Title

Decreased Cyclic AMP Levels in Dendritic Cells: Effects on Cell Signaling and Role in Allergic Inflammation

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Decreased Cyclic AMP Levels in Dendritic Cells: Effects on Cell Signaling and Role in Allergic Inflammation

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Amy Michelle Chinn is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

I dedicate this dissertation to my Mom and Dad, who have always lovingly supported me all these years, advocating for me when I needed it and always standing by my side. Your constant love and guidance have shaped me to be the person I am today, and I am so blessed and beyond grateful to be your daughter.

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LIST OF ABBREVIATIONS

18S	18S ribosomal RNA
6MB	N6-Monobutyryladenosine-3′,5′-cyclic monophosphate
8ME	8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate
β_2-AR	β_2-adrenergic receptor
Δ*Gnas*	CD11c$^{\Delta \mathit{Gnas}}$
A33	PDE4B-specific inhibitor
AKAP	A-kinase anchoring protein
ATF-1	Activating transcription factor 1
BAL	Bronchoalveolar lavage
cAMP	3’,5’-cyclic adenosine monophosphate or cyclic AMP
cGMP	Cyclic guanosine monophosphate
COPD	Chronic obstructive pulmonary disease
CPT	8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic monophosphate
CRE	cAMP response element
CREB	Cyclic AMP response element binding protein
CREM	cAMP response element modulator
Ct	Cycle threshold
DC	Dendritic cell
dCt	Delta cycle threshold
DE	Differential expression
DMSO	Dimethyl sulfoxide
Epac	Exchange protein directly activated by cAMP or Rap guanine nucleotide exchange factor
FDR	False discovery rate
G_i	$G\alpha_i$ subunit
GO	Gene Ontology

GPCR	G protein-coupled receptor
G_s	$G\alpha_s$ subunit
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL-4	Interleukin-4
IL-5	Interleukin-3
IL-13	Interleukin-13
IL-17A	Interleukin-17A
LABA	Long-acting β_2-adrenergic receptor agonist
MDL-12,330A	Adenylyl cyclase inhibitor
MHC-II	Major histocompatibility complex, class II
mRNA	Messenger RNA
MRP4	Multidrug resistance-associated protein 4 or ATP-binding cassette transporter ABCC4
N6	N6-Phenyladenosine-3',5'-cyclic monophosphate
ND	Not detected
OT-II	B6.Cg-Tg(TcraTcrb)425Cbn/J
Ova	Ovalbumin
PDE	Phosphodiesterase
PDE3B	Phosphodiesterase 3B
PDE4A	Phosphodiesterase 4A
PDE4B	Phosphodiesterase 4B
PDE4C	Phosphodiesterase 4C
PDE4D	Phosphodiesterase 4D
PGE_2	Prostaglandin E_2
PKA	Protein Kinase A
PKI	Protein kinase inhibitor
qPCR	Real-time quantitative PCR
RNA-Seq	RNA-Sequencing
Ro 20-1724	PDE4 inhibitor

SABA	Short-acting β_2-adrenergic receptor agonists
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
Th17	Type 17 helper T cell
Treg	Regulatory T cell
WT	Wildtype

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ABSTRACT OF THE DISSERTATION

Decreased Cyclic AMP Levels in Dendritic Cells: Effects on Cell Signaling and Role in Allergic Inflammation

by

Amy Michelle Chinn

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2019

Professor Paul A. Insel, Chair

Dendritic cells (DCs) are crucial targets for modulating allergic disease due to their role in initiating helper T cell activation and differentiation. CD11c$^{\Delta Gnas}$ (Δ*Gnas*) mice have a DC-specific deletion of the Gα subunit of the heterotrimeric ($\alpha\beta\gamma$) GPT binding protein Gα_s, which activates adenylyl cyclase to produce cyclic AMP (cAMP). Isolated Δ*Gnas* DCs have aberrant cAMP signaling, induce type II helper T cell (Th2) differentiation, and mediate the development of allergic asthma *in vivo*.

The studies presented in this dissertation investigate the mechanism by which cells respond to atypical cAMP signaling, and in particular decreased levels of cAMP, and how in DCs this mediates Th2 inflammation which contributes to the development of allergic disease.

