesting that autocrine/paracrine secretion and sensing of IL-4 by conventional $CD4^+$ T cells is required at approximately 48 hours following TCR activation to promote Th2 differentiation. To test if restoration of IL-4 levels within this initial 48-hour window was sufficient to restore Th2 differentiation in DGKζ KO T cells, exogenous IL-4 was added at either 0, 24, 48, 72, or 96 hours after TCR activation. Indeed, we found that addition of exogenous IL-4 within 48 hours following TCR activation was sufficient to restore Th2

differentiation in DGKζ KO T cells (Fig. 2.2, E-H). These data reveal that the loss of DGKζ attenuates Th2 differentiation by likely impairing early TCR-mediated, T-cell intrinsic IL-4 production.

DGKζ KO mice are protected from OVA-induced allergic airway inflammation and AHR

To test whether this reduction in Th2 differentiation would correlate with protection against asthma in DGKζ KO mice, WT and DGKζ KO mice were subjected to an OVA-induced allergic asthma mouse model. In line with our *in vitro* data, OVA-challenged DGKζ KO mice exhibited significantly reduced total inflammatory cell and eosinophil numbers in the bronchoalveolar lavage (BAL) fluid, decreased BAL Th2 cytokine levels, and diminished OVA-specific IgG1 serum antibody levels compared to OVA-challenged WT controls (Fig. 2.3, B-D). This correlated with decreased inflammatory infiltrates in the lungs of OVA-challenged DGKζ KO mice (Fig. 2.3, E). Importantly, AHR was almost completely abolished in OVA-challenged DGKζ KO compared to WT mice (Fig. 2.3, A). In contrast to our *in vitro* data, OVA-challenged DGKζ KO mice did not display a shift towards a heightened Th1 response, since no difference in BAL IFNγ or OVA-specific IgG2a serum antibody levels was observed compared to WT controls (Fig. 2.3, C and D).

Although DGKζ is the predominant isoform that controls DAG-mediated signaling in T cells, another DGK isoform known as DGKα also contributes to this process (*170*). DGKα KO T cells displayed an enhancement of Th1 differentiation and a partial but significant attenuation of Th2 differentiation when stimulated through their TCR and expanded *in vitro* (Fig. 2.4, A and B). In accordance, DGKα KO mice showed a partial but significant reduction in OVA-induced AHR and airway inflammation compared to WT

controls (Fig. 2.4, C-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 DGKζ is independently mediated by separate compartments

The near complete abolition of AHR in DGKζ KO mice despite a significant but partial reduction in airway inflammation prompted us to test whether DGKζ deficiency in non-hematopoietic cells also contributed to protection against OVA-induced allergic asthma. The hematopoietic compartment of lethally irradiated WT and DGKζ KO mice was reconstituted with bone marrow cells from either WT or DGKζ KO mice. Similar to DGKζ KO mice, DGKζ KO→DGKζ KO BM chimeric mice showed reduction in eosinophilic inflammation, Th2 cytokine levels in the BAL fluid, and AHR responses compared to WT→WT BM chimeric mice. Surprisingly, however, we found that the reduction in inflammation and AHR was mediated by two separate cell compartments. While WT mice reconstituted with DGKζ KO bone marrow cells displayed significantly reduced eosinophilic inflammation and Th2 cytokine levels in the BAL fluid, they were not protected against OVA-induced AHR (Fig. 2.5, A-C). In contrast, DGKζ KO mice reconstituted with WT bone marrow cells were completely protected from OVA-induced AHR despite the presence of eosinophilic inflammation and Th2 cytokines in the BAL fluid (Fig. 2.5, A-C). These data suggested that DGKζ deficiency in hematopoietic cells contributes to reduced airway inflammation, while DGKζ deficiency in non-hematopoietic cells leads to protection against AHR.

DGKζ deficiency in T cells protects from OVA-induced airway eosinophilia and partially attenuates OVA-induced Th2 differentiation

To more precisely interrogate the impact of DGKζ deficiency in hematopoietic cells in OVA-induced airway inflammation, DGKζ was conditionally deleted in hematopoietic cells or T cells using a Vav-inducible Cre (Vav-Cre DGKζ$^{fl/fl}$ mice) or CD4-inducible Cre (CD4-Cre DGKζ$^{fl/fl}$ mice), respectively. Vav-Cre DGKζ$^{fl/fl}$ mice displayed significantly attenuated OVA-induced eosinophil accumulation and IL-4 levels in the BAL fluid, while the OVA-induced AHR response was completely intact (Fig. 2.5, D-F). Similarly, CD4-Cre DGKζ$^{fl/fl}$ mice showed a significant reduction in OVA-induced eosinophil accumulation and IL-4 levels in the BAL fluid (Fig. 2.5, G and H).

Interestingly, while IL-4 levels were diminished in Vav-Cre DGKζ$^{fl/fl}$ and CD4-Cre DGKζ$^{fl/fl}$ mice following OVA challenge, IL-5 and IL-13 levels were relatively intact in these mice (Fig. 2.5, F and H). To test if the loss of DGKζ in T cells selectively attenuated the ability of T cells to produce IL-4 after Th2 differentiation *in vivo*, we adoptively transferred a mixture of CD45.1$^+$CD45.2$^+$ WT and CD45.2$^+$ DGKζ KO OT-II CD4$^+$ T cells into naïve CD45.1$^+$ WT mice followed by OVA immunization. OT-II T cells express a transgenic T cell receptor specific for the $OVA_{323-339}$ peptide presented on the MHC Class II I-A^b molecule. In accordance with the Vav-Cre DGKζ$^{fl/fl}$ and CD4-Cre DGKζ$^{fl/fl}$ data, we found that the proportion of DGKζ KO OT-II T cells expressing IL-4 was significantly diminished while the proportions of DGKζ KO OT-II T cells expressing IL-5 and IL-13 was relatively unaltered compared to WT OT-II T cells following OVA sensitization (Fig. 2.5, I). These data suggest that the loss of DGKζ selectively impairs the ability of T cells to produce IL-4 in a T cell-intrinsic manner during Th2 differentiation *in vivo*. In addition, we observed that the frequency of DGKζ KO OT-II T cells expressing IFNγ was significantly increased compared to WT OT-II T cells following OVA sensitization, suggesting that Th1 differentiation was enhanced in the absence of DGKζ

in vivo (Fig. 2.5, I). Overall, these data demonstrate that DGKζ deficiency inhibits the development of eosinophilic airway inflammation independently of AHR in a T cell-intrinsic manner by partially attenuating Th2 differentiation *in vivo.*

DGKζ deficiency in airway smooth muscle cells protects from OVA-induced AHR

Airway hyperresponsiveness is regulated 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 (*177-179*). Furthermore, DGKζ has been shown to be expressed in sensory neurons arising from the dorsal root ganglion and in smooth muscle cells (*180-184*). To identify the non-hematopoietic cell type that was responsible for protection against OVA-induced AHR in the absence of DGKζ, DGKζ was conditionally deleted in sensory neurons (Pirt-Cre DGKζ$^{fl/fl}$) and smooth muscle cells (Myh11-Cre DGKζ$^{fl/fl}$). Pirt-Cre DGKζ$^{fl/fl}$ mice exhibited similar OVA-induced AHR compared to control mice (Fig. 2.6, A). However, Myh11-Cre DGKζ$^{fl/fl}$ mice were protected from OVA-induced AHR, despite unaltered airway inflammation (Fig. 2.6, B-D). Moreover, tracheal rings isolated from either DGKζ KO or Myh11-Cre DGKζ$^{fl/fl}$ mice displayed significantly attenuated methacholine-induced contractile forces as compared to WT or Myh11-Cre controls (Fig. 2.6, E-G). These data demonstrate that DGKζ regulates airway smooth muscle cell contraction in a cell-intrinsic manner to promote allergen-induced AHR independently of inflammation.

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 next tested whether increased ERK signaling was responsible for the effect of DGKζ deficiency on Th2 differentiation. To this end, we assessed Th differentiation of naïve

DGKζ KO $CD4^+$ T cells activated through their TCR in the presence of the U0126, a pharmacological inhibitor of MEK1/2 (the kinase that phosphorylates and activates ERK). Indeed, we found that inhibition of ERK signaling was sufficient to restore Th2 differentiation in DGKζ KO T cells (Fig. 2.7, A). In addition, while 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. 2.7, A). In contrast, Th1 differentiation in WT and DGKζ KO T cells was attenuated in the presence of U0126, thus establishing that enhanced TCR-mediated DAG signaling impairs Th2 differentiation and promotes Th1 differentiation in an ERK-dependent manner (Fig. 2.7, A).

We next took a gain-of-function approach to test whether the enhancement of ERK signaling in T cells was sufficient to attenuate OVA-induced airway inflammation. Sevenmaker (ERK^{SEM}) 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 (*121*). Similar to DGKζ KO T cells, ERK^{SEM} T cells displayed enhanced Th1 differentiation and attenuated Th2 differentiation following TCR stimulation and expansion *in vitro* (Fig. 2.7, B and C). Furthermore, ERK^{SEM} mice displayed significantly reduced eosinophilia and Th2 cytokine release in the airways following OVA challenge compared to WT controls, thus demonstrating that the enhancement of ERK signaling is sufficient to inhibit Th2 differentiation and protect from OVA-induced airway inflammation *in vivo* (Fig. 2.7, E and F). Importantly, similar to the Vav-Cre $DGK\zeta^{fl/fl}$ mice, ERK^{SEM} mice displayed intact OVA-induced AHR compared to WT controls despite attenuated OVA-induced type 2 airway inflammation (Fig. 2.7, D). Overall, these data demonstrate that

the regulation of DAG signaling by DGKζ controls Th2 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 next tested whether DGK could represent a novel target for the prevention and treatment of asthma. While there are no known selective and potent inhibitors of DGKζ, a pan-DGK inhibitor, R59949 that is relatively selective for DGKα is commercially available (*185, 186*). DGKα and DGKζ are expressed in both T cells and smooth muscle cells (*170, 171, 182*). Since DGKα KO mice have a partial but significant reduction in OVA-induced airway inflammation and AHR, we tested whether pharmacological inhibition of DGKα kinase activity by R59949 could block OVA-induced type 2 airway inflammation and AHR. Mice were systemically treated with R59949 during the late sensitization and airway challenge phases of the murine model of OVA-induced asthma. Compared to vehicle-treated mice, R59949-treated mice displayed significantly attenuated AHR, and reduced eosinophilia and Th2 cytokines in the BAL fluid (Fig. 2.8, A-C). To test whether DGKα inhibition could block type 2 airway inflammation and AHR after allergen-specific T cell responses have already been established, OVA-sensitized mice were systemically treated with R59949 only during the airway challenge phase of the OVA-induced asthma model. Compared to vehicle-treated mice, R59949-treated mice again exhibited significantly reduced OVA-induced AHR (Fig. 2.8, D). In contrast, treatment with R59949 during the airway challenge phase failed to alter type 2 airway inflammation following OVA challenge (Fig. 2.8, E and F). Together, these data suggest that DGK can be pharmacologically targeted to reduce AHR and airway inflammation. However, while 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 DGKα by R59949 affects human airway smooth muscle contraction. Human airway smooth muscle cells were pre-treated with or without R59949 and stimulated with the non-selective M3 receptor agonist, carbachol. The phosphorylation of myosin light chain (MLC), a critical step in smooth muscle cell contraction, was reduced by R59949 (Fig. 2.8, G). 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. Following overnight incubation with R59949, carbachol-induced bronchoconstriction was significantly decreased (Fig. 2.8, H). Overall, these data highlight that acute inhibition of DGK is sufficient to protect from the development of OVA-induced airway inflammation and AHR and is sufficient to attenuate OVA-induced AHR in presensitized mice, suggesting that DGK is a potential therapeutic target for the prevention and treatment of asthma.

Discussion

Our findings demonstrate that the regulation of diacylglycerol signaling by DGKζ and DGKα plays a critical role in a mouse model of allergen-induced asthma. Genetic ablation of DGKζ or DGKα resulted in protection from OVA-induced airway inflammation and AHR. Using bone marrow chimeras, we demonstrated that the attenuation of AHR is due to the loss of DGKζ in the radioresistant compartment while the reduction of airway inflammation is due to the loss of DGKζ in the radiosensitive compartment, thus highlighting that DGKζ regulates these processes in separate compartments and

independently of each other. Generation of cell-type specific conditional DGKζ KO mice revealed that the protection from OVA-induced airway inflammation was mediated by the loss of DGKζ in T cells, while the attenuation of OVA-induced AHR was facilitated by the absence of DGKζ in airway smooth muscle cells. Finally, acute loss of DGK activity through pharmacological blockade with a DGKα inhibitor was sufficient to protect from the induction of murine allergen-induced asthma, attenuate allergen-induced AHR in mice with established allergen-induced immune responses, and reduce carbachol-induced bronchoconstriction of human airways, thus establishing that modulating DGK activity represents a viable therapeutic strategy for the treatment of asthma.

Given that DAG acts as a positive signal transduction molecule downstream of activating receptors, one might predict that the inhibition of DGK would always lead to an enhancement of immune responses. Our findings highlight the idea that augmenting DAG-mediated signaling does not necessarily lead to increased activation. In settings of Th1 and cell-mediated immune responses, increased DAG signaling caused by the loss of DGKζ augments the function of T cells and NK cells (*171-174*). In contrast, mast cell degranulation during allergic responses is inhibited in the absence of DGKζ (*175*). Thus, targeting DGKζ is immunomodulatory, i.e., it is immunostimulatory or immunosuppressive depending on the context. Our data presented here reinforce this notion, as the loss of DGKζ inhibits Th2-mediated inflammation.

We demonstrated that the loss of DGKζ attenuated the differentiation of naïve T cells into Th2 phenotype *in vitro* and suppressed eosinophilic inflammation and Th2 cytokine release in the BAL fluid of asthmatic mice *in vivo*. Although DGKζ KO mice displayed a reduction in the BAL levels of all major Th2 cytokines (IL-4, IL-5, and IL-13), only IL-4 but not IL-5 or IL-13 was reduced in the airways of Vav-Cre DGKζ$^{fl/fl}$ and CD4-

Cre DGKζ$^{fl/fl}$ mice. In accordance, DGKζ KO OT-II cells displayed a selective impairment in their potential to produce IL-4 but not IL-5 or IL-13 compared to co-transferred WT OT-II cells following OVA sensitization *in vivo*. These results suggest that the loss of DGKζ selectively impairs the ability of T cells to produce IL-4 in a T cell-intrinsic manner during Th2 differentiation *in vivo*. This could potentially explain the selective reduction in BAL IL-4 levels seen in the hematopoietic-specific and T cell-specific DGKζ KO mice following OVA challenge *in vivo*. It is still unclear why WT mice reconstituted with DGKζ KO bone marrow cells showed a reduction in all three Th2 cytokines. The bone marrow reconstitution studies may need to be interpreted with caution given the unknown effects of irradiation, reconstitution efficiency, and increased age of bone marrow-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 attenuate Th2 differentiation by enhancing ERK phosphorylation. We observed that partial attenuation of ERK signaling by U0126 restored Th2 differentiation in DGKζ KO T cells. Furthermore, T cells from ERK^{SEM} mice showed increased Th1 and decreased Th2 differentiation *in vitro*, which correlated with protection from OVA-induced type 2 airway inflammation *in vivo*. Thus, the effect of DGKζ deficiency on the inhibition of Th2 differentiation is dependent on increased ERK activation. However, the mechanism by which DGK affects acetylcholine-induced smooth muscle cell contraction is still unclear. Similar to TCR signaling, activation of muscarinic type 3 receptors, which are GPCRs that signal through Gαq proteins, leads to PLC-dependent DAG generation and ERK activation (*187, 188*). Thus, it is possible that the attenuation of smooth muscle contraction in DGKζ KO airway smooth muscle cells is also ERK-dependent. However,

previous work has shown that ERK signaling promotes rather than suppresses carbachol-induced contraction in ileal smooth muscle cells and α_1-adrenergic receptor-induced contraction, which is another Gαq-coupled GCPR, in vascular smooth muscle cells (*189, 190*). Interestingly, previous studies have shown that a rise in intracellular Ca^{2+} initiated by PLC critically mediates the crossbridge cycling of actin and myosin to drive smooth muscle contraction and DAG maintains contraction by inhibiting a negative regulator of the crossbridge cycling (*191-193*). Consequently, one might expect that the lack of DGKζ would exacerbate bronchoconstriction. However, the diminution of smooth muscle contraction by the loss of DGKζ suggests that other biological mechanisms are involved.

Importantly, our study demonstrates that AHR and eosinophilic airway inflammation are separate and distinct processes that mediate the development of asthma and can exist independently of each other. In accordance, recent clinical trials have shown that while inhibition of type 2 cytokine signaling is efficacious in reducing eosinophilia and decreasing the frequency of asthma exacerbations in asthmatic patients, these approaches failed to alter impaired baseline lung function and histamine-induced airway responses, thus implying that suppressing inflammation is not sufficient to reverse AHR and airway smooth muscle dysfunction in asthma (*194, 195*). Our findings formally demonstrate the novel concept that airway eosinophilic inflammation and AHR are regulated independently of each other and reveal that DGKζ plays a central role in the induction of these processes during the development of asthma. We envision targeting DGKζ as a novel therapeutic strategy that will promote the prevention and resolution of asthma by suppressing both the immune and non-immune responses that drive the disease.