In Chapter One, I review current knowledge of DCs, asthma, the cAMP pathway, and the role of both DCs and cAMP in the pathophysiology of asthma.

In Chapter Two, I present a transcriptomic analysis of RNA-Sequencing of WT and Δ*Gnas* DCs to identify pathways with altered expression in Δ*Gnas* DCs. Δ*Gnas* DCs have an increase in microtubule-associated genes. Increased tubulin expression appears to be mediated by decreased Protein Kinase A (PKA) activation, indicating that decreased cAMP levels is responsible for the increased microtubule expression in Δ*Gnas* DCs. I theorize that changes in microtubule function contribute to Δ*Gnas* DCs' increased Th2 induction.

In Chapter Three, I demonstrate that Δ*Gnas* DCs have decreased basal cAMP levels and investigate how cells compensate for chronically reduced cAMP concentrations. Δ*Gnas* DCs have decreased gene expression of PKA RIIβ, multiple GPCRs, and the phosphodiesterases PDE4B and PDE4D in response to lower cAMP levels. Experiments show that PDE4B is a regulator of cAMP levels in DCs and its expression rises and falls via PKA in an attempt to return the cell to cAMP homeostasis. Furthermore, the high expression of PDE4B makes it a novel target to raise cAMP levels in DCs and reduce Th2 differentiation.

Together, these data indicate that raising cyclic AMP levels in DCs is an effective approach to reduce Th2 inflammation and identifies PDE4B as a novel target in DCs to decrease Th2 inflammation for the treatment of allergic disease.

CHAPTER ONE: INTRODUCTION TO DENDRITIC CELLS, ALLERGIC ASTHMA, THE CYCLIC AMP PATHWAY, AND THEIR ROLE IN ALLERGIC INFLAMMATION

Introduction

This dissertation examines the role of the second messenger cyclic AMP in dendritic cells and how decreased cellular levels of cyclic AMP affect dendritic cell function and contribute to the pathogenesis of allergic asthma.

Dendritic Cells

Dendritic cells (DCs) originate from macrophage-dendritic cell progenitors (MDP) in the bone marrow; MDPs give rise to both dendritic cells and monocytes[1,2]. In the dendritic cell lineage, MDPs become common dendritic progenitor (CDP) cells which give rise to two distinct DC populations: conventional DCs and plasmacytoid DCs. Conventional DCs are professional antigen-presenting cells and in this capacity induce immune responses and form the bridge between the innate and adaptive immune responses[3–7]. Plasmacytoid DCs form a small subset of DCs that have a similar lineage but different function; they secrete large amounts of type I interferon in response to viral infection. Signaling by conventional DCs is the focus of this dissertation.

Conventional DCs are commonly identified by the surface marker CD11c due to their high expression of this integrin. DCs reside in peripheral (non-lymphoid) tissues where they internalize both self and non-self antigens. These antigens are then processed and the resulting peptides (fragments of the original internalized antigen) are loaded onto MHC class II (MHC-II) molecules: this process is termed antigen presentation. DCs are inefficient at antigen presentation until they undergo maturation after encountering a danger signal, which is usually pathogen-associated[8]. After maturation, antigen presentation becomes very efficient and the expression of T cell co-stimulatory molecules increases on the cell surface. In this way, DCs are the most efficacious antigen-presenting cells and far more so than macrophages[8,9]. In parallel, DCs migrate out of the peripheral tissues where they reside and move to secondary lymphoid organs where T cells and B cells are located. Adaptive immune responses are initiated when T cells or B cells bind to their cognate antigen presented by DCs on an MHC-II molecule. By linking antigen uptake, antigen presentation, and cell migration with a danger signal, antigen presentation is restricted to antigens taken up during maturation, thereby favoring antigens that could be pathogen-related. Moreover, DCs represent crucial targets for modulating diseases influenced by aberrant immune cell activation.

Allergic Diseases and Asthma

Allergic diseases are hypersensitivity disorders in which the immune system reacts to environmental substances typically considered harmless. Common allergic diseases include allergic asthma, atopic dermatitis, food allergies, and allergic rhinitis. Many children who are atopic undergo a progression of allergic diseases, with the development of atopic dermatitis in infancy and then subsequently developing food allergy, asthma, and allergic rhinitis: this progression is termed the atopic march[10,11]. DCs contribute to the pathogenesis of allergic disease when they prime immune responses to harmless environmental antigens.