Figures

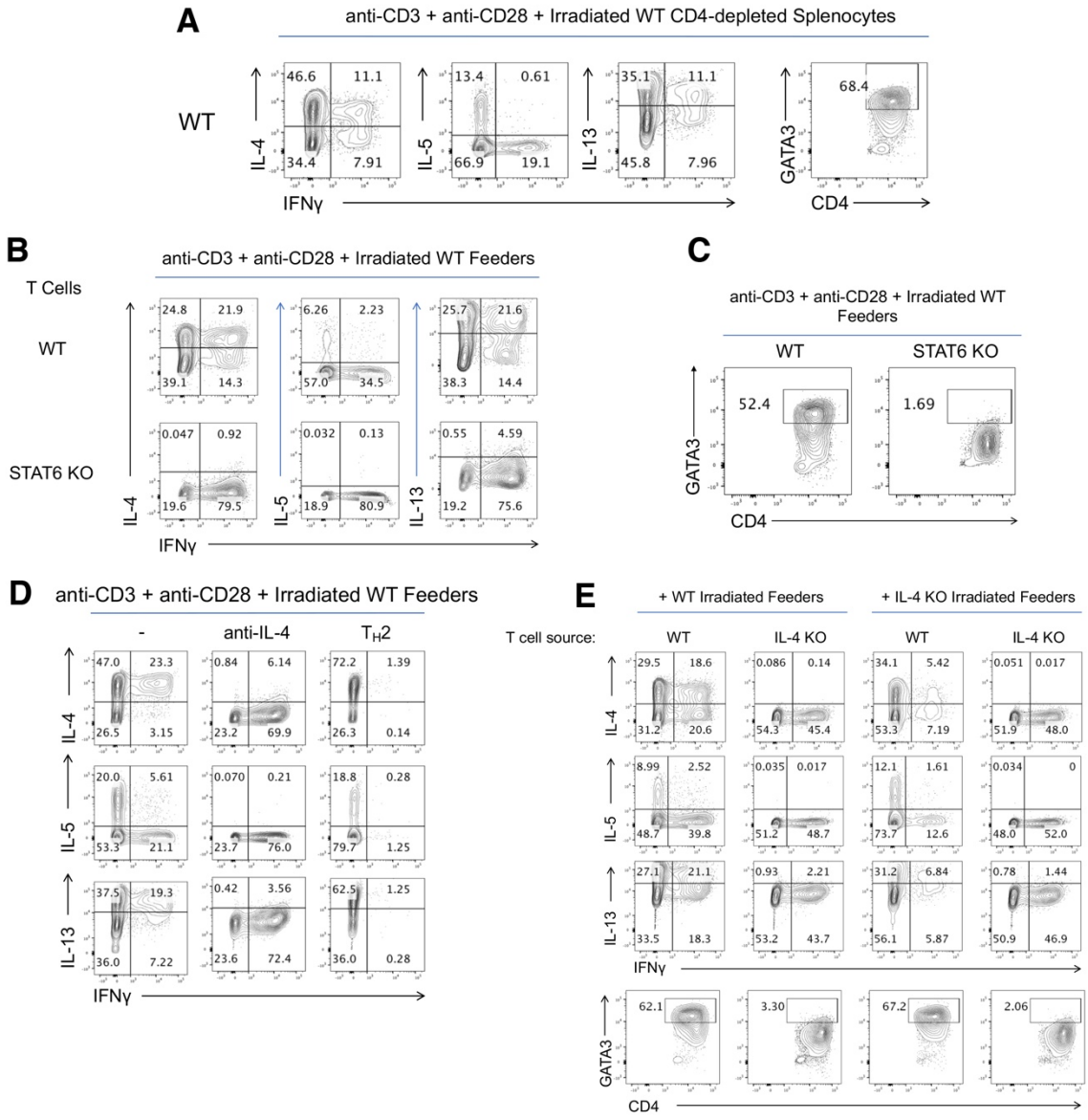

Figure 2.1. Conventional CD4$^+$ T cells produce IL-4 following TCR activation to drive Th2 differentiation under nonpolarizing conditions *in vitro*. (**A**)**,** Frequency of cytokine-producing and GATA3-expressing WT CD4$^+$ T cells after activation with anti-CD3 and anti-CD28 in the presence of irradiated WT CD4-depleted splenocytes (nonpolarizing conditons) for 5 days *in vitro*. (**B**)**,** Frequency of cytokine-producing and (**C**)**,** GATA3-expressing WT and STAT6 KO CD4$^+$ T cells after activation with anti-CD3 and anti-CD28 under nonpolarizing conditions for 5 days *in vitro*. (**D**)**,** Frequency of cytokine-producing WT CD4$^+$ T cells after activation with anti-CD3 and anti-CD28 in the presence of media alone, anti-IL-4 antibody, or T_H2 (anti-IL12 + anti-IFNγ + IL-4)

conditions for 5 days *in vitro*. (**E**)**,** Frequency of cytokine-producing and GATA3-expressing WT or IL-4 KO $CD4^+$ T cells after activation with anti-CD3 and anti-CD28 in the presence of either irradiated WT or IL-4 KO CD4-depleted splenocytes for 5 days *in vitro*. Data are representative of at least 3 independent experiments.

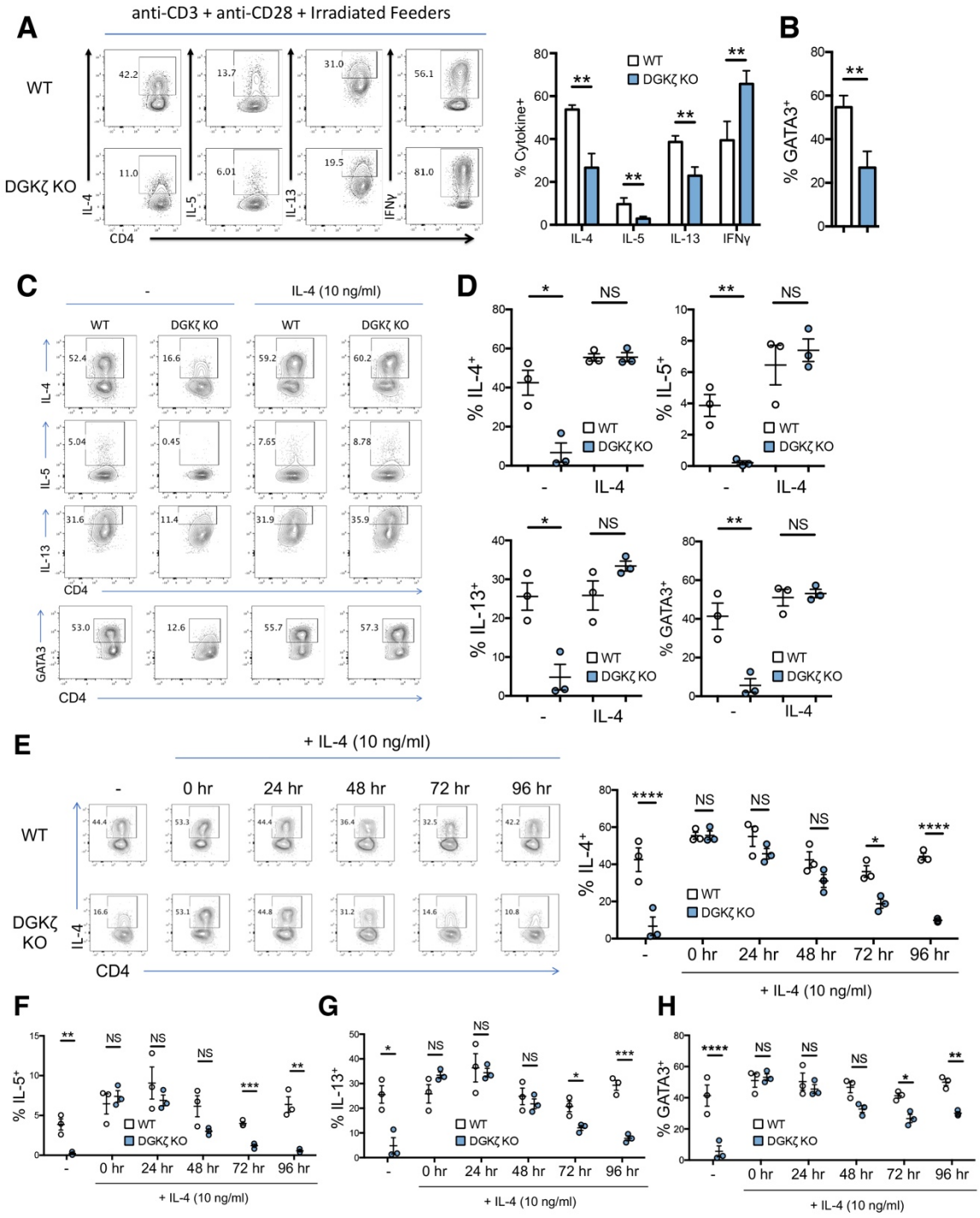

Figure 2.2. DGKζ KO T cells display impaired Th2 differentiation *in vitro*. (**A**), Frequency of cytokine-producing and (**B**), GATA3-expressing WT and DGKζ KO CD4$^+$ T cells after activation with anti-CD3 and anti-CD28 under nonpolarizing conditions for 5 days *in vitro*. (**C**), Frequency of cytokine-producing WT and DGKζ KO CD4$^+$ T cells after

activation with anti-CD3 and anti-CD28 under nonpolarizing conditions in the absence or presence of exogenous IL-4 for 5 days *in vitro*. (**E-G**)**,** Frequency of cytokine-producing and (**H**)**,** GATA3-expressing WT and DGKζ KO $CD4^+$ T cells untreated or treated with exogenous IL-4 at various timepoints following activation with anti-CD3 and anti-CD28 under nonpolarizing conditions for 5 days *in vitro*. Data are pooled from 5 independent experiments (A and B) or representative of 2 independent experiments (C-H). Data are represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, NS = not significant [two-sided unpaired Student's t-test, (C) and (D)**;** Two way ANOVA with Bonferroni's post-test, (A); One way ANOVA with Tukey's post-test, (B) and (E)].

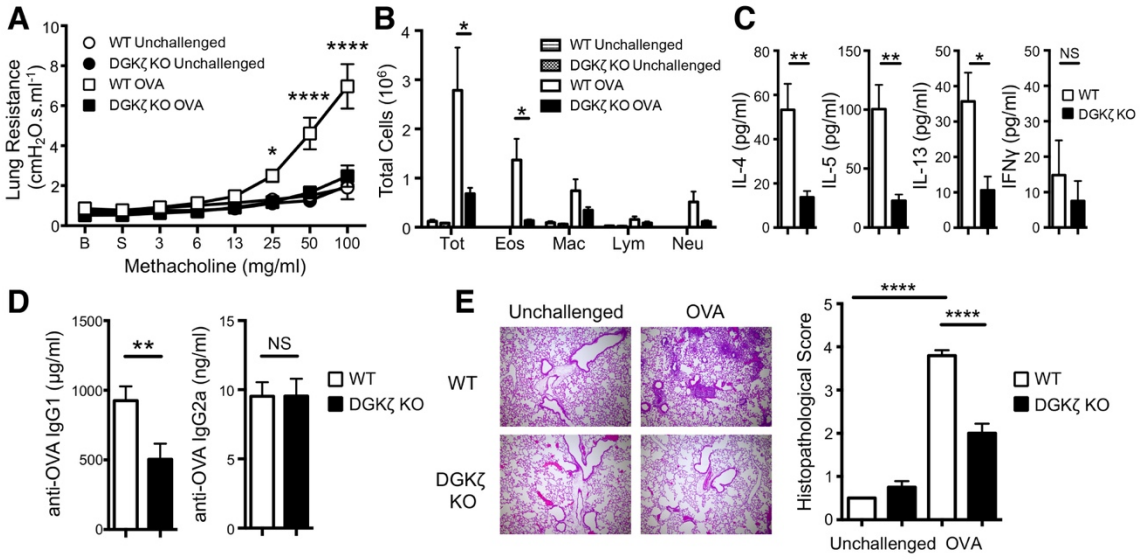

Figure 2.3. DGKζ KO mice are protected from OVA-induced allergic airway inflammation and airway hyperresponsiveness (AHR). (**A**), Changes in lung resistance after methacholine challenge and (**B**)**,** total number of eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu) in the bronchoalveolar lavage (BAL) fluid of unchallenged or OVA-challenged WT and DGKζ KO mice [(A), n = 6-9 mice/group; (B), n = 6 mice/unchallenged group, n = 19-22 mice/challenged group); done in collaboration with Wen Lu and Amanda Schmidt Paustian]. (**C**) and (**D**)**,** BAL fluid Th1 and Th2 cytokine levels and OVA-specific IgG1 and IgG2a serum antibody levels in OVA-challenged WT and DGKζ KO mice [(n = 14-16 mice/group); data in (C) was done in collaboration with Wen Lu and Amanda Schmidt Paustian]. (**E**)**,** Representative images (at 20x magnification) and compiled histopathological scores of hematoxylin and eosin (H&E) staining of unchallenged or OVA-challenged WT and DGKζ KO mice (n = 4-5 mice/group). (A) through (C) was done in collaboration with Wen Lu and Moyar Ge. Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, NS = not significant [two-sided unpaired Student's t-test, (C) and (D)**;** Two way ANOVA with Bonferroni's post-test, (A); One way ANOVA with Tukey's post-test, (B) and (E)].

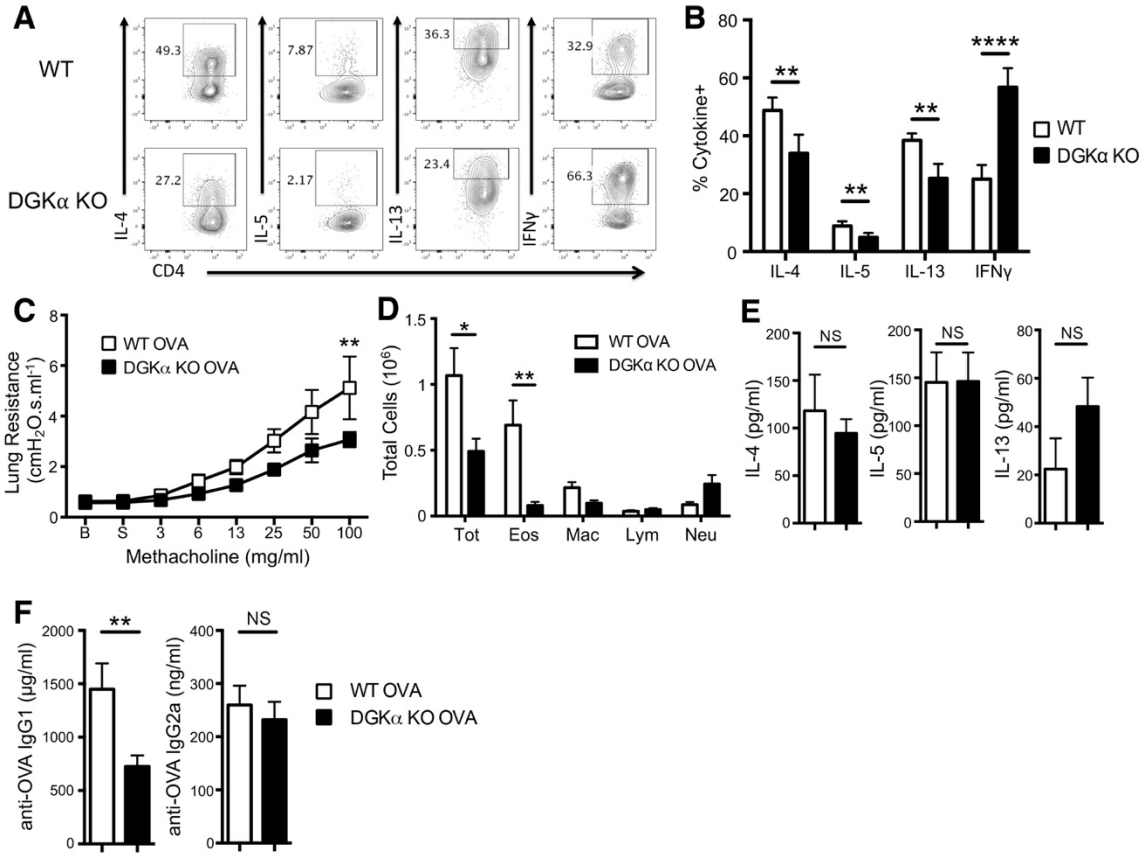

Figure 2.4. DGKα KO mice are partially protected from OVA-induced allergic airway inflammation and AHR. (**A** and **B**)**,** Frequency of cytokine-producing WT and DGKα KO CD4$^+$ T cells after activation with anti-CD3 and anti-CD28 under nonpolarizing conditions for 5 days *in vitro* (n = 11 mice/group). **C,** Changes in lung resistance in response to methacholine, (**D**)**,** Total number of eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu), and (**E**)**,** bronchoalveolar lavage (BAL) fluid Th2 cytokine levels in OVA-challenged WT and DGKα KO [(C), n = 10 mice/group; (D), n = 15-17 mice/group; (E), n = 13-14 mice/group]. (**F**)**,** OVA-specific IgG1 and IgG2a serum antibody levels in OVA-challenged WT and DGKζ KO mice (n = 13-14 mice/group). (A) through (F) was done in collaboration with Wen Lu and Moyar Ge. Data are pooled from at least 3 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant [Two way ANOVA with Bonferroni's post-test, (C); two-sided paired Student's t-test, (B); two-sided unpaired Student's t-test, (D) through (F)].

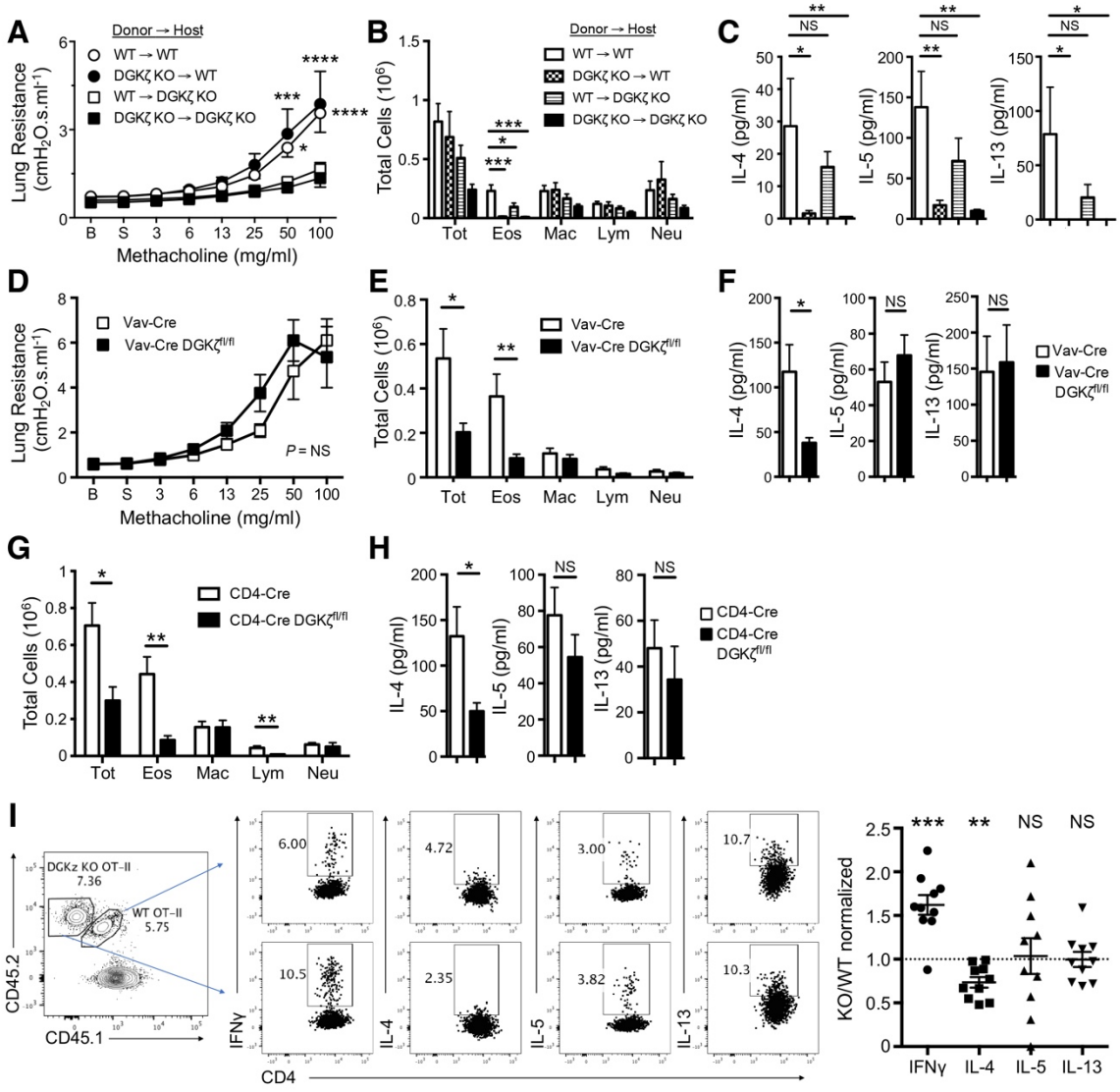

Figure 2.5. Hematopoietic and non-hematopoietic cells differentially contribute to OVA-induced airway inflammation and AHR in the absence of DGKζ. (**A**)**,** Changes in lung resistance in response to methacholine challenge and (**B** and **C**)**,** bronchoalveolar lavage (BAL) cell counts [total (Tot), eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu)], and BAL cytokine levels of OVA-challenged WT and DGKζ KO BM chimeras (n = 7-9 mice/group). (**D**-**F**)**,** Changes in lung resistance following methacholine challenge, BAL cell counts, and BAL cytokine levels in OVA-challenged Vav-Cre DGKζ$^{fl/fl}$ mice and Vav-Cre controls [(D), n = 7 mice/group; (E and F), n = 13-14 mice/group]. (**G** and **H**)**,** BAL cell counts and BAL cytokine levels in OVA-challenged CD4-Cre DGKζ$^{fl/fl}$ mice and CD4-Cre controls [(G), n = 12-13 mice/group; (H), n = 16-17 mice/group]. (**I**)**,** Representative flow cytometry plots and KO/WT ratios of adoptively transferred cytokine-producing WT OT-II and DGKζ KO OT-II CD4$^+$ T cells (pregated on CD4$^+$Vβ5$^+$ live singlets) in the spleen of OVA-sensitized

congenically disparate WT hosts following restimulation with PMA/ionomycin *ex vivo* (n = 10 mice/group). (A) through (H) was done in collaboration with Wen Lu, Moyar Ge, and Amanda Schmidt Paustian. Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant [Two way ANOVA with Bonferroni's post-test, (A) and (D); One way ANOVA with Tukey's post-test, (B); One way ANOVA with Dunn's post-test, (C); two-sided unpaired Student's t-test, (E) through (H); one-sided Student's t-test, (I)].

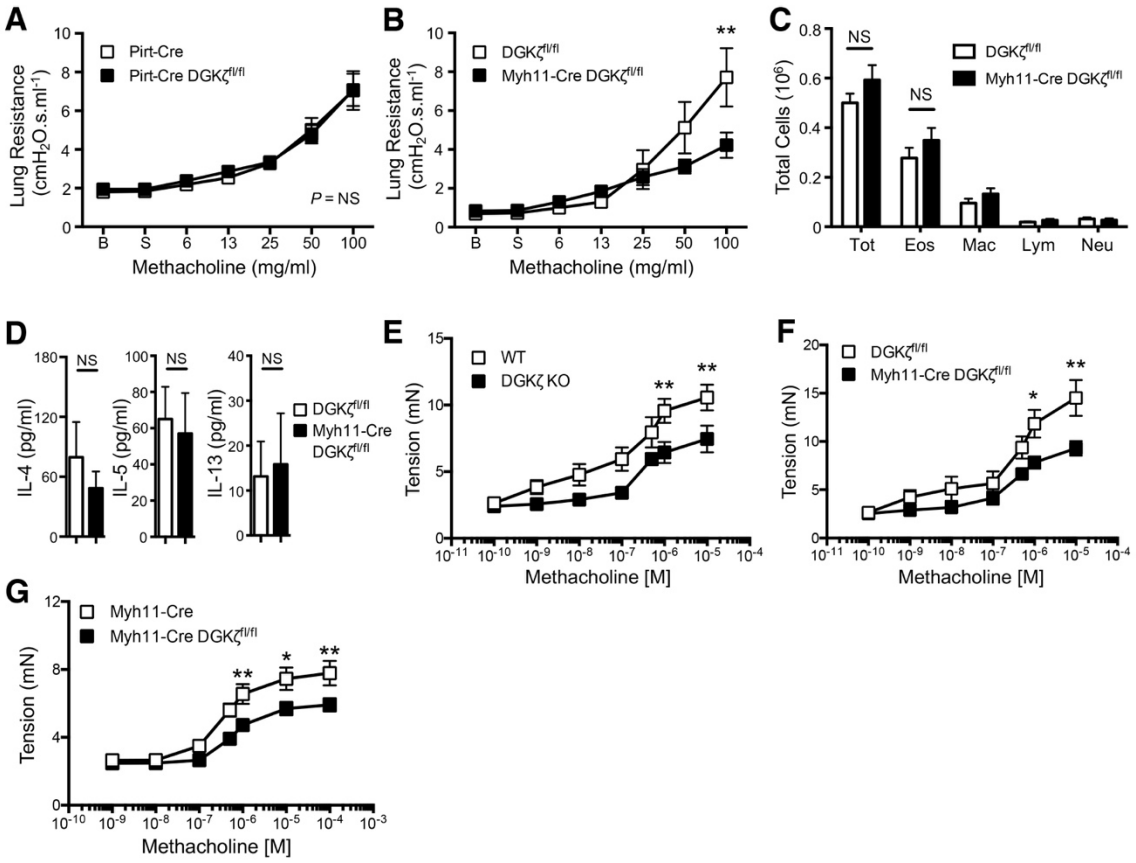

Figure 2.6. The loss of DGKζ in airway smooth muscle cells protects against AHR. (**A** and **B**)**,** Changes in lung resistance in response to methacholine challenge of OVA-challenged Pirt-Cre DGKζ$^{fl/fl}$, Myh11-Cre DGKζ$^{fl/fl}$, and their respective WT control mice [(A), n = 8-11 mice/group; (B), n = 9-14 mice/group, (A) and (B) was done in collaboration with Nadan Wang and Deepak Deshpande]. (**C**)**,** Total number of eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu), and (**D**)**,** Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid of OVA-challenged Myh11-Cre DGKζ$^{fl/fl}$ mice and DGKζ$^{fl/fl}$ controls (n = 18-21 mice/group). (**E-G**)**,** Contractile forces generated from DGKζ KO, Myh11-Cre DGKζ$^{fl/fl}$, Myh11-Cre and DGKζ$^{fl/fl}$ control tracheal rings in response to methacholine [n = 6-11 mice/group, done in collaboration with Deepak Deshpande]. Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, NS = not significant [Two way ANOVA with Bonferroni's post-test, (A), (B), (E) through (G); two-sided unpaired Student's t-test, (C) and (D)].

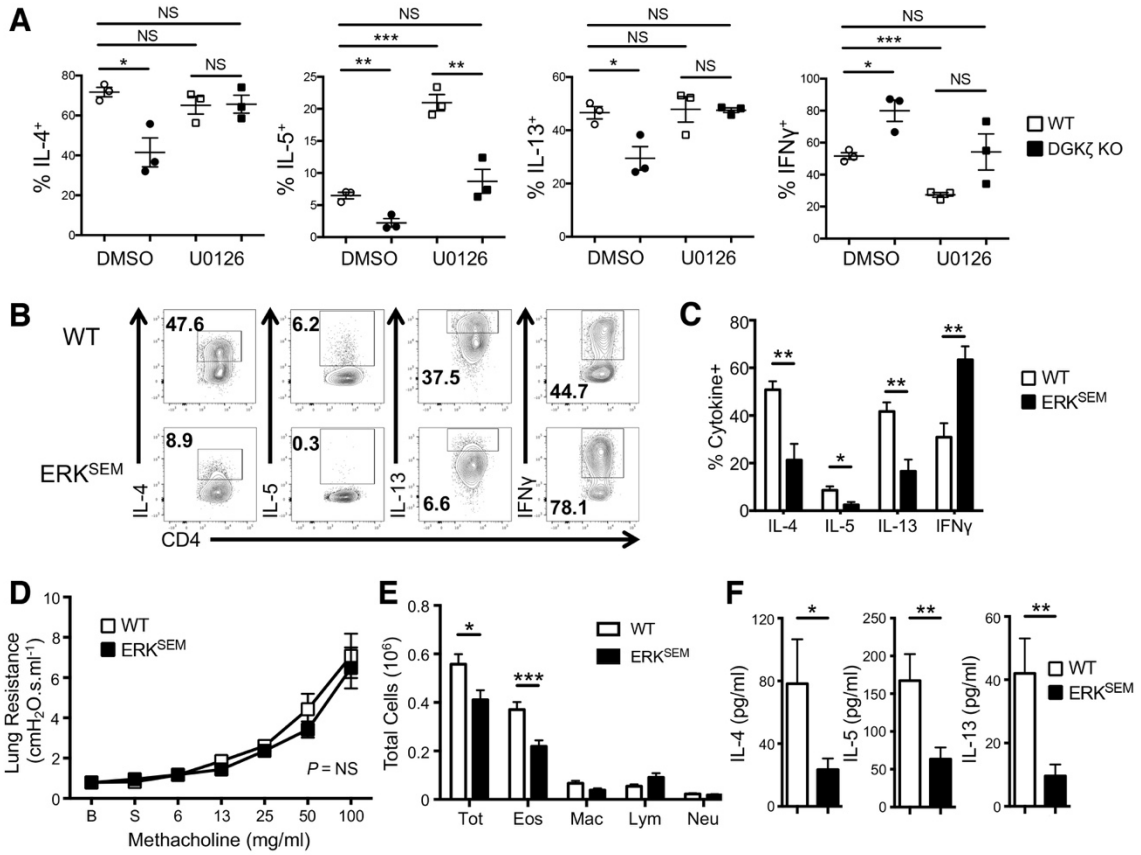

Figure 2.7. Enhancement of ERK signaling in T cells is sufficient to protect from OVA-induced allergic airway inflammation but insufficient to protect from OVA-induced AHR. (**A**)**,** Frequency of cytokine-producing WT and DGKζ KO $CD4^+$ T cells or (**B** and **C**)**,** WT and sevenmaker transgenic (ERK^{SEM}) $CD4^+$ T cells after pretreatment with either vehicle control or U0126 followed by activation with anti-CD3 and anti-CD28 under nonpolarizing conditions for 5 days *in vitro* [(A), n = 3 mice/group; (B and C), n = 6 mice/group]. (**D**-**F**)**,** Changes in lung resistance following methacholine challenge, bronchoalveolar lavage (BAL) cell counts [total (Tot), eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu)], and BAL Th2 cytokine levels in OVA-challenged WT and ERK^{SEM} mice [(D), n = 8-9 mice/group; (E and F), n = 21-26 mice/group]. Data are representative of 2 independent experiments (A) or pooled from at least 2 independent experiments (C-G). (D) was done in collaboration with Nadan Wang and Deepak Deshpande. Data are represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant [two-sided unpaired Student's t-test, (A), (E), and (F); Two way ANOVA with Bonferroni's post-test, (D); two-sided paired Student's t-test, (C)].

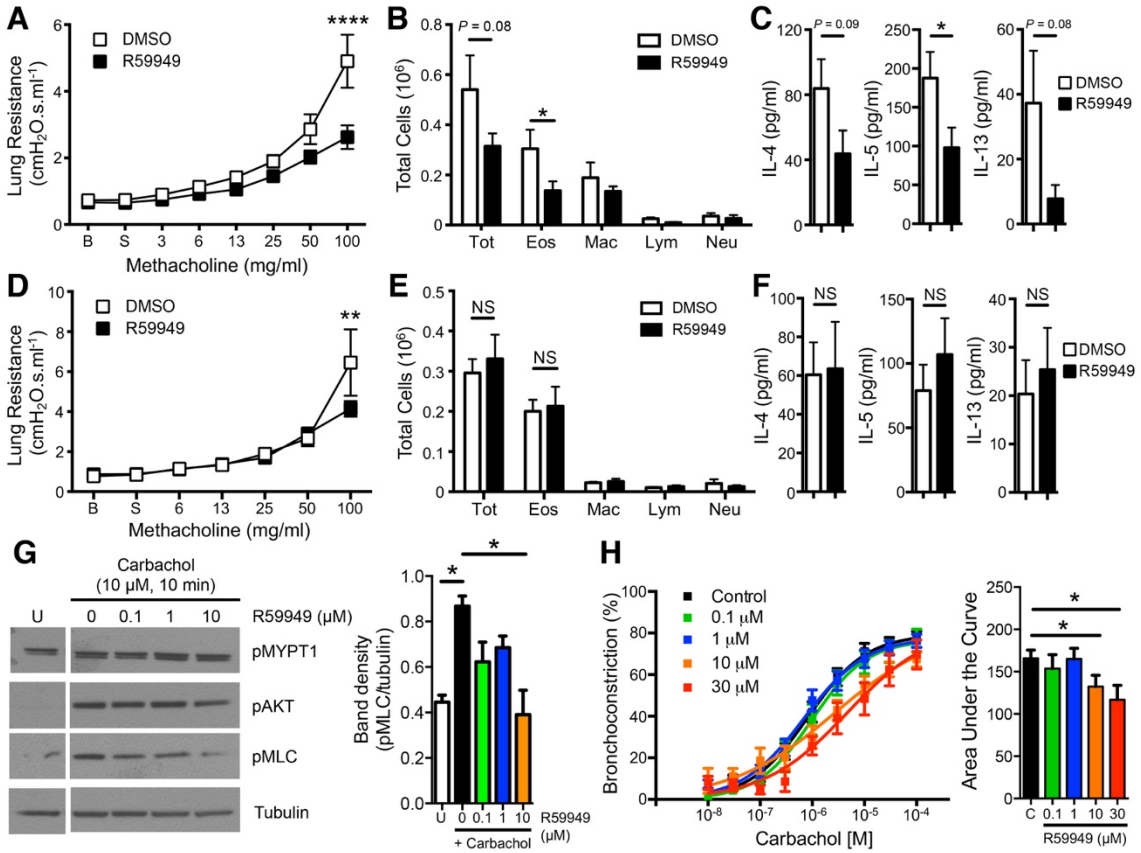

Figure 2.8. Pharmacological inhibition of diacylglycerol kinase (DGK) protects against OVA-induced allergic airway inflammation and AHR. (**A**-**C**)**,** Changes in lung resistance following methacholine challenge, bronchoalveolar lavage (BAL) cell counts [Total (Tot), eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu)], and BAL cytokine levels in OVA-challenged mice treated with either vehicle control or R59949 during late sensitization/airway challenge phase or (**D**-**F**)**,** or treated with either vehicle control or R59949 only during the airway challenge phase of the OVA-induced asthma model [(A), n = 7-8 mice/group, done in collaboration with Moyar Ge; (B), n = 15-17 mice/group; (C), n = 10-11 mice/group; (D-F), n = 8-10 mice/group, (D) was done in collaboration with Nadan Wang and Deepak Deshpande]. (**G**), Western blotting of phospho-myosin light chain (pMLC), phospho-protein kinase B (pAkt), and phospho-myosin phosphatase target subunit 1 (pMYPT1) in lysates of carbachol-stimulated human airway smooth muscle (HASM) cells pretreated with vehicle vs. R59949 [n = 3 donors, done in collaboration with Cynthia Koziol-White from the lab of Reynold Panettieri Jr.]. (**H**)**,** Carbachol-induced bronchoconstriction of human precision cut lung slices (PCLS) pretreated with vehicle vs. R59949 [n = 9-23 slices/condition from 3-7 donors/condition; done in collaboration with Cynthia Koziol-White from the lab of Reynold Panettieri Jr.]. Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS

= not significant [Two way ANOVA with Bonferroni's post-test, (A); two-sided unpaired Student's t-test, (B) through (E)].