Asthma

Asthma is an inflammatory disorder of the airways characterized by chronic airway inflammation and airway hyperresponsiveness[12]. Airway hyperresponsiveness is defined as increased sensitivity to constrictor agonists administered via the inhalation route. This sensitivity results in increased bronchoconstriction (constriction due to smooth muscle contraction around the bronchioles) and increased mucus production, which together lead to narrowing of the airway. Characteristic asthma symptoms include shortness of breath, chest tightness, wheezing, and coughing. Asthma is defined as having variable expiratory airflow limitation, with patient symptoms varying in intensity over time[12]. Asthma is diagnosed in patients who have both a history of and documented clinical evidence of variable lung function. Clinical lung function tests are measured by a spirometer, usually in conjunction with an inhaled administered challenge such as methacholine (a muscarinic cholinergic receptor agonist) to trigger bronchoconstriction, a bronchodilator (e.g., short-acting beta-adrenergic receptor agonists (SABA)) to demonstrate reversibility, or an exercise test (as some types of asthma are induced by exercise).

The word asthma is derived from the Greek word *aazein* which means “to pant” and records of asthma by the clinician Aretaeus of Cappadocia can be found as early as the first century A.D.[13,14]. Though an ancient disease, the prevalence of asthma increased dramatically in the mid-to-late 1970s; surveys in the UK estimated that the prevalence of asthma among children more than doubled between the 1970s and the late 1990s[15,16]. In the 2000s, asthma prevalence continued to increase, but at a slower rate, and it appears to have reached a plateau since 2009[17]. The hygiene hypothesis was thought to explain this rise in asthma prevalence and proposed that the cause may be increased cleanliness and decreased microbial exposure. However, recent data has shown that the hygiene hypothesis, while explaining some increases in allergic diseases, does not specifically explain the increase in asthma. It has been proposed instead that microbial exposure unaffected by personal hygiene plays a role in the increased prevalence of asthma[18–20].

Asthma is a common disease with prevalence varying widely among countries, from as low as 1.0% in Vietnam to as high as 21.5% in Australia[21]. Overall, the global prevalence of clinical asthma cases is 4.5%, affecting an estimated 235 million people worldwide[12,21,22]. It is the most common noncommunicable disease in children[22]. The increase in prevalence of allergic diseases has imposed a significant public health challenge. The economic costs associated with asthma are among the highest compared to other chronic diseases, exceeding those of HIV and tuberculosis combined[23,24]. It was estimated that the economic burden of asthma in the United States in 2013 was $81.9 billion[25]. Unlike certain other allergic diseases, asthma is a public health challenge in all countries independent of a country's level of development[22,23].

Asthma is a heterogenous disease and can be thought of as a syndrome characterized by asthmatic symptoms and variable airflow limitation. Within this syndrome are different asthma phenotypes, i.e., disease characteristics independent of molecular mechanism, and different endotypes, i.e., distinct disease mechanisms that give rise to that phenotype[26,27]. Commonly described asthma phenotypes include allergic asthma, non-allergic asthma, late-onset asthma, asthma with persistent airflow limitation, and obesity-associated asthma[12,27]. Allergic asthma is the most prevalent asthma phenotype.

It has been proposed that instead of asthma phenotypes, asthma endotypes should direct therapy[28–30]. Although asthma endotype classification is still being debated and not widely agreed upon, one proposed classification system divides endotypes according to the type of predominant cellular inflammatory mediator, as determined by sputum cytology and/or peripheral blood cell analysis: eosinophilic, neutrophilic, mixed-granulocytic (which has features of both the eosinophilic and neutrophilic), and paucigranulocytic (not related to eosinophilic nor neutrophilic) asthma[27,31]. An alternative classification system that is gaining increased acceptance divides asthma cases into either type 2 (type 2 helper T cell (Th2)-high) or non-type 2 (Th2-low) asthma[32–34]. Th2-high asthma is strongly associated with allergic asthma and increased eosinophilic inflammation. There is a wide overlap between patients classified as having Th2-high asthma and those with eosinophilic asthma, but they are not the same and the criteria for each is different[35] (**Figure 1.1**).