CHAPTER 3: DIACYLGLYCEROL KINASE ZETA REGULATES PAPAIN-INDUCED TYPE 2 AIRWAY INFLAMMATION

Introduction

Asthma is a chronic respiratory disease that is characterized by airway inflammation and obstruction that is initiated at mucosal surfaces where inhaled allergens contact the lung epithelium. While common allergens, such as house dust mite (HDM), papain, and *Alternaria alternata*, are heterogeneous, a common component of these allergens are molecules with protease activity. Proteases in inhaled allergens damage the epithelial barrier and trigger the production of IL-25, IL-33, and TSLP by cells residing in the epithelium (*196-199*). These epithelial-derived cytokines activate tissue resident immune cells, which include group 2 innate lymphoid cells (ILC2) that produce IL-5 and IL-13, basophils that produce IL-4, and mast cells that release vasoactive amines and inflammatory lipid mediators (*200-205*). These epithelial-derived cytokines further prime lung dendritic cells to process antigen, migrate to the draining lymph nodes, and activate allergen-specific $CD4^+$ T cells (*206*).

Inhalation of papain, a plant-derived cysteine protease, has been shown to cause occupational asthma in humans (*207*). Intranasal administration of papain in mice triggers the activation of ILC2s and the induction of airway eosinophilia in the airways in an IL-33-dependent manner (*200*). Damage elicited by the protease activity of papain can lead to necrosis of IL-33-expressing cells, resulting in the release of bioactive IL-33 stored in the nucleus into the extracellular milieu. However, cell death-independent mechanisms for IL-33 release can occur in response to extracellular ATP and mechanical stress (*208, 209*). While the enzymatic activity of papain has been shown to be important for the initiation of type 2 immune responses in the lung, the molecular signals that regulate the sensing of protease allergen-derived enzymatic activity by the

epithelial barrier in the airways to trigger the release of IL-33 to elicit ILC2-mediated inflammation are not completely understood.

Diacylglycerol kinases (DGK) are a family of enzymes that convert diacylglycerol (DAG) to phosphatidic acid (PA) via phosphorylation to terminate DAG-mediated signaling (*64, 73*). There are 10 different isoforms comprising 5 different classes of DGKs, each of which control different cellular functions based on their distinct structural motifs and subcellular localization (*64, 73-76*). The zeta isoform of DGK (DGKζ) is widely expressed in the hematopoietic and non-hematopoietic compartments and is important for regulating diverse cellular processes in both of these compartments (*176*). In the previous chapter, we demonstrated that DGKζ plays an immunomodulatory role in type 2 immune responses by positively controlling Th2 differentiation through the regulation of TCR-mediated ERK signaling to promote type 2 airway inflammation. Furthermore, the loss of DGKζ has been shown to attenuate FcεRI-mediated degranulation of mast cells and consequently, DGKζ KO mice display impaired IgE-mediated anaphylactic responses *in vivo (132)*. However, whether DGKζ plays an immunomodulatory role in ILC2-mediated inflammatory responses is unknown.

Here, we report that the loss of DGKζ protects from papain-induced type 2 airway inflammation. Papain-challenged DGKζ KO displayed reduced ILC2 accumulation, decreased IL-5 and IL-13 release, and attenuated eosinophilia in the airways. Unexpectedly, protection from papain-induced airway inflammation was not due to the loss of DGKζ in the hematopoietic compartment because hematopoietic-specific DGKζ KO mice exhibited intact papain-induced type 2 airway inflammation. Interestingly, IL-33-mediated inflammation was also intact in DGKζ KO mice, suggesting that the impairment in papain-mediated airway inflammation is not due to defective responses to IL-33 in

DGKζ KO mice. However, we found that IL-33 mRNA levels were significantly lower in DGKζ KO mice and this correlated with decreased frequencies of IL-5-producing lung ILC2s at homeostasis. Yet, conditional loss of DGKζ in alveolar type II cells , sensory neurons, or smooth muscle cells did not recapitulate the protection from papain-induced airway inflammation seen in global DGKζ KO mice, suggesting that DGKζ controls the regulation of IL-33 through an unknown non-hematopoietic cell type.

Results

DGKζ KO mice are protected from papain-induced type 2 airway inflammation

In the previous chapter, we demonstrated that DGKζ controls Th2 differentiation *in vitro* and promotes the generation of Th2 cells to drive OVA-induced airway inflammation *in vivo*. We wanted to examine whether DGKζ also controlled the generation and function of ILC2s in the lung. To test this, lung ILC2s were isolated and characterized from naïve DGKζ KO mice. While total numbers and GATA3 expression in lung ILC2s were normal in DGKζ KO mice, DGKζ KO lung ILC2 displayed significantly impaired ability to produce IL-5 and IL-13 following *ex vivo* stimulation with PMA and ionomycin (Fig. 3.1 and Fig. 3.2, A). To test if ILC2-mediated inflammatory responses were impaired in DGKζ KO mice, WT and DGKζ KO mice were subjected to a mouse model of acute allergen-induced airway inflammation elicited by intranasal administration of papain for 5 consecutive days. In line with our *in vitro* data, papain-challenged DGKζ KO exhibited significantly reduced ILC2 accumulation in the bronchoalveolar lavage (BAL) fluid and the lung parenchyma (Fig. 3.2, B). Furthermore, proliferation of lung ILC2s in response to papain was diminished in DGKζ KO mice as determined by decreased frequency and total number of $Ki67^+$ lung ILC2s (Fig. 3.2, C). Additionally, we observed a decreased frequency of $IL\text{-}5^+IL\text{-}13^+$ ILC2s in papain-challenged DGKζ KO

mice, implying that ILC2 cytokine effector function in response to papain was diminished in DGKζ KO mice (Fig. 3.2, D). This correlated with decreased BAL Th2 cytokine levels in the lungs of papain-challenged DGKζ KO mice (Fig. 3.2, E). Production of IL-5 and IL-13 by ILC2 is critical for early eosinophil recruitment and activation into the airways and the lung parenchyma following intranasal papain administration (*200*). Indeed, papain-challenged DGKζ KO mice displayed reduced accumulation of eosinophils the BAL and lung parenchyma (Fig. 3.2, F). Overall, the loss of DGKζ attenuates papain-induced type 2 airway inflammation.

Protection from papain-induced airway inflammation in the absence of DGKζ is not mediated by the hematopoietic compartment.

The initiation of type 2 immune responses by intranasal administration of papain is mediated by cellular responses from both the hematopoietic and non-hematopoietic compartments. To test if DGKζ deficiency in the hematopoietic cells was sufficient to provide protection from papain-induced airway inflammation, DGKζ was conditionally deleted in hematopoietic cells using a Vav-inducible Cre (Vav-Cre DGKζ$^{fl/fl}$ mice). To our surprise, Vav-Cre DGKζ$^{fl/fl}$ mice displayed intact papain-induced eosinophilia in the airways and in the lung parenchyma (Fig. 3.3, A). Furthermore, accumulation of lung ILC2s in response to papain was unchanged while the accumulation of ILC2s in the BAL was significantly enhanced in Vav-Cre DGKζ$^{fl/fl}$ mice (Fig. 3.3, B). Moreover, the induction of BAL Th2 cytokines in the airway of hematopoietic-specific conditional DGKζ KO mice was intact and slightly enhanced following papain exposure (Fig. 3.3, C). These data suggested that protection from papain-induced airway inflammation was not driven by loss of DGKζ in the hematopoietic compartment but rather due to loss of DGKζ in the non-hematopoietic compartment.

Airway inflammation driven by intranasal administration of IL-33 is intact in DGKζ KO mice

Tissue-derived cytokines, IL-33 and TSLP, have been shown to be released from epithelial cells in response to papain *in vitro* and *in vivo (200, 210-212)*. However, papain-induced eosinophilic inflammation is largely dependent on IL-33 *in vivo (205, 211)*. Indeed, papain-induced influx of eosinophils, ILC2 accumulation, and Th2 cytokine release in the airways was almost completely abrogated in mice lacking the IL-33 receptor, ST2, but remained relatively intact in TSLPR KO mice, thus confirming that papain-induced eosinophilic inflammation is predominantly mediated by IL-33 (Fig. 3.4, A-C). To test if protection from papain-induced airway inflammation in the absence of DGKζ was due to impaired responses to IL-33, DGKζ KO mice were subjected to intranasal administration of IL-33 for 3 consecutive days. In contrast to the papain treatment, DGKζ KO exhibited similar accumulation of eosinophils and ILC2s in the BAL following IL-33 treatment compared to control mice (Fig. 3.4, D and E). In addition, IL-33-mediated accumulation of lung eosinophils and ILC2s was enhanced in DGKζ KO mice compared to WT controls (Fig. 3.4, D and E). However, BAL IL-5 and IL-13 levels in DGKζ KO mice following IL-33 administration was comparable to levels seen in WT controls (Fig. 3.4, F). To further test if loss of DGKζ impacted responses to IL-33, ILC2s were sorted from the lungs of naïve WT and DGKζ KO mice and stimulated with IL-33 in combination with IL-2 for 72 hours. DGKζ KO exhibited similar production of IL-5 and IL-13 in response to stimulation with IL-33 and IL-2 (Fig. 3.5, A). Furthermore, the frequency of IL-5$^+$ and IL-13$^+$ DGKζ KO ILC2s in response to IL-33 stimulation following *in vitro* expansion was comparable to responses from WT ILC2s (Fig. 3.5, B and C). Overall, these data demonstrated that protection from papain-induced airway

inflammation in DGKζ KO mice is not due to diminished responses to IL-33 released following papain challenge.

Loss of DGKζ in alveolar type II cells does not protect from papain-induced airway inflammation

The induction of potent type 2 airway inflammation in response to IL-33 administration but not to papain challenge in DGKζ KO mice suggested that the loss of DGKζ might be affecting IL-33 expression, processing, or release. To test if the absence of DGKζ impacted the regulation of lung IL-33 levels at homeostasis, we measured IL-33 mRNA levels from whole lung homogenates obtained from naïve WT and DGKζ KO mice. Surprisingly, we found significantly lower levels of IL-33 mRNA transcripts as well as diminished levels of IL-25 and TSLP mRNA in DGKζ KO lungs compared to WT controls (Fig. 3.6, A). Tissue-resident lung ILC2 constitutively produce IL-5 at homeostasis in a manner that is partially dependent on IL-33 but not IL-25 or TSLP, implying the existence of a homeostatic pool of IL-33 released in the absence of inflammation (*213*). We observed that the frequency of DGKζ KO lung ILC2s producing IL-5 at homeostasis was significantly decreased compared to WT controls, thus suggesting that homeostatic release of IL-33 was potentially diminished in the absence of DGKζ (Fig. 3.6, B).

Alveolar type II cells are a predominant source of pulmonary IL-33 postnatally and during adult life under homeostatic conditions (*214*). Furthermore, DGKζ has been shown to be expressed in alveolar type II cells present in the lung epithelium (*215*). To test if the loss of DGKζ in alveolar type II cells was sufficient to protect from papain-induced airway inflammation, DGKζ was conditionally deleted in alveolar type II cells using the Nkx2.1-inducible Cre (Nkx2.1-Cre DGKζ$^{fl/fl}$ mice). However, we found that

papain-induced eosinophilia, ILC2 accumulation, and the induction of BAL Th2 cytokines was unaltered in Nkx2.1-Cre DGKζ$^{fl/fl}$ mice (Fig. 3.7, A-C).

Sensory neurons arising from the dorsal root ganglion (DRG) have been shown to express the ST2 receptor and respond to IL-33 to produce neuropeptides, such neuromedin U (NMU), that can promote ILC2 responses following infection with *Nippostronglus brasiliensis* or during HDM-mediated type 2 inflammatory responses in the lung (*216-218*). Additionally, airway smooth muscle cells have been shown to express IL-33 mRNA transcript and potentially IL-33 protein that is upregulated during asthmatic inflammation *in vivo (219)*. To test if the loss of DGKζ in either of these cell types was responsible for protection from papain-induced airway inflammation, DGKζ was conditionally deleted in DRG sensory neurons using the Pirt-inducible Cre (Pirt-Cre DGKζ$^{fl/fl}$) or in smooth muscle cells using the Myh11-inducible Cre (Myh11-Cre DGKζ$^{fl/fl}$). However, similar to the Nkx2.1-Cre DGKζ$^{fl/fl}$ mice, Pirt-Cre DGKζ$^{fl/fl}$ and Myh11-Cre DGKζ$^{fl/fl}$ displayed comparable induction of eosinophils and ILC2 in the airways and the lung parenchyma following papain challenge as compared to their respective Cre controls (Fig. 3.7, D and E). Overall, these data indicate that DGKζ potentially regulates papain-induced airway inflammation by regulating IL-33 levels in the lung during homeostasis and inflammation but not directly through alveolar type II cells, DRG sensory neurons, or airway smooth muscle cells.

Discussion

Our findings demonstrate that the regulation of diacylglycerol signaling by DGKζ plays a critical role in a mouse model of protease-mediated allergic airway inflammation. Genetic ablation of DGKζ resulted in protection from papain-induced type 2 airway inflammation. Generation of hematopoietic-specific DGKζ KO mice revealed that protection from

papain-induced airway inflammation was not due to the actions of DGKζ in the hematopoietic compartment. Importantly, bypassing papain with administration of IL-33 elicited comparable inflammation in DGKζ KO mice compared to WT controls, suggesting that the defect in type 2 immune responses mounted against papain is upstream of IL-33 release. Indeed, DGKζ KO mice displayed significantly decreased IL-33 mRNA levels, which correlated with decreased ILC2-mediated production of IL-5 at homeostatic conditions in the lung. Surprisingly, the loss of DGKζ in alveolar type II cells, DRG sensory neurons, or airway smooth muscle cells failed to protect from papain-induced ILC2 responses *in vivo*, suggesting that DGKζ is required in another cell type from the non-hematopoietic lineage to regulate papain-induced airway inflammation.

As a negative regulator of DAG-mediated signaling, one might predict that the loss of DGKζ would universally lead to immune activation. Interestingly, however, the inhibition of DGKζ does not only enhance but also suppresses selective immune responses. In settings of Th2 responses, increased DAG signaling suppresses the degranulation of DGKζ KO mast cells resulting in protection from IgE-mediated anaphylactic responses *in vivo (132)*. Furthermore, augmenting TCR-mediated DAG signaling impairs Th2 differentiation in DGKζ KO T cells through an ERK-dependent manner to suppress OVA-induced airway inflammation *in vivo*. While these studies demonstrate the role of DGKζ in type 2 immune cells, our data demonstrates that DGKζ deficiency can also impair type 2 airway inflammation by potentially regulating the function of the non-hematopoietic compartment to suppress ILC2 activation in the airways. Overall, our data reinforces the notion that the loss of DGKζ inhibits Th2 inflammation through multiple mechanisms.

The mechanism by which DGKζ protects from papain-induced airway inflammation is not entirely clear. We began our studies by hypothesizing that DGKζ would impact ILC2 function in a cell-intrinsic manner. However, ILC2 responses following *in vitro* treatment with IL-33, the major effector cytokine released following papain challenge, were relatively normal. Furthermore, deletion of DGKζ in the hematopoietic compartment failed to provide protection from papain-induced airway ILC2 accumulation and eosinophilia, suggesting that DGKζ regulates papain-induced airway inflammation through an ILC2-extrinsic and non-hematopoietic manner. While we observed that IL-33 mRNA levels were significantly diminished in DGKζ KO mice, conditional deletion of DGKζ in alveolar type II cells, the predominant source of pulmonary IL-33, failed to protect from papain-induced airway inflammation. These results imply that either alveolar type II cells are not the relevant source of IL-33 for potentiating papain-induced airway inflammation *in vivo* or DGKζ regulates IL-33 in alveolar type II cells through an indirect mechanism by influencing the function of secondary cell type, such a fibroblast or stromal cell, involved promoting papain-induced IL-33 release. In agreement with the former, recent work has demonstrated that adventitial stromal cells residing in adventitial cuff structures are sources of IL-33 and TSLP in the lung airways and serve as a niche for tissue-resident ILC2s to help support ILC2 responses during helminth infection *in vivo (220)*. However, whether adventitial stromal cells constitute the relevant source of pulmonary IL-33 to drive ILC2 activation in response to papain and whether DGKζ regulates this response remains to be seen. The mechanism by which papain elicits IL-33 release is not very well understood. Some studies suggest that IL-33, which is constitutively stored in the nucleus, is primarily released through necrosis of cells damaged by the protease activity of papain despite no direct evidence of this process *in vivo*. Furthermore, IL-33 can be released through cell-

death independent mechanisms through either mechanical stress or ATP release (*208, 209*). Thus, it is also possible that papain enzymatic activity elicits IL-33 release from alveolar type II cells or other IL-33 sources indirectly through mechanical sensing by other cells, such as fibroblasts.

In summary, our study demonstrates that an enzyme involved in DAG metabolism is important for protease-mediated airway inflammatory responses. Our data suggest that DGKζ might function to regulate activation of the non-hematopoietic compartment by papain enzymatic activity to prompt the release of IL-33 and initiate type 2 immune responses in the airways. We propose that DGKζ might represent a novel target for the treatment of allergic airway inflammatory diseases driven by common environmental proteases.

Figures

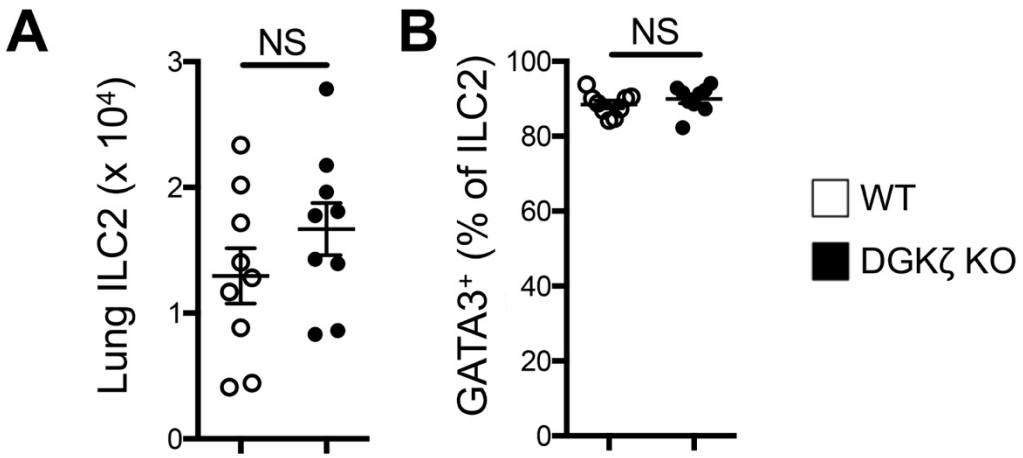

Figure 3.1. Normal numbers and GATA3 expression of DGKζ KO lung ILC2s. (**A**), Total numbers of type 2 innate lymphoid cells (ILC2) (gated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets with Lin^- = $CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) from naïve WT and DGKζ KO lungs (n = 9 mice/group). (**B**), Frequency of lung ILC2s (pregated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets) expressing GATA3 from naïve WT and DGKζ KO mice. (n = 9 mice/group). Data are pooled from 3 independent experiments and represented as mean ± SEM; NS = not significant [two-sided unpaired Student's t-test, (A) and (B)].

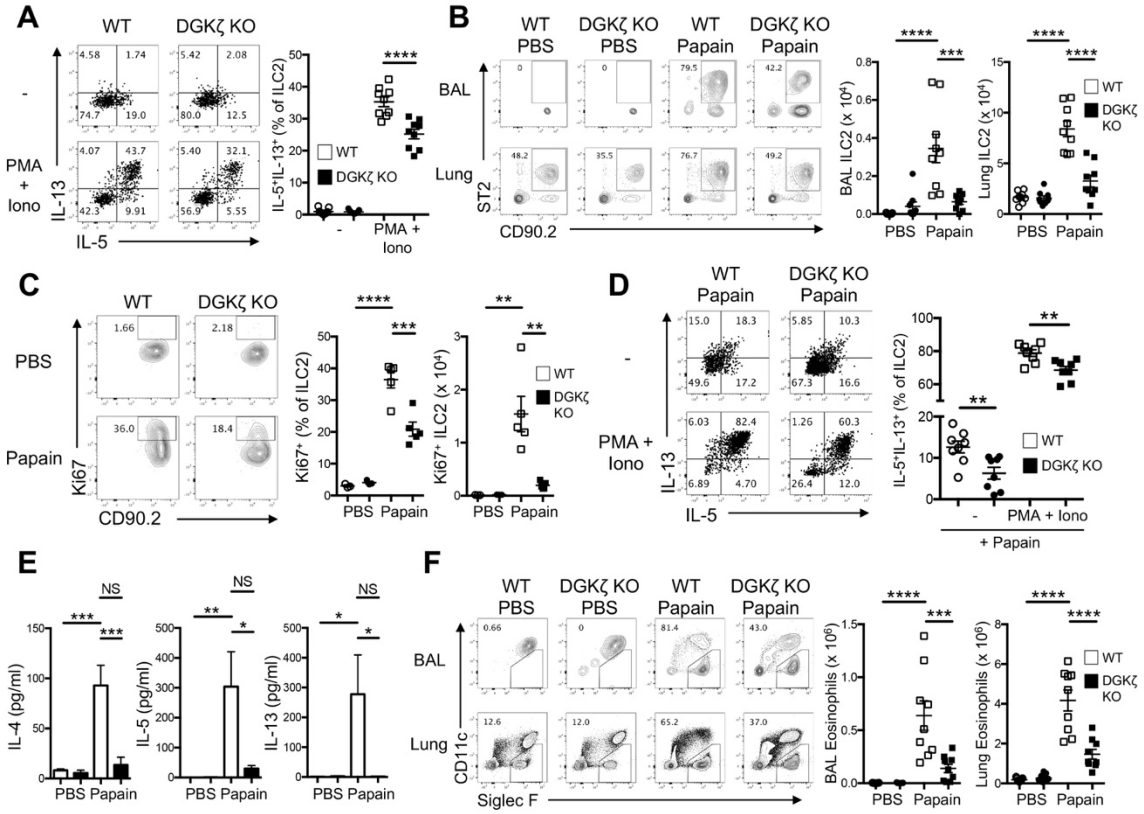

Figure 3.2. DGKζ KO mice are protected from papain-induced type 2 airway inflammation. (**A**)**,** Representative flow cytometry plots and enumeration of the frequencies of WT and DGKζ KO lung ILC2 (pregated on $CD45^+Lin^-CD90^+ST2^+$ live singlets with Lin^- = $CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) expressing IL-5 and IL-13 following stimulation with media alone or PMA + ionomycin *ex vivo* (n = 9 mice/group). (**B**)**,** Representative flow cytometry plots (pregated on $CD45^+Lin^-$ live singlets) of the frequencies and total cell numbers of BAL and lung ILC2 from WT or DGK KO mice intranasally challenged with PBS or papain for 5 days (n = 9 mice/group). (**C**)**,** Representative flow cytometry plots and enumeration of the frequencies and total numbers of WT and DGKζ KO lung ILC2 (pregated on $CD45^+Lin^-CD90^+ST2^+$ live singlets) expressing Ki67 following intranasal challenge with PBS or papain for 5 days (n = 3-5 mice/group). (**D**), Representative flow cytometry plots and enumeration of the frequencies of WT and DGKζ KO lung ILC2 (pregated on $CD45^+Lin^-CD90^+ST2^+$ live singlets) expressing IL-5 and IL-13 following restimulation with media alone or PMA + ionomycin after intranasal challenge with papain for 5 days (n = 8 mice/group). (**E**)**,** BAL cytokine levels in PBS or papain-challenged WT and DGKζ KO mice (n = 6 mice/group). (**F**)**,** Representative flow cytometry plots (pregated on $CD45^+Ly6G^-$ live singlets) of the frequencies and total cell numbers of BAL and lung eosinophils from WT or DGK KO mice intranasally challenged with PBS or papain for 5 days (n = 9 mice/group). Data are pooled from at least 2 independent experiments (A, B, E, and F) or representative of 2 independent experiments (C-D). Data are represented as mean ± SEM; * $P < 0.05$, ** P

< 0.01, *** $P < 0.001$, **** $P < 0.0001$, NS = not significant [One way ANOVA with Tukey's post-test, (A) through (F)].

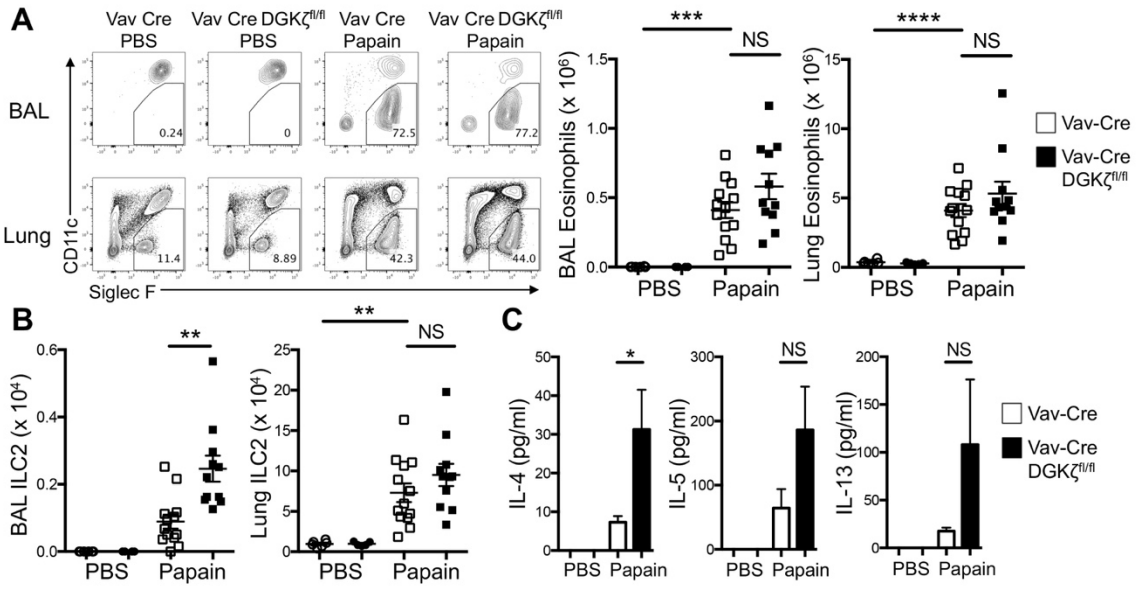

Figure 3.3. Protection from papain-induced airway inflammation in the absence of DGKζ is not mediated by the hematopoietic compartment. (**A**)**,** Representative flow cytometry plots (pregated on $CD45^+Ly6G^-$ live singlets) of the frequencies and total cell numbers of BAL and lung eosinophils from Vav-Cre and Vav-Cre $DGK\zeta^{fl/fl}$ mice intranasally challenged with PBS or papain for 5 days (n = 5-13 mice/group). (**B**)**,** Total cell numbers of BAL and lung ILC2s (gated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets with Lin^- = $CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) from Vav-Cre or Vav-Cre $DGK\zeta^{fl/fl}$ mice intranasally challenged with PBS or papain for 5 days (n = 5-13 mice/group). (**C**)**,** BAL cytokine levels in PBS or papain-challenged Vav-Cre or Vav-Cre $DGK\zeta^{fl/fl}$ mice (n = 2-3 mice/group for PBS challenged mice, n = 7-10 mice/group for papain challenged mice). Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS = not significant [One way ANOVA with Tukey's post-test, (A) and (B); two-sided unpaired Student's t-test, (C)].

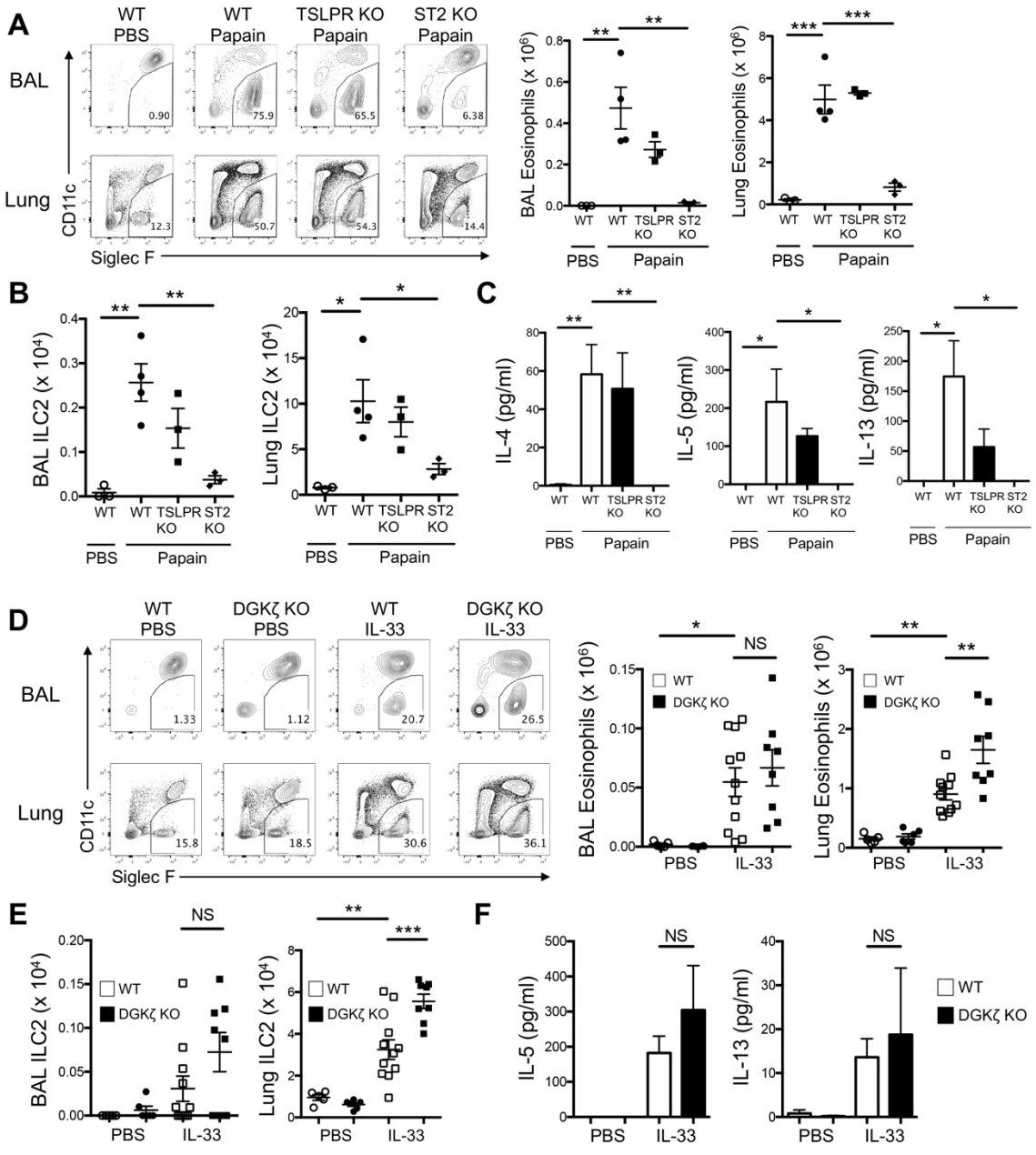

Figure 3.4. Airway inflammation driven by intranasal administration of IL-33 is intact in DGKζ KO mice. (**A**)**,** Representative flow cytometry plots (pregated on $CD45^+Ly6G^-$ live singlets) of the frequencies and total cell numbers of BAL and lung eosinophils from WT, TSLPR KO, and ST2 KO mice intranasally challenged with PBS or papain for 5 days (n = 3-4 mice/group). (**B**)**,** Total cell numbers of BAL and lung ILC2s (gated on $CD45^+Lin^-CD90.2^+CD25^+$ live singlets with Lin^- = $CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) from WT, TSLPR KO, and ST2 KO mice intranasally

challenged with PBS or papain for 5 days (n = 3-4 mice/group). (**C**)**,** BAL cytokine levels in PBS or papain-challenged WT, TSLPR KO, and ST2 KO mice (n = 3-7 mice/group). (**D**)**,** Representative flow cytometry plots (pregated on $CD45^+Ly6G^-$ live singlets) of the frequencies and total cell numbers of eosinophils and (**E**)**,** total cell numbers of ILC2 (gated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets) from the BAL and lungs of WT and DGKζ KO mice intranasally challenged with PBS or IL-33 for 3 days (n = 5-6 mice/group for PBS-challenged mice, n = 8-11 mice/group for IL-33-challenged mice). (**F**)**,** BAL cytokine levels in PBS or IL-33-challenged WT and DGKζ KO mice (n = 4-8 mice/group). Data are representative of 2 independent experiments (A and B) or pooled from at least 2 independent experiments (B-F). Data are represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant [One way ANOVA with Tukey's post-test, (A) through (F)].

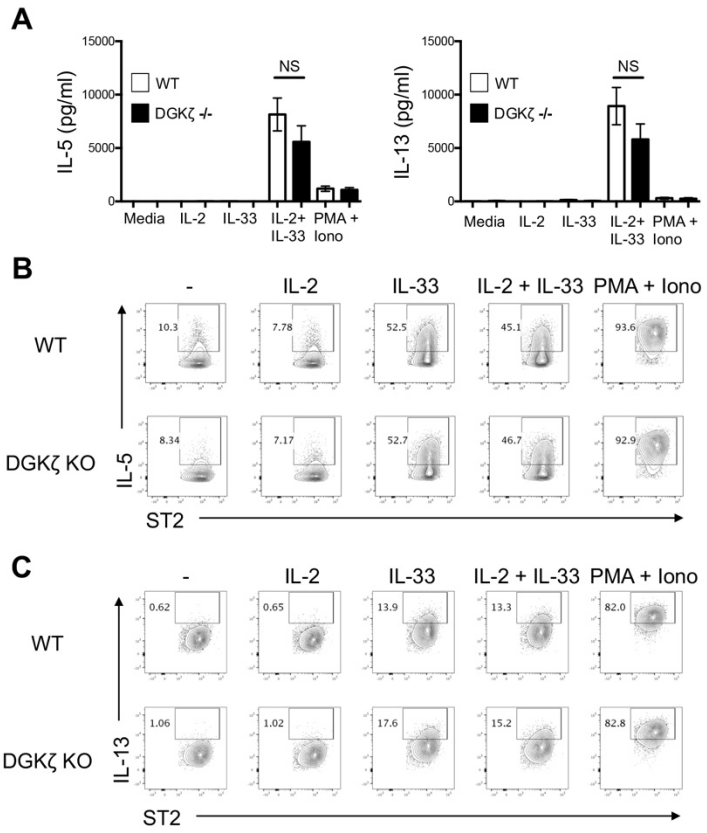

Figure 3.5. DGKζ KO lung ILC2s produce IL-5 and IL-13 in response to IL-33 stimulation normally. (**A**)**,** IL-5 and IL-13 production from FACS-sorted WT and DGKζ KO lung ILC2s stimulated with media alone, IL-2, IL-33, IL-2 + IL-33, or PMA + ionomycin for 72 hours in vitro (n = 5 replicates, each replicate consisting of ILC2s sorted from 5 pooled lungs). (**B** and **C**), Representative flow cytometry plots of *in vitro* lung ILC2 (pregated on $CD45.2^{+}Lin^{-}ST2^{+}$) expressing IL-5 and IL-13 following *in vitro* expansion for 7 days followed by restimulation with media alone, IL-2, IL-33, IL-2 + IL-33, or PMA + ionomycin for 4 hours. Data are pooled from 5 independent experiments (A) or representative of 2 independent experiments (B-F). Data are represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = not significant [One way ANOVA with Tukey's post-test, (A) through (F)].