Pathophysiology of Asthma

Dendritic cells are the key antigen-presenting cells in asthma and initiate immune responses to various allergens. As noted above, upon maturation, DCs migrate from peripheral tissues to the secondary lymphoid organs and present processed antigens (in this case, processed allergens) to T cells and B cells. If a T cell can bind strongly to the antigenic peptide-MHC II complex on the DC cell surface, it will be activated. This is the first of the two signals that are required for naïve T cell activation. The second signal is the antigen-nonspecific interaction between co-stimulatory molecules present on both the helper T cell and the DC. When a naïve helper T cell is activated, it differentiates into a helper T cell subset depending on the cytokine environment and activation of particular transcription factors and signaling transducer and activator of transcription (STAT) proteins[36]. Functionally distinct helper T cell subsets are characterized by the cytokines they secrete and their master transcriptional regulator[37] (**Table 1.1**).

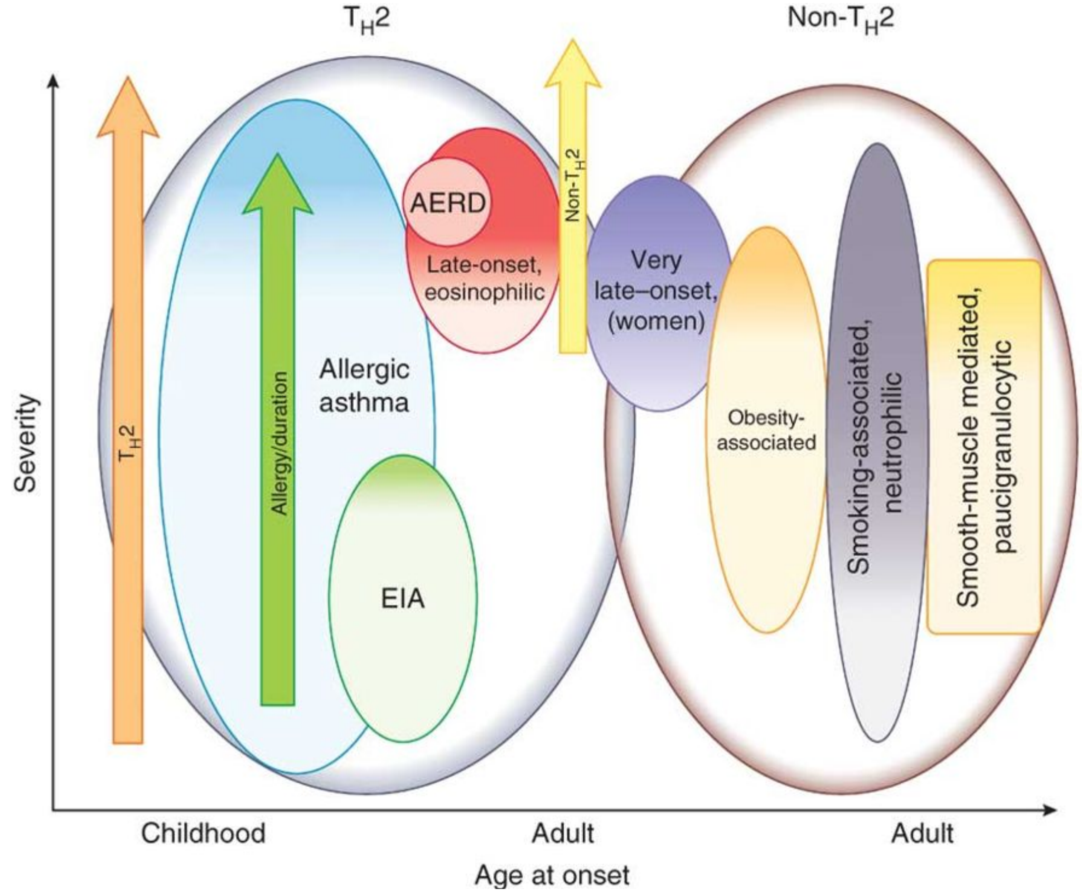

Figure 1.1: Asthma Phenotypes Within the Th2-High (T_H2) and Th2-Low (Non-T_H2) Endotype Groupings. The size of the ellipses represents the relative proportion of affected individuals with that particular asthma phenotype. Color intensity represents the range of asthma severity, with brighter colors indicating more severe disease. AERD, aspirin-exacerbated respiratory disease; EIA, exercise-induced asthma. Reprinted by permission from Springer Nature: Nature Medicine, "Asthma phenotypes: the evolution from clinical to molecular approaches", Sally Wenzel, Copyright 2012[29]. Copyright has been obtained.