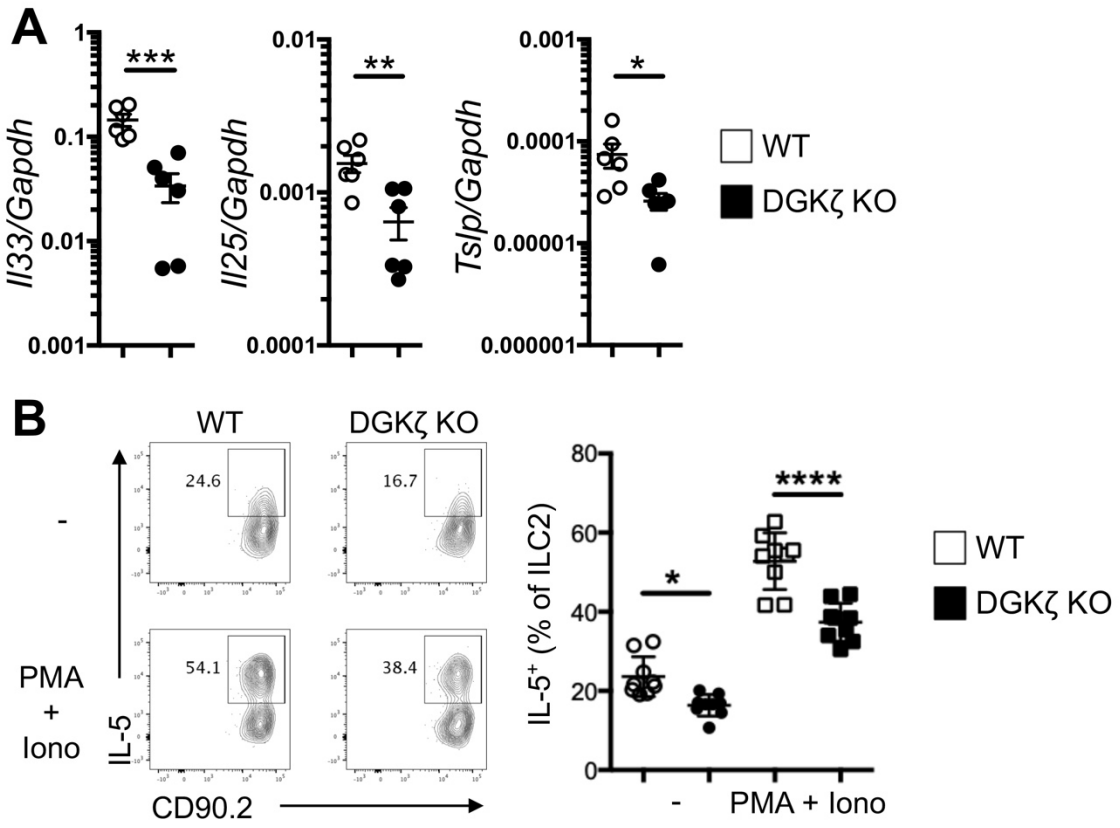

Figure 3.6. DGKζ KO mice display reduced IL-33 mRNA levels at homeostasis. (**A**), *IL33*, *IL25*, *Tslp* mRNA expression in lung tissue of naïve WT and DGKζ KO mice. Values shown were determined by RT-PCR and are normalized to *Gapdh* [n = 6 mice/group; done in collaboration with Li-Yin Hung]. (**B**), Representative flow cytometry plots and enumeration of the frequencies of WT and DGKζ KO lung ILC2 (pregated on $CD45^+Lin^-CD90^+ST2^+$ live singlets) expressing IL-5 following stimulation with media alone or PMA + ionomycin *ex vivo* (n = 9 mice/group). Data are pooled from at least 2 independent experiments and represented as mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, NS = not significant [two-sided unpaired Student's t-test, (A); One way ANOVA with Tukey's post-test, (B)].

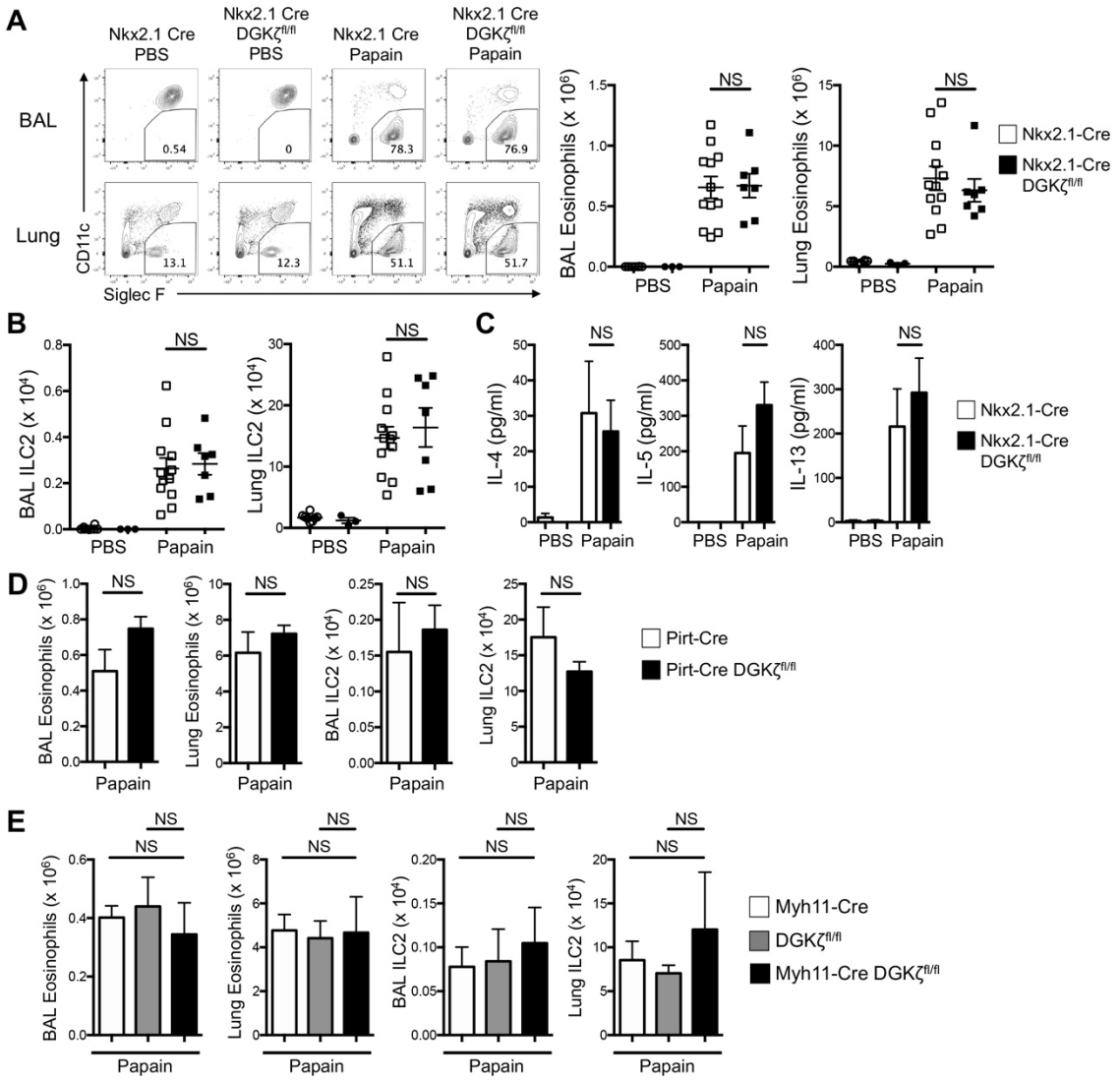

Figure 3.7. Loss of DGKζ in alveolar type II cells does not protect from papain-induced airway inflammation. (**A**)**,** Representative flow cytometry plots (pregated on $CD45^+Ly6G^-$ live singlets) of the frequencies and total cell numbers of eosinophils and (**B**) total cell numbers of ILC2s (gated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets with Lin^- = $CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) from the BAL and lungs of Nkx2.1-Cre and Nkx2.1-Cre DGK$\zeta^{fl/fl}$ mice intranasally challenged with PBS or papain for 5 days (n = 3-8 mice/group for PBS challenged mice, n = 7-11 mice/group for papain challenged mice). (**C**)**,** BAL cytokine levels in PBS or papain-challenged Nkx2.1-Cre and Nkx2.1-Cre DGK$\zeta^{fl/fl}$ mice (n = 3-6 mice/group for PBS challenged mice, n = 5-8 mice/group for papain challenged mice). (**D**)**,** Total cell numbers of eosinophils (gated on $CD45.2^+Ly6G^-CD11c^-$Siglec F^+ live singlets) and ILC2s (gated on $CD45^+Lin^-CD90.2^+ST2^+$ live singlets) in the BAL and lungs of Pirt-Cre and Pirt-Cre DGK$\zeta^{fl/fl}$ or (**E**) Myh11-Cre, DGK$\zeta^{fl/fl}$, and Myh11-Cre DGK$\zeta^{fl/fl}$ mice intranasally challenged with PBS or papain for 5 days (n = 3-5 mice/group). Data are pooled from at least 2 independent experiments (A-C) or representative of 1 independent experiment (D and E). Data are represented as mean ±

SEM; NS = not significant [two-sided unpaired Student's t-test, (A) through (D); One way ANOVA with Tukey's post-test, (E)].

CHAPTER 4: DISCUSSION

Overview

This thesis aimed to further our understanding of how TCR-driven signal transduction instructs Th2 differentiation of naïve $CD4^+$ T cells. The notion that strong TCR signaling blocks Th2 differentiation has existed since the late 1990's, but the majority of the previous studies demonstrating this phenomenon were carried out *in vitro* using TCR transgenic T cells with single specificities and by modulating TCR signaling either through altering TCR:peptide-MHC affinity or cognate peptide concentration. It was unclear whether selective enhancement of specific TCR-mediated signal transduction pathways could suppress Th2 differentiation *in vitro* and block Th2 inflammation *in vivo* in a polyclonal setting. Through genetic deletion of DGKζ, a negative regulator of TCR signaling, we were able to demonstrate that enhancement of DAG-mediated signaling could attenuate Th2 differentiation *in vitro.* Moreover, we were able to show that this effect translated to protection against a Th2-mediated disease as mice lacking DGKζ exhibited decreased type 2 airway inflammation in a mouse model of allergic asthma. Further investigation of signaling pathways uncovered that the selective enhancement of ERK signaling downstream of DAG was sufficient to inhibit Th2 differentiation and protect from Th2-mediated airway inflammation *in vivo*. This thesis is the first to show that the specific enhancement of DAG signaling downstream of the TCR is sufficient to attenuate Th2 differentiation and protect from Th2-mediated disease in an ERK-dependent manner.

Additionally, through our studies, we uncovered that DGKζ plays an additional role in regulating smooth muscle contraction and allergen-induced AHR independently of its effects on airway inflammation. We observed that in addition to blunted type 2

inflammation, mice lacking DGKζ were almost completely resistant to airway hyperresponsiveness (AHR) *in vivo* in a mouse model of allergic asthma. A more rigorous analysis revealed that the mechanisms by which DGKζ deficiency protected against allergic asthma was more complex than originally hypothesized. Surprisingly, we found that the mechanisms by which DGKζ protected against airway inflammation and AHR were separable. Conditional deletion of DGKζ in T cells led to decreased type 2 airway inflammation with no attenuation of AHR. In contrast, conditional deletion of DGKζ in airway smooth muscle cells led to diminished AHR with no attenuation of airway inflammation. Furthermore, we found that loss of DGKζ diminished methacholine-induced tracheal smooth muscle contractile force generation, thus demonstrating that DGKζ directly regulates smooth muscle contraction in a cell-intrinsic and inflammation-independent manner. Our findings reveal that the inflammatory and AHR components of asthma are not as interdependent as generally believed and that DGKζ simultaneously control both processes through distinct mechanisms.

Finally, we also demonstrate a novel role for DGKζ in regulating protease allergen-mediated type 2 airway inflammation through the actions of DGKζ on the non-hematopoietic compartment. We find that genetic ablation of DGKζ was sufficient to protect from papain-induced airway inflammation. However, the loss of DGKζ in the hematopoietic compartment was not able to protect from airway inflammation following papain treatment. Further analysis revealed that protection from papain in the absence of DGKζ might potentially be due to an impairment in IL-33 production/release in response to papain. However, further studies are needed to fully understand the mechanism by which DGKζ influences protease allergen-driven type 2 immune responses in the lung.

Collectively, this thesis offers many novel findings and insights into the cell signals that drive Th2 differentiation and allergic airway inflammation. Furthermore, we demonstrate that enhancement of DAG signaling does not always lead to immune activation, as generally believed. Additionally, this thesis highlights that DGKζ can also play an immunomodulatory role in the non-hematopoietic compartment to regulate type 2 immune-mediated disease. The following sections discuss the implications of our findings and further questions that remain from this work.

DAG-driven signal transduction blocks Th2 development

Several reports have indicated that strong and prolonged TCR signaling blocks the differentiation of naïve $CD4^+$ T cells into the Th2 lineage. By inhibiting various MAPK pathways using small molecule inhibitors, the degree of TCR-mediated ERK activation was determined to be a key regulator of this process (*60*). Through genetic deletion of a negative regulator of DAG signaling or through knock-in of a hypersensitive ERK mutant, we were able to demonstrate that selective enhancement of TCR-mediated ERK signaling was sufficient to attenuate Th2 differentiation.

Strong TCR-mediated ERK signaling is thought to attenuate Th2 differentiation by independently blocking early GATA3 induction and IL-2R-mediated STAT5 signaling to impair early IL-4 transcription. However, the molecular nature by which TCR-mediated ERK activity blocks these processes is not well understood. Jorritsma et al. have suggested that strong TCR-mediated ERK signaling results in preferential skewing of Th differentiation away from Th2 differentiation by altering ratio of AP-1 complexes from predominantly JunB-JunB homodimers to JunB-cFos heterodimers following TCR activation (*58*). However, Yamane et al. reported that activation of 5C.C7 TCR transgenic T cells with low and high concentrations of pPCC (conditions that favor Th2

and Th1 differentiation respectively) induced comparable levels of nuclear c-Fos expression (*60*). Furthermore, activation of 5C.C7 T cells with high peptide concentrations elicited higher expression of nuclear JunB expression at 24 hours following TCR activation, thus arguing against altered ratios of AP-1 complexes as a driver of differential early IL-4 production (*60*).

Additionally, further experiments will be needed to determine if the kinase activity of ERK directly dampens IL-2R-mediated STAT5 phosphorylation. However, the role of ERK in regulating STAT5 activity is controversial. Previous studies have shown that ERK1/2 physically complexes with STAT5a in Chinese hamster ovary cells and that the kinase activity of ERK1/2 is required for enhancement of STAT5a transcriptional activity through phosphorylation of Ser780 on STAT5a (*221*). It is possible that the kinase activity of ERK could also work upstream of STAT5 to dampen rather than promote IL-2R-mediated STAT5 phosphorylation. Interestingly, TCR activation of naïve $CD4^+$ T cells has been shown to transiently inhibit phosphorylation and activation of multiple components of the IL-4R signaling pathway, including IL-4Rα, Jak1, Jak3, STAT6, and IRS-2, following ligation with IL-4 (*222*). TCR-mediated suppression of cytokine receptor signaling was not limited to IL-4R signaling but also included IL-2R-mediated STAT5 activation, IL-6R-mediated signaling, and IFNα-mediated signaling (*222*). Importantly, PLCγ-dependent calcineurin and Ras-MAPK pathways were both involved in TCR-mediated suppression of cytokine signaling in a manner that was independent of new protein synthesis (*222*). Interestingly, inhibition of ERK activation using U0126 could restore IL-4-mediated phosphorylation of STAT6 and IRS2 following TCR ligation, suggesting that ERK activity is critical for regulating cytokine receptor responsiveness in response to TCR stimulation (*222*). Furthermore, cytokine receptor responsiveness

returned ~12 hours post TCR activation and became resistant to inhibition by subsequent TCR ligation (*222*). However, inhibition of Th2 differentiation by strong and prolonged TCR-mediated ERK signaling is likely not mediated by alterations in IL-4R responsiveness because Th2 differentiation was restored in DGKζ KO T cells following addition of exogenous IL-4. However, it is possible that the strength of TCR-mediated ERK signaling could delay the timing in which responsiveness of other cytokine receptors (e.g. IL-2 receptor) is restored in order to drive preferential Th differentiation toward a particular Th subset.

Moreover, the mechanism by which weak TCR-mediated signaling elicits early GATA3 induction is also not very well understood. Previous work has shown that NF-κB p50 subunit is required for GATA3 expression at late timepoints following TCR activation under Th2 polarization conditions (*223*). However, the requirement for the NF-κB p50 subunit and other members of the NK-κB for early GATA3 expression and IL-4 transcription is unknown. Furthermore, the molecular mechanism by which strong TCR-mediated ERK signaling blocks GATA3 mRNA induction is unknown. Our findings reveal that augmenting TCR-mediated DAG signaling by genetic deletion of DGKζ is sufficient to impair Th2 differentiation. The loss of DGKζ in T cells enhances both the NF-κB and ERK signal transduction pathways following TCR activation. However, inhibition of ERK signaling using U0126 was sufficient to restore Th2 differentiation in DGKζ, suggesting that enhancement of TCR-mediated ERK signaling dominantly controls Th1 versus Th2 fate decision in DGKζ KO T cells. However, we do not know whether enhancement of NF-κB signaling also contributes to this process. Analysis of the role of the NF-κB pathway on Th differentiation has been hampered by the lack of mice with mutations in components of the NF-κB pathway that lead to inducible augmentation of NF-κB

signaling following TCR stimulation rather than constitutive activation of the pathway in the absence of TCR signaling. However, further experiments will be needed using inhibitors of the PKC-NF-κB pathway to determine if augmentation of the NF-κB pathway also contributes to influencing Th2 differentiation in DGKζ KO T cells.