Table 1.1: Helper T cell subsets. CD4$^+$ helper T cell subsets, their master transcriptional regulators, and the cytokines these cells secrete[36].

Helper T cell Subset	Master Transcriptional Regulator	Cytokines Secreted
Th1 (Type 1)	T-bet	IFN-γ, Lfα, IL-2
Th2 (Type 2)	GATA3	IL-4, IL-5, IL-10, IL-13
Th17 (Type 17)	RORγt	IL-17A, IL-17F, IL-21, IL-22
Treg (Regulatory)	FOXP3	IL-10, TGF-β

Naïve helper T cells are driven to differentiate into type 2 helper T cells (Th2 cells) by the presence of the cytokine interleukin-4 (IL-4) during stimulation by their cognate antigen and by activation of the transcription factor GATA binding protein 3 (GATA3) and the signal transducer STAT6[36,38] (**Table 1.1**). Th2 cells are a central mediator of inflammation due to their secretion of the inflammatory cytokines IL-4, IL-5, and IL-13. In this way, Th2 differentiation works as a positive feedback loop with Th2 cells secreting IL-4, which in turn promotes other naïve helper T cells to differentiate into Th2 cells. Atopic and asthmatic patients have increased levels of IL-4 in their serum and bronchoalveolar lavage (BAL) fluid[39,40]. Allergic disease is generally thought to be mediated by an overactive Th2 arm of the immune system.

IL-4 and IL-13 promote immunoglobulin class-switching in B cells to produce Immunoglobulin E (IgE). IgE antibodies bind to the high affinity IgE receptor FcεRI on mast cells, thereby sensitizing them to respond in an antigen-specific manner when the host is again exposed to the allergen in question[41–43]. IgE-FcεRI crosslinking immediately triggers mast cell degranulation leading to the release of histamine and leukotrienes which results in bronchoconstriction, increased mucus production, vasodilation, and the recruitment of other inflammatory cells[43]. IL-13 causes airway hyperresponsiveness, and both IL-4 and IL-13 promote increased mucus production[44,45] (**Figure 1.2**).

IL-5 regulates eosinophil differentiation, activation, and survival and is a strong eosinophil chemoattractant[46]. IL-4 and IL-13 further promote eosinophil

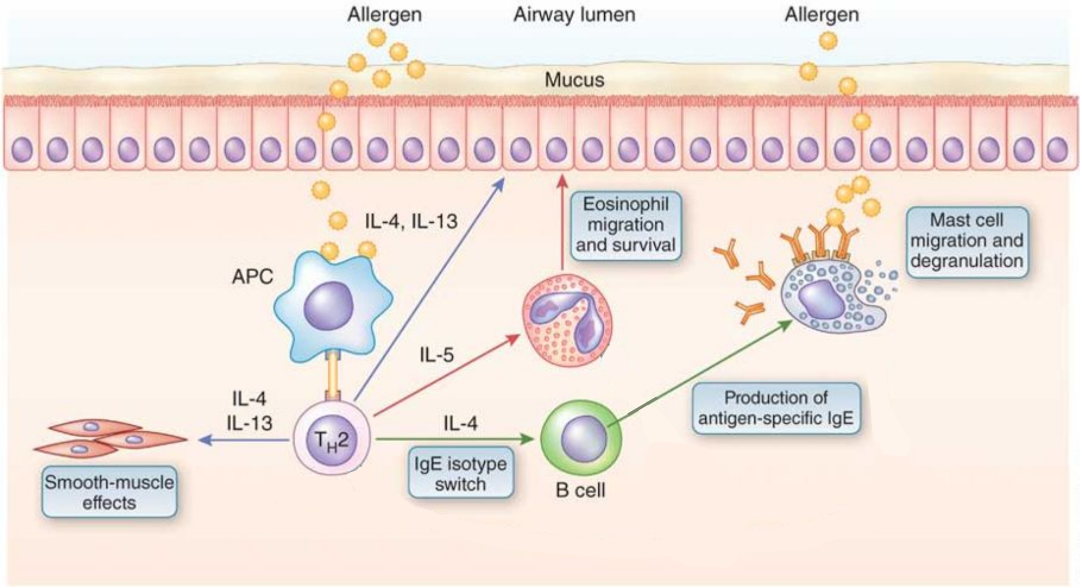

Figure 1.2: Contribution of Th2 Cytokines to Asthma Pathology. Dendritic cells (APC) present antigens (allergens) to helper T cells. Activated Th2 cells secrete IL-4, IL-5, and IL-13 which promote eosinophil migration and survival, airway hyperresponsiveness, increased mucus production, and IgE isotype class switching in B cells. IgE class switching leads to IgE production and results in mast cell degranulation and release of histamine. APC, antigen presenting cell [dendritic cells]. Adapted by permission from Springer Nature: Nature Medicine, “Asthma phenotypes: the evolution from clinical to molecular approaches”, Sally Wenzel, Copyright 2012[29]. Copyright has been obtained.

recruitment by up-regulating vascular cell adhesion molecule-1 (VCAM-1) expression[46,47]. Eosinophils have large specific granules (termed secondary granules) that contain large amounts of inflammatory mediators including proteins, cytokines, chemokines, and enzymes[44,48]. Major basic protein (MBP) is one of the predominant substances in eosinophil secondary granules and produces airway hyperreactivity in primates and rats[49,50]. Eosinophils secrete Th2 cytokines which further the inflammatory cycle and also produce transforming growth factor beta (TGF-β) which contributes to airway remodeling[44]. Chronic airway inflammation regulates the airway remodeling process, a process that includes airway smooth muscle cell hypertrophy and hyperplasia, increased collagen and fibronectin deposition, and goblet cell hyperplasia which together result in increased airway obstruction due to thickening of the airway and increased mucus production[51,52] (**Figure 1.2**).

Th2-low asthma encompasses diverse asthma pathophysiologies, including neutrophilic, paucigranulocytic, obesity-associated, and asthma related to environmental exposure[48]. Gene expression analyses have suggested that the Th2-low endotype can be divided into Th17-high and Th2/Th17-low patterns[53]. Th17 cells induce neutrophil recruitment through secretion of the cytokines IL-17A and IL-17F; increased levels of IL-17A in sputum is connected to increased neutrophil recruitment[54–56]. Importantly, airway neutrophilia is associated with asthma severity and correlated with corticosteroid resistance, one of the cornerstones of asthma therapy[48,53,57].

Animal Models of Asthma

Many species have been used in animal models of asthma, including mice, rats, guinea pigs, cats, and dogs. Mice are the most commonly used species and in particular, the BALB/c strain which is predisposed to produce Th2 immune responses[58,59]. Mice do not develop asthma spontaneously but asthma-like symptoms can be induced with model antigens used in the lab (e.g., ovalbumin (Ova) or aeroallergens (e.g., house dust mite) which is a sensitizing antigens in humans).

Animal models consist of two phases: sensitization and challenge[60]. Sensitization is performed by administering the antigen to the animal through the intraperitoneal, subcutaneous, or intranasal routes of delivery. After a period of time, the animal is challenged with the antigen used for sensitization, thereby provoking a strong immune response. Both acute and chronic allergen challenge models are employed, differing in the length of time over which an animal is challenged: up to 30 days in acute models and for a minimum of 5 weeks in chronic models[61]. Acute allergen challenge models replicate many features of asthma, including increased IgE levels, airway inflammation, goblet cell hyperplasia, and airway hyperresponsiveness. However, these models do not reproduce the characteristic chronic inflammation and airway remodeling, and many of the asthmatic features resolve a few weeks after the last antigen challenge. In contrast, chronic allergen challenge models more closely reproduce human asthma and some models have persistent airway remodeling, airway hyperresponsiveness, and lung inflammation. However, there is limited airway smooth muscle hypertrophy and recruitment of mast cells in chronic allergen challenge models, and inflammation is not restricted to the airways. Overall, mouse models of asthma reproduce many, but not all, of the characteristic features of asthma seen in humans, and their low cost and ease of genetic manipulation make them an advantageous model to study[60].