Regulation of airway hyperresponsiveness by DGK independent of eosinophilic airway inflammation

Through our studies on the role of DGKζ on Th2 differentiation and allergen-induced airway inflammation, we uncovered that DGKζ also plays a critical role in airway smooth muscle contraction and allergen-induced airway hyperresponsiveness. Allergic asthma is a chronic airway inflammatory disease characterized by eosinophilic airway inflammation and reversible airflow obstruction mediated in part by hypercontractility of bronchial smooth muscle cells. The current paradigm argues that airway inflammation drives the non-immune abnormalities observed in asthma. However, several reports have hinted that the role of eosinophilic inflammation on the development of airway hyperresponsiveness is more complex than originally thought. Systemic immunization with OVA and aluminum hydroxide followed by intranasal challenge with OVA elicits AHR in the airways of challenged mice in an IgE/mast-cell independent manner (*224, 225*). Cohn et al. demonstrated that transfer of IL-4 KO OVA-specific Th2 cells into naïve WT recipient mice was sufficient to induce AHR despite markedly reduced airway eosinophilia in comparison to transfer of WT OVA-specific Th2 cells, thus indicating that IL-4 and eosinophils were not essential for the induction of OVA-induced AHR (*160*). Furthermore, Corry et al. demonstrated that administration of neutralizing antibodies against IL-4 or IL-5 during the effector phase had no impact on AHR induction despite almost complete abrogation in OVA-induced eosinophilia, thus arguing that eosinophils

are dispensable for allergen-induced AHR (*163*). These results likely reflect the important role of IL-13 in OVA-induced AHR given that IL-13 KO mice exhibited significantly impaired OVA-induced AHR despite intact OVA-induced airway eosinophilia (*226*). However, it is important to note that that all of the previous studies were performed in mice generated on the BALB/c background and that species-specific differences in the mechanisms that regulate allergen-induced AHR do exist. In support of this, Walsh et al. reported that eosinophil-deficient ΔdblGATA1 mice on the C57BL/6 but not the BALB/c background displayed diminution in OVA-induced AHR, thus highlighting that the factors that control OVA-induced airway disease can be different depending on the species (*227*). Nevertheless, these results suggest that inflammation is important in initiating the development of allergen-induced airway hyperresponsiveness.

Our findings add to this complexity by demonstrating that OVA-induced AHR can develop normally even in the presence of diminished eosinophilia and Th2 cytokine release in the airways. While the mechanism by which OVA-induced AHR is preserved in hematopoietic-specific DGKζ KO mice might be due to retained IL-13 induction in the BAL, the factors that explain why OVA-induced AHR remains intact despite attenuated OVA-induced eosinophilic airway inflammation in DGKζ KO bone marrow chimeras and ERK^{SEM} mice are not entirely clear. One potential explanation is that the induction of allergen-induced AHR is simply less sensitive to the reduction in type 2 cytokines than the process of allergen-induced airway eosinophilia. However, an alternative explanation could be that other T cell-derived factors, such as IL-3, could contribute to the induction of OVA-induced AHR given that IL-3 KO mice display significantly attenuated OVA-induced AHR despite intact OVA-induced airway eosinophilia following OVA challenge (*228*).

Regardless of the inflammatory factors important in initiating OVA-induced AHR, our studies indicate that DGKζ is essential for the development of AHR independently of its role in regulating airway inflammation. However, the mechanism by which the loss of DGKζ protects from allergen-induced AHR is not entirely clear. Previous studies have shown that a rise in intracellular Ca^{2+} initiated by PLC critically mediates the crossbridge cycling of actin and myosin to drive smooth muscle contraction and DAG maintains contraction by inhibiting a negative regulator of the crossbridge cycling (*191-193*). Consequently, one might expect that the lack of DGKζ would exacerbate bronchoconstriction. However, the diminution of smooth muscle contraction by the loss of DGKζ suggests that other biological mechanisms are involved. Indeed, preliminary data from our collaborators indicate that inhibition of DGK kinase activity in human ASM cells significantly impairs IP_3 generation and Ca^{2+} flux following activation with methacholine, thus resulting in decreased phosphorylation of myosin light chain and decreased ASM contraction (data not shown). Exactly how the loss of DGKζ activity leads to impaired methacholine-induced Ca^{2+}responses is not fully understood but could involve enhanced negative feedback of PKC isoforms on PLC activity, similar to what is seen during FcεRI-mediated activation of DGKζ KO mast cells (*132*).

Potential caveats of our results are that the deletion efficiencies of DGKζ in the hematopoietic compartment in Vav-Cre DGKζ$^{fl/fl}$ mice, in the T cell compartment in CD4-Cre DGKζ$^{fl/fl}$ mice, in airway smooth muscle cells in Myh11-Cre DGKζ$^{fl/fl}$ mice, and in dorsal root ganglion sensory neurons in the Pirt-Cre DGKζ$^{fl/fl}$ mice are unknown. Therefore, we cannot fully rule out the possibility that the phenotypes that we see in our conditional DGKζ KO mice is due to DGKζ not being effectively deleted in these cell

types. However, confirmation of the deletion efficiencies of DGKζ using our various Cre systems are currently ongoing.

Importantly, our findings demonstrate that OVA-induced eosinophilic airway inflammation and AHR are separable processes and that DGKζ is a common regulator of both processes. Furthermore, these results suggest that while the initiation of the molecular processes that lead to allergen-induced AHR might be inflammation-dependent, the establishment of AHR is largely independent of eosinophilic inflammation. In support of this, An et al. demonstrated that primary human airway smooth muscle (ASM) cells isolated from asthmatic donors displayed significantly enhanced baseline cell traction forces and augmented contraction in response to M_3-muscarinic receptor and H_1-histamine receptor activation than non-asthmatic ASM cells (*229*). These differences persisted even upon subsequent passaging of asthmatic ASM cells, suggesting that the development of asthma generates cell-intrinsic changes in airway smooth muscle cells to potentiate hypercontractile responses in an inflammation-independent manner (*229*). Moreover, recent clinical trials have shown that while inhibition of type 2 cytokine signaling is efficacious in reducing eosinophilia and decreasing the frequency of asthma exacerbations in asthmatic patients, these approaches failed to alter impaired baseline lung function and histamine-induced airway responses, thus implying that suppressing inflammation is not sufficient to reverse AHR and airway smooth muscle dysfunction in asthma (*194, 195*). While the reasons for this are not clear, it is tempting to speculate that the inflammation-independent nature of airway smooth muscle dysfunction seen in asthmatic patients might be due to epigenetic changes that occur in ASMs during the development of asthma that then drive hyperactive responses to subsequent contractile triggers. While the current therapeutics

strategies have been focused on targeting the biological pathways that promote airway inflammation, our data suggests that designing strategies to prevent and reverse airway smooth muscle dysfunction, which is the core abnormality seen in both Th2-high and Th2-low asthmatics, is equally as important. DGKζ potentially represents a novel therapeutic target to promote the prevention and resolution of asthma by suppressing both the immune and non-immune responses that drive the disease.

Regulation of protease-mediated airway inflammation by DGKζ

Through our studies, we identified that DGKζ also regulates type 2 airway inflammation mediated by protease allergens, such as papain. However, the means by which DGKζ protects from papain-induced airway inflammation is distinct in comparison to the role of DGKζ in OVA-induced airway inflammation because DGKζ deficiency is required in the non-hematopoietic compartment rather than the hematopoietic/T cell compartments to elicit protection. The mechanism by which DGKζ controls papain-induced airway inflammation is not entirely clear but potentially revolves around the regulation of IL-33, which is necessary for driving inflammation in the airways following papain challenge. Surprisingly, DGKζ deficiency in alveolar type II cells, which comprise the predominant source of pulmonary IL-33, was not sufficient to provide protection from airway inflammation in response to papain. These data suggest that either DGKζ regulates IL-33 production/release from alternative cellular sources to drive papain-induced inflammation in the lung or that DGKζ indirectly regulates IL-33 production/release from alveolar type II cells through another non-hematopoietic cell type that is involved in sensing protease allergens and promoting IL-33 release from alveolar type II cells. However, potential caveats of these results are that the deletion efficiencies of DGKζ in the hematopoietic compartment in Vav-Cre DGKζ$^{fl/fl}$ mice, in

alveolar type II cells in Nkx2.1-Cre DGKζ$^{fl/fl}$ mice, in airway smooth muscle cells in Myh11-Cre DGKζ$^{fl/fl}$ mice, and in dorsal root ganglion sensory neurons in the Pirt-Cre DGKζ$^{fl/fl}$ mice are unknown. Therefore, we cannot fully rule out the possibility that the phenotypes that we see in our conditional DGKζ KO mice is due to DGKζ not being effectively deleted in these cell types. However, confirmation of the deletion efficiencies of DGKζ using our various Cre systems are currently ongoing.

While alveolar type II cells constitute a major source of IL-33, other lung cells, such as CD31^{+} endothelial cells, PDGFRα-GFP^{+} adventitial stromal cells, EpCAM^{-}CD31^{-} Sca1^{-} stromal cells, and certain subsets of myeloid cells, also express IL-33 that could be functionally relevant in regulating IL-33-mediated responses in the airways (*220*). Indeed, Dahlgren et al. have shown that ILC2s reside around adventitial stromal cells located in adventitial cuff structures present in the lung (*220*). Furthermore, adventitial stromal cells express both IL-33 and TSLP to help support ILC2 maintenance in the lung (*220*). In turn, ILC2-mediated production of IL-13 drives adventitial stromal cell expansion and augments IL-33 expression (*220*). Depletion of adventitial stromal cells during infection with *Nippostrongylus brasiliensis* impairs ILC2 and Th2 cell accumulation and function in the lung partially in an IL-33-dependent manner, thus suggesting that this adventitial stromal cell-ILC2 circuit is functionally relevant for regulating ILC2 responses at homeostasis and during inflammation (*220*). However, while the authors showed that ILC2s remain localized around adventitial cuff structures following papain challenge, whether depletion of adventitial stromal cells or loss of adventitial stromal cell-derived IL-33 impairs ILC2 responses during papain-mediated lung inflammation was not addressed (*220*). Furthermore, the Cre-expressing mice that we utilized for our studies (Nkx2.1-Cre, Pirt-Cre, and Myh11-Cre) likely do not display

Cre activity in adventitial stromal cells and would not delete DGKζ in these cells upon crossing these Cre-expressing mice to DGKζ$^{fl/fl}$ mice. Further experiments involving the generation of Gli-creERT2 DGKζ$^{fl/fl}$ and Gli-creERT2 IL-33$^{fl/fl}$ mice to conditionally delete DGKζ and IL-33 in adventitial stromal cells will be required to determine the role of DGKζ and IL-33 in regulating papain-induced airway inflammation by adventitial stromal cells.

How the regulation of DAG-mediated signaling by DGKζ influences protease-mediated airway inflammation remains a mystery. However, DAG-driven signal transduction pathways have been previously implicated in the regulation of tissue-derived cytokines involved in type 2 inflammation. Deletion of IKKβ, a catalytic subunit of the IKK complex required for activation of the canonical NF-κB pathway, in intestinal epithelial cells significantly diminished transcription of TSLP and impaired the induction of protective type 2 immunity to *Trichuris muris* infection (*230*). In contrast, deletion of IKKα, a catalytic subunit of the IKK complex involved in activation of both the canonical and non-canonical NF-κB pathways, enhanced TSLP induction and impaired innate immunity to *Citrobacter rodentium* infection by impairing IL-22 secretion from ILC3s (*231*). While enhancing activation of the canonical NF-κB pathway by augmenting DAG signaling would be expected to enhance TSLP transcription, it is possible that prolonged DAG signaling can also inhibit the NF-κB pathway by downregulating components of the canonical NF-κB pathway as seen in human colonic epithelial cells stimulated with PMA, a DAG analogue resistant to DGK activity (*232*). Whether DAG signal transduction pathways also influence IL-33 regulation is unknown. However, IL-33 has been shown to upregulate DGKζ in cardiomyocytes but the functional relevance of this pathway and whether this also occurs in airway cells exposed to IL-33 are not clear (*85*).

It is also possible that DGKζ regulates the production and release of IL-33 by regulating cell-death pathways. Protease allergens, such as papain, are thought to promote cell damage to IL-33-expressing cells residing in the lung epithelium, resulting in necrotic cell death and release of constitutively-produced IL-33 that is stored in the nucleus of these cells (*233*). However, direct evidence that necrotic cell death drives the release of IL-33 in response to protease allergens is lacking. Interestingly, we found that RIPK3 KO mice, which cannot undergo programmed necroptosis, displayed intact papain-induced ILC2 accumulation and eosinophilia in the lungs, thus ruling out a role for programmed necroptotic cell death in IL-33 release (data not shown).

Additionally, it is also possible that the loss of DGKζ might promote protection from papain-induced airway inflammation by altering the type of cell death that IL-33-expressing cells undergo. Zhang et al. found that NLRP3 activating signals induce the production of DAG to promote the recruitment of DAG effector molecule PKD (*234*). The activity of PKD was critical for activation of NLRP3 inflammasome to cleave pro-caspase 1 to caspase-1 (*234*). In addition, we know that caspase-1 plays a regulatory role in IL-33 biology by cleaving biologically active full-length IL-33 into biological inactive fragments during inflammasome activation (*235*). In line with these results, caspase-1KO and NLRP3 KO mice have been shown to display enhanced airway eosinophilia in response to the protease allergen, HDM (*236*). The enhanced eosinophilic airway inflammation was associated with increased IL-33 protein levels in lung at baseline and following HDM treatment in caspase-1 KO mice, thus implying that NLRP3-mediated activation of capase-1 is critical for dampening IL-33-mediated airway inflammation following HDM challenge (*236*). However, we observed that DGKζ KO bone marrow derived macrophages (BMDM) exhibited reduced NLRP3-mediated inflammasome

activation rather than enhanced activation (data not shown). Whether DGKζ deficiency also dampens inflammasome activation in IL-33-expressing cells is not known but, based on the BMDM data, we argue that the regulation of inflammasome activation to control papain-induced release of bioactive IL-33 is not likely to be the mechanism by which DGKζ protects from papain-induced airway inflammation. Further studies will need to be done to determine how DGKζ acts to promote protease-mediated type 2 airway inflammation in the non-hematopoietic compartment.

Broader implications of these pathways in sites outside of the lung

While these findings indicate that targeting DGKζ might be potentially beneficial for the treatment of diseases that involve aberrant type 2 inflammation and pathological smooth muscle function, DGKζ inhibition might also have detrimental effects in the lung or other tissue compartments that require activation of these functions to protect the host during infection or to maintain tissue homeostasis. For example, type 2 immune responses are critical in protecting from helminth infections (*237*). In addition, smooth muscle contraction in the gut is important for the expulsion of helminth parasites and impairments in intestinal smooth muscle contraction have been shown to increase worm burden (*238-240*). Therefore, the loss of DGKζ activity would be expected to impair rather than promote host responses against helminths. Interestingly, smooth muscle contraction is also important during childbirth (*241*). While global DGKζ KO female mice can give birth and have relatively normal litter sizes, smooth muscle-specific DGKζ KO females cannot give birth and die prior to delivery (unpublished results). These results suggest that DGKζ might play a critical role in promoting delivery during parturition but that compensatory factors also exist in regulating this process. It is tempting to speculate that DGKζ might actually play a broader role in non-homeostatic contractile responses,

such as those seen during AHR, intestinal smooth muscle-mediated helminth expulsion, and uterine smooth muscle-mediated delivery of progeny during childbirth.

Conclusions

The work presented in this thesis describes how regulation of TCR-mediated DAG signaling by DGKζ influences Th2 differentiation and Th2-mediated inflammation. Through these studies, DGKζ was uncovered to a play a broader role in regulating immune and non-immune responses associated with type 2 inflammation. Although Th2 differentiation has been studied extensively for decades, many questions still remain on the key requirements for instructing the generation of this lineage. Further studies will be needed to delineate the cellular and molecular pathways that are critical for the development and function of Th2 cells and utilize this knowledge to control type 2 immune responses in clinical settings.

APPENDIX

Material and Methods

Chapter 2

Mice

C57BL/6, B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrCrl (CD45.1^{+}), and C57BL/6-Tg(TcraTcrb)425Cbn/Crl (CD45.2^{+} WT OT-II) mice were purchased from The Jackson Laboratories or Charles River Laboratories. Generation of DGKζ knockout (KO), DGKζ floxed (DGKζ$^{fl/fl}$), and DGKα KO mice were described previously (*170, 242, 243*). CD45.1^{+}CD45.2^{+} WT OT-II mice were generated by crossing CD45.2^{+} WT OT-II to CD45.1^{+} WT mice. CD45.2^{+} DGKζ KO mice were crossed to CD45.2^{+} WT OT-II mice to generate CD45.2^{+} DGKζ KO OT-II mice. B6.Cg-*Commd10*$^{Tg(Vav1-icre)A2Kio}$/J (Vav-Cre) and Tg(Cd4-cre)1Cwi/BfluJ (CD4-Cre) mice were purchased from the Jackson Laboratories and crossed to DGKζ$^{fl/fl}$ mice to generate Vav-Cre DGKζ$^{fl/fl}$ and CD4-Cre DGKζ$^{fl/fl}$ mice, respectively. B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J (Myh11-Cre) mice were purchased from The Jackson Laboratories and crossed to DGKζ$^{fl/fl}$ mice to generate Myh11-Cre DGKζ$^{fl/fl}$ mice. Pirt-Cre mice were crossed to DGKζ$^{fl/fl}$ mice to generate Pirt-Cre DGKζ$^{fl/fl}$ mice (*244*). IL-4 KO and STAT6 KO mice were purchased from The Jackson Laboratories. Sevenmaker (ERK^{SEM}) mice were provided by Laurence Samuelson from the National Institutes of Health and were originally developed by Stephen Hedrick from the University of California, San Diego (*121*). Unless otherwise specified, all mice were 7-12 weeks old at the time of use, were housed in pathogen-free conditions and 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 non-viable cells. Intracellular cytokine staining was performed with the BD Cytofix/Cytoperm Kit according to the manufacturer's protocol. Flow cytometry was performed with a LSR II or FACSCanto flow cytometer (BD Biosciences). For cell sorting, freshly isolated splenocytes were stained with FITC-conjugated anti-CD4 (GK1.5, eBioscience) and anti-FITC MACS beads (Miltenyi Biotec) and, subsequently, passed through MACS columns (Miltenyi Biotec) according to the manufacturer's protocol to enrich for $CD4^+$ T cells. MACS-enriched $CD4^+$ T cells were subjected to cell surface staining prior to cell sorting. FACS was performed with a FACSAria cell sorter (BD Biosciences). Data were analyzed and plotted with FlowJo software (TreeStar). For flow cytometry, we utilized the following fluorochrome-conjugated antibodies: BV605 anti-CD11b (M1/70, Biolegend), APC anti-CD11c (N418, Tonbo Biosciences), unconjugated anti-CD16/CD32 (2.4G2, BD Biosciences), PE Cy-7 anti-CD19 (eBio1D3, eBioscience), APC eF780 anti-CD25 (PC61.5, eBioscience), PE Cy7 anti-CD3 (17A2, Biolegend), FITC anti-mouse CD4 (RM4-5, eBioscience; GK1.5, eBioscience), PE anti-CD44 (IM7, eBioscience), APC Cy7 anti-CD45.1 (A20, BD Pharmingen), AF700 anti-CD45.2 (104, eBioscience), PE Cy7 anti-B220 (RA3-6B2, Biolegend), Pacific Blue anti-mouse CD45RB (C363-16A, Biolegend), BV421 anti-F4/80 (BM8, Biolegend), PerCP eF710 anti-GATA3 (TWAJ, eBioscience), PE Cy7 anti-IFNγ (XMG1.2, Biolegend), PerCP eF710 anti-IL-13

(eBio13A, eBioscience), BV711 anti-IL4 (11B11, Biolegend), APC anti-IL-5 (TRFK5, Biolegend), PerCP Cy5.5 anti-Ly6G (HK1.4, Biolegend), PE anti-Siglec F (E50-2440, BD Biosciences), FITC anti-Vα2 (B20.1, BD Pharmingen), and PE anti-Vβ5.1, 5.2 (MR9-4, BD Pharmingen). For western blots, we utilized the following antibodies: anti-pMLC (Ser 19) (3671, Cell Signaling Technologies), anti-pAkt (Ser473) (9271, Cell Signaling Technologies), anti-MYPT1 (Thr696) (5163, Cell Signaling Technologies), and anti-α-Tubulin (DM1A) (3873, Cell Signaling Technologies).