Therapies for Asthma

The mainstays of current asthma therapy are inhaled corticosteroids and β_2-adrenergic receptor (β_2-AR) agonists (**Figure 1.3**). The current recommendation for treatment of mild asthma is low-dose inhaled corticosteroids (ICS, e.g., fluticasone, budesonide, etc.) with the addition of a long-acting β_2-AR agonist (LABA, e.g., formoterol, salmeterol, etc.) for patients whose asthma is not controlled by ICS treatment alone. Together, ICS and LABA are used as long-term controller medications to control symptoms and reduce airway inflammation[12]. Leukotriene receptor antagonists (e.g., montelukast, zafirlukast, etc.) are an alternative to low-dose ICS-LABA treatment. Medium-dose ICS is used in patients whose asthma is uncontrolled by low-dose ICS. Short-acting β_2-AR agonists (SABA, e.g., albuterol) are used as needed as reliever (rescue) medications for quick relief of asthma symptoms. Asthma treatment is considered successful when a reliever medication is no longer needed to control asthmatic symptoms. Patients with severe, uncontrolled asthma can use high dose ICS-LABA as a controller medication,

Figure 1.3: Recommended Approach to Asthma Management for Adults and Adolescents. Therapies for asthma consist of both 1) long-term controller medications for symptom control and reducing airway inflammation and 2) reliever medications for quick relief of asthma symptoms. Box 3-5A from "Global Strategy for Asthma Management and Prevention" by Global Initiative for Asthma, 2019[12]. Available fromwww.ginasthma.org, published in Fontana, WI, USA. Copyright has been obtained.

Box 3-5A

Adults & adolescents 12+ years

Personalized asthma management:
Assess, Adjust, Review response

Confirmation of diagnosis if necessary
Symptom control & modifiable risk factors (including lung function)
Comorbidities
Inhaler technique & adherence
Patient goals

Treatment of modifiable risk factors & comorbidities
Non-pharmacological strategies
Education & skills training
Asthma medications

Symptoms
Exacerbations
Side-effects
Lung function
Patient satisfaction

Asthma medication options:
Adjust treatment up and down for individual patient needs

	STEP 1	**STEP 2**	**STEP 3**	**STEP 4**	**STEP 5**
PREFERRED CONTROLLER to prevent exacerbations and control symptoms	As-needed low dose ICS-formoterol *	Daily low dose inhaled corticosteroid (ICS), or as-needed low dose ICS-formoterol *	Low dose ICS-LABA	Medium dose ICS-LABA	High dose ICS-LABA Refer for phenotypic assessment ± add-on therapy, e.g.tiotropium, anti-IgE, anti-IL5/5R, anti-IL4R
Other controller options	*Low dose ICS taken whenever SABA is taken†*	*Leukotriene receptor antagonist (LTRA), or low dose ICS taken whenever SABA taken †*	*Medium dose ICS, or low dose ICS+LTRA #*	*High dose ICS, add-on tiotropium, or add-on LTRA #*	*Add low dose OCS, but consider side-effects*
PREFERRED RELIEVER	As-needed low dose ICS-formoterol *		As-needed low dose ICS-formoterol ‡		
Other reliever option	*As-needed short-acting β_2 -agonist (SABA)*				

* Off-label; data only with budesonide-formoterol (bud-form)
† Off-label; separate or combination ICS and SABA inhalers
‡ Low-dose ICS-form is the reliever for patients prescribed bud-form or BDP-form maintenance and reliever therapy
\# Consider adding HDM SLIT for sensitized patients with allergic rhinitis and FEV >70% predicted

with various add-on treatments. These include: long-acting muscarinic cholinergic receptor antagonists (LAMA, e.g., tiotropium), anti-IgE treatment (omalizumab), antibiotics (azithromycin), anti-interleukin-5/5R treatment (mepolizumab), anti-interleukin-4Rα, and bronchial thermoplasty[12] (**Figure 1.3**).

Despite all of these available medications, it is estimated that 50% of adults and 38% of children have asthma that is uncontrolled by treatment regimens[62]. Uncontrolled asthma is defined as poor symptom control and/or frequent exacerbations; these patients have a significantly decreased quality of life[12]. 2.5 - 5% of all asthma patients have severe, uncontrolled disease, yet this population accounts for 37.5% of all asthma-related direct costs[63,64]. Finding new, effective asthma therapies thus represents an unmet medical need.

The Cyclic AMP Pathway and its Regulation

Cyclic AMP (3',5'-cyclic adenosine monophosphate or cAMP) is a ubiquitous signaling molecule found in organisms across all of the three domains of cellular life: archaea, bacteria, and eukarya. Discovered in 1958 by Earl Sutherland in canine tissue, it was the first second messenger to be described[65,66]. Subsequently, cAMP signaling was found to be important in the function of bacteria and archaea; for example, its contribution to the pathogenicity of *Pseudomonas aeruginosa* and *Vibrio cholerae* is well-documented[67,68]. In eukaryotes, cAMP is involved in a wide variety of cellular processes such as proliferation, metabolism, and cell death, in numerous cell types, including immune cells, neural cells, and smooth muscle cells among others.

The Cyclic AMP Pathway

In eukaryotes, the cAMP pathway is canonically activated when agonists bind to $G\alpha_s$-coupled G protein-coupled receptors (GPCRs) on the cell surface. GPCRs are the largest family of receptors with over >800 GPCRs in the human genome[69]. They are seven-transmembrane proteins and the signal transducers between the first (i.e., hormone, neurotransmitter, etc.) and second messenger systems. GPCRs are critical regulators of cellular functions and physical processes, are involved in a wide variety of diseases, and are also the target of ~35% of approved drugs[70]. Their tissue-selective expression and accessibility from the extracellular space contribute to their utility as drug targets[71].

GPCRs couple to heterotrimeric ($\alpha\beta\gamma$) GTP binding (G) proteins. In its inactive state, the $G\alpha$ subunit binds GDP. Upon binding of an activating ligand (agonist) to a GPCR, the GPCR undergoes a conformational change and activates the $G\alpha$ subunit to exchange GDP with GTP, thereby triggering the dissociation of the $G\alpha$ and $G\beta\gamma$ subunits[72]. Both the $G\alpha$ and $G\beta\gamma$ dimer initiate signaling cascades. Multiple $G\alpha$ subunits that activate different signaling pathways: $G\alpha_s$ and $G\alpha_i$ signal through the cAMP pathway, $G\alpha_q$ activation leads to an increase in cellular calcium and diacylglycerol, and $G\alpha_{12/13}$ activates the

GTPase Rho. The Gα subunit Gα_s activates adenylyl cyclase to catalyze the conversion of adenosine triphosphate (ATP) into cAMP. Agonists of Gα_i-coupled GPCRs have the opposite effect with Gα_i inhibiting adenylyl cyclase resulting in reduced cellular cAMP levels (**Figure 1.4**).

Regulators of G Protein Signaling (RGS proteins) promote GTP hydrolysis to inactivate G proteins and turn off GPCR-mediated signaling pathways. The inactivated Gα protein, now bound to GDP again, then binds to the G$\beta\gamma$ dimer and another GPCR at the cell membrane, ready to initiate signaling cascades again.

cAMP generated by adenylyl cyclase mediates its effects primarily via protein kinase A (PKA or cAMP-dependent protein kinase) and the exchange protein directly activated by cAMP (Epac or Rap guanine nucleotide exchange factor), as well as through cyclic nucleotide-gated channels[73]. PKA is a holoenzyme and exists as a tetramer consisting of two regulatory and two catalytic subunits. Each regulatory (R) subunit has two cAMP Binding Domains. When four cAMP molecule bind to the two R subunits, the R subunits undergo a conformational change, releasing the two activated catalytic (C) subunits[74]. These C subunits then proceed to phosphorylate substrates with the consensus sequence Arg-Arg-X-Ser/Thr[75]. Epac is a guanine nucleotide exchange factor (GEF) and has a cAMP Binding Domain that is homologous to those of PKA regulatory subunits. When bound to cAMP, Epac