CD4$^+$ T cell differentiation assays

MACS-enriched CD4$^+$ T cells were sorted for naïve T cells (CD4$^+$CD45RB$^+$CD25$^-$CD44^{lo}). Sorted naïve CD4$^+$ T cells were plated (30,000 cells/well) in tissue culture media [MEMα (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-Glutamine, 12.5 mM HEPES (Life Technologies), 22.9 µM β-mercaptoethanol (Bio-Rad)] and activated with soluble anti-CD3 (10 µg/ml; 145-2C11; BD Pharmingen) and soluble anti-CD28 (3 µg/ml; 37.51; BD Pharmingen) in the presence of irradiated (5000 rads) CD4-depleted splenocytes (150,000 cells/well). In some experiments, sorted naïve CD4$^+$ T cells were pretreated with either DMSO or the MEK1/2 inhibitor, U0126 (0.4 µM, Cell Signaling), for 30 min prior to TCR activation. In other experiments, sorted naïve CD4$^+$ T cells were activated under T_H2 conditions [anti-IL-12 (10 µg/ml; C15.6; Biolegend), anti-IFNγ (10 µg/ml; XMG1.2; Biolegend), IL-4 (10 ng/ml; Peprotech)]. On day 5 post-activation, T cells were restimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of brefeldin A (5 µg/ml; Cell Signaling) for 5 hours. Following 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 (i.p.) injection of 10 µg OVA (Sigma-Aldrich) and 2.25 mg of Imject™ Alum (ThermoFisher Scientific), which contains aluminum hydroxide and magnesium hydroxide with inactive stabilizers, in 200 µl PBS on day 0 and day 14. Following sensitization, mice were intranasally (i.n.) challenged with 10 µg OVA in 20 µl PBS on days 28-30. Airway responses to methacholine were measured approximately 16 hours after the last i.n. challenge. Mice were anesthetized with an i.p injection of ketamine (87.5 mg/kg, Hospira Inc.) and xylazene (12.5 mg/kg, Akorn Inc.), cannulated via the trachea, and attached to a lung mechanics analyzer (FlexiVent, SCIREQ Inc.). Airway responses were measured following the administration of increasing doses of methacholine through the use of a nebulizer as we previously described (*245*).

Following measurement of airway mechanics, the lung was flushed 3 times with 0.7 ml of MACS buffer [PBS with 5% bovine serum albumin (BSA; Fisher Bioreagents) and 2 mM EDTA (Invitrogen)] containing complete Mini, EDTA-free protease inhibitor cocktail (Roche). The BAL fluid was centrifuged at 6797 x g at 4° C for 2 min in a microcentrifuge. BAL supernatant was collected and stored at -80° C prior to 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 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 (ThermoFisher 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 (*246-248*). For analysis by flow cytometry, BAL cells were stained with antibodies against cell surface markers and identified by the following gating schemes: eosinophils ($Ly6G^-CD11c^-Siglec\ F^+$), neutrophils ($CD11b^+Ly6G^+$), macrophages ($Ly6G^-F4/80^+$), and lymphocytes ($CD3^+$ or $CD19^+$ or $B220^+$). All cells were pregated on $CD45^+$ live cells.

Following BAL fluid collection, murine lungs were injected with 10% buffered formalin (Fisher Healthcare) though 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 (*249, 250*). 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.

Adoptive transfer of WT and DGKζ KO OT-II

MACS-enriched $CD4^+$ T cells isolated from spleens and lymph nodes of $CD45.1^+CD45.2^+$ WT OT-II TCR transgenic mice and $CD45.2^+$ DGKζ KO OT-II TCR transgenic mice were sorted for naïve T cells ($CD4^+CD45RB^+CD25^-CD44^{lo}$). Sorted naïve $CD45.1^+CD45.2^+$ WT OT-II and $CD45.2^+$ DGKζ KO OT-II cells were transferred intravenously into $CD45.1^+$ WT hosts at a 1:1 ratio (400,000 WT OT-II:400,000 DGKζ OT-II). One day post-transfer, mice were subjected to OVA sensitization. Five days after

the second i.p. OVA/Alum immunization, splenic $CD4^+$ T cells were isolated via MACS-enrichment and restimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of brefeldin A (5 µg/ml; Cell Signaling) for 5 hours. Following restimulation, activated $CD4^+$ T cells were washed twice with PBS and prepared for cell surface and intracellular cytokine staining.

Generation of bone marrow chimeric mice

Donor bone marrow (BM) cells from $CD45.1^+$ WT or $CD45.2^+$ DGKζ KO donor mice were transferred (3-4 x 10^6 BM cells) intravenously into lethally irradiated (1100 rads) $CD45.1^+$ WT or $CD45.2^+$ DGKζ KO hosts. 10 weeks post-bone marrow transfer, mice were subjected to OVA sensitization and challenge.

Murine tracheal ring contraction

Airway contractility in isolated murine tracheal rings was determined using multi-wire myograph (ADInstruments) as described previously (*251*). Tracheal rings were mounted on the myograph, bathed in Krebs-Henseleit (K-H) buffer (pH 7.4, 37°C and 95% O_2/5% CO_2), 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 was performed using Chart 7 software.

Airway contraction measurements in human precision cut lung slices (PCLS)

Human PCLS preparation and bronchoconstriction experiments were performed as previously described (*252*). In brief, healthy whole lungs from non-asthmatic 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 μm (Precisionary Instruments VF300 Vibratome). Lung slices were rested in Ham's F12 medium in 12-well tissue culture plates at 37° C for two days post-isolation and washed three times with fresh media to rid airways of agarose on day 1 and 2 during the resting period. Next, slices were incubated overnight (18 hours) with DMSO or DGK inhibitor, R59949 (Sigma-Aldrich). Following 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, 40x magnification) connected to a live video feed (Evolution QEi 32-0074A-130). Suitable airways on slices were chosen based on 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 lumenal area were measured using Image-Pro Plus software (version 6.0, Media Cybernetics) as previously described (*252*). Area under curve (AUC) was calculated from the dose-response curves generated.

Human airway smooth muscle (HASM) isolation, culture, and immunoblotting

Primary HASM were generated from non-diseased tracheas received from the National Disease Research Interchange (Philadelphia, PA). HASM culture was performed as previously described (*253*). 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 1-5 passages prior to use.

For immunoblotting analysis, HASM cells were grown to confluence in 12 well tissue culture plates. Cells were serum starved for 24 hours prior to stimulation. Cells

were incubated overnight (18 hours) with DMSO or R59949. Following overnight incubation, cells were stimulated with 10 μM carbachol for 10 min. After stimulation, cells were fixed with perchloric acid, scraped, pelleted, and lysed in RIPA buffer. Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting for phosphorylated myosin light chain (pMLC), phospho-Akt (pAkt), and phosphorylated myosin light chain phosphatase (pMYPT1) was performed using tubulin as a loading control. The antibodies used for immunoblotting are listed in Supplemental Table 2.

***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 10 mg/kg of DGK inhibitor, R59949 (Sigma-Aldrich), in 50% polyethylene glycol (PEG) 400 (Sigma-Aldrich) solution beginning immediately after the second i.p. OVA/Alum immunization and ending before the last i.n. OVA challenge (late sensitization and challenge phases) or beginning immediately before the first i.n. OVA challenge and ending before the last i.n. OVA challenge (challenge phase only).

Statistical analysis

P values were calculated using unpaired or paired two-tailed Student's t-test, one-way ANOVA with Tukey's post-test or Dunn's post-test analysis, or two-way ANOVA with Bonferroni's post-test analysis as indicated in the figure legends. Graphical representation and statistical analysis of data were performed with Prism 6 software (GraphPad).

Chapter 3

Mice

C57BL/6 were purchased from Charles River Laboratories. Generation of DGKζ knockout (KO) and DGKζ floxed (DGKζ$^{fl/fl}$) were described previously (*170, 242, 243*). B6.Cg-*Commd10*$^{Tg(Vav1\text{-}icre)A2Kio}$/J (Vav-Cre) were purchased from the Jackson Laboratories and crossed to DGKζ$^{fl/fl}$ mice to generate Vav-Cre DGKζ$^{fl/fl}$. C57BL/6J-Tg(Nkx2-1-cre)2Sand/J (Nkx2.1-Cre) mice were purchased from The Jackson Laboratories and crossed to DGKζ$^{fl/fl}$ mice to generate Nkx2.1-Cre DGKζ$^{fl/fl}$ mice. B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J (Myh11-Cre) mice were purchased from The Jackson Laboratories and crossed to DGKζ$^{fl/fl}$ mice to generate Myh11-Cre DGKζ$^{fl/fl}$ mice. Pirt-Cre mice were crossed to DGKζ$^{fl/fl}$ mice to generate Pirt-Cre DGKζ$^{fl/fl}$ mice (*244*). TSLPR KO mice were provided by Warren Leonard from the National Institutes of Health (*254*). *Il1rl1*$^{-/-}$ mice (ST2 KO) were provided by Edward Behrens from the University of Pennsylvania and were originally developed by Andrew McKenzie at the University of Cambridge (*199*). Unless otherwise specified, all mice were 7-12 weeks old at the time of use, were housed in pathogen-free conditions and 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 non-viable cells. Intracellular cytokine

staining was performed with the BD Cytofix/Cytoperm Kit according to the manufacturer's protocol. Intracellular transcription factor staining was performed with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) according to manufacturer's protocol. Flow cytometry was performed with a LSR II or FACSCanto flow cytometer (BD Biosciences). For cell sorting, freshly isolated cells from lung tissue were subjected to cell surface staining prior to cell sorting. FACS was performed with a FACSAria cell sorter (BD Biosciences). Data were analyzed and plotted with FlowJo software (TreeStar). For flow cytometry, we utilized the following fluorochrome-conjugated antibodies: PerCP Cy5.5 anti-Ly6G (HK1.4, Biolegend), APC anti-CD11c (N418, Tonbo Biosciences), AF700 anti-CD45.2 (104, eBioscience), PE anti-Siglec F (E50-2440, BD Biosciences), BV421 anti-F4/80 (BM8, Biolegend), BV605 anti-CD11b (M1/70, Biolegend), PE anti-CD25 (PC61, Biolegend), PE Cy7 anti-CD3 (17A2, Biolegend), PE Cy7 anti-CD5 (53-7.3, eBioscience), PE Cy7 anti-mouse CD11b (M1/70, Biolegend), PE Cy7 anti-mouse CD11c (N418, Biolegend), PE Cy-7 anti-CD19 (eBio1D3, eBioscience), PE Cy7 anti-B220 (RA3-6B2, Biolegend), PE Cy7 anti-NK1.1 (PK136, Biolegend), PE Cy7 anti-CD49b (DX5, Biolegend), APC eF780 anti-CD90.2 (53-2.1, eBioscience), PE anti-CD127 (A7R34, Tonbo Biosciences), biotinylated anti-ST2 (DJ8, MD Biosciences), PE Cy7 anti-IFNγ (XMG1.2, Biolegend), PerCP eF710 anti-IL-13 (eBio13A, eBioscience), BV711 anti-IL4 (11B11, Biolegend), APC anti-IL-5 (TRFK5, Biolegend), PerCP Cy5.5 anti-Ly6G (HK1.4, Biolegend), unconjugated anti-CD16/CD32 (2.4G2, BD Biosciences). PerCP eF710 anti-GATA3 (TWAJ, eBioscience), PerCP eF710 anti-Ki67 (SolA15, eBioscience), and BV421 Streptavidin (405225).

Papain-induced allergic airway inflammation model

For induction of acute type 2 airway inflammation, mice were intranasally (i.n.) challenged with either 20 µl PBS alone or 30 µg papain (Calbiochem) in 20 µl PBS daily for 5 days, and euthanized on day 6 for analysis.

Intranasal treatment with IL-33

For induction of acute IL-33-mediated inflammation., mice were intranasally (i.n.) challenged with either 20 µl PBS alone or 200 ng IL-33 (Peprotech) in 20 µl PBS daily for 3 days, and euthanized on day 4 for analysis.

Isolation of cells from the BAL and lung tissue

Following euthanasia, mice were cannulated via the trachea and the lung was flushed once with 0.7 ml of MACS buffer [PBS with 5% bovine serum albumin (BSA; Fisher Bioreagents) and 2 mM EDTA (Invitrogen)] containing complete Mini, EDTA-free protease inhibitor cocktail (Roche). The BAL fluid was centrifuged at 6797 x g at 4° C for 2 min in a microcentrifuge. BAL supernatant was collected and stored at -80° C prior to analysis. The remaining BAL cell pellet was treated with erythrocyte lysis buffer (ELB), pelleted, and resuspended in PBS for analysis by flow cytometry.

For isolation of cells from lung tissue, lungs were perfused with 5 ml of PBS through the right ventricle of the heart prior to removal. Lung lobes were cut into small pieces with scissors and digested with 0.1 Wünsch units/ml of Liberase TM (Roche) and 25 µg/ml of DNase I (Roche) in 5 ml of 1x Hank's Balance Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} (Corning) for 1 hour at 37° C on a mechanical shaker (180 rpm). Samples

were passed through 70-μm Nylon filter paper, pelleted, and treated with ELB buffer. Cells were washed and resuspended in PBS for analysis by flow cytometry.

Detection of BAL cytokines

For measurement of cytokine concentrations in cell-free BAL fluid, mouse-specific IL-4 (BD Biosciences), IL-5 (Biolegend), and IL-13 (eBioscience) ELISA kits were used according to the manufacturer's protocol.

ILC2 restimulation, isolation, and in vitro expansion

For detection of cytokine-production from ILC2s from naïve, PBS, or papain-challenged mice, single-cell suspensions of cell isolated from lung tissue were resuspended in tissue culture media [MEMα (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-Glutamine, 12.5 mM HEPES (Life Technologies), 22.9 μM β-mercaptoethanol (Bio-Rad)], plated in 96 well-plates, and stimulated with either media alone or with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (5 μg/ml; Cell Signaling) for 4 hours. Following stimulation, activated lung cells were washed twice with PBS and prepared for cell surface and intracellular cytokine staining.

For ILC2 isolation, lung cells were pooled at least 5 mice per group and lung ILC2s were FACS-sorted from the lineage-negative ($CD3^-CD5^-CD11b^-CD11c^-CD19^-B220^-NK1.1^-DX5^-$) $CD90^+ST2^+$ population. Sorted ILC2s were resuspended in tissue culture media and plated at a density of 10,000 cells/well in 96-well plates. ILC2 were stimulated with media alone, IL-2 (1000 U/ml; Peprotech), IL-33 (10 ng/ml; Peprotech),

or PMA (100 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 72 hours. Cell-free supernatant were collected and analyzed for IL-5 and IL-13 cytokines by ELISA.

For *in vitro* expansion, FACS-sorted lung ILC2s were resuspended in tissue culture media and plated at a density of 10,000 cells/well in 96-well plates. ILC2 were expanded with IL-2 (1000 U/ml; Peprotech) and IL-33 (10 ng/ml; Peprotech) for 5 days. Expanded ILC2 were then washed, replated, and rested in IL-2 (1000 U/ml; Peprotech) for 2 days. Following resting, expanded ILC2s were washed, plated (50,000 cells/well), and restimulated with media alone, IL-2 (1000 U/ml; Peprotech), IL-33 (10 ng/ml; Peprotech), or PMA (100 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (5 μg/ml; Cell Signaling) for 4 hours. Following restimulation, activated ILC2s were washed twice with PBS and prepared for cell surface and intracellular cytokine staining.

Quantitative Real-Time PCR

Total RNA was harvested from lung or intestinal tissues with the use of RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (500 ng) was reverse-transcribed with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One to four diluted cDNA samples were added to SsoAdavanced SYBR Green Supermix (Bio-Rad, Hercules, CA), and real-time PCR reactions were run on CFX96 Real-Time PCR detection system (Bio-Rad). Gene expression is normalized to *Gapdh*, and data are presented as means ± SEM from the replicates. Primers used in this study included the following: *Gapdh* forward: 5′-AGGTCGGTGTGAACGGATTTG-3′, and reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′; *Il25* forward: 5′-

GCTGTTGCTGAAGAAGGTAGT-3′, and reverse: 5′-TTCAAGTCCCTGTCCAAC-3′; *Il33* forward: 5′-TCCCAACAGAAGACCAAAG-3′, and reverse: 5′-GATA-CTGCCAAGCAAGGAT-3′;

Statistical analysis

P values were calculated using unpaired two-tailed Student's t-test or one-way ANOVA with Tukey's post-test as indicated in the figure legends. Graphical representation and statistical analysis of data were performed with Prism 6 software (GraphPad).

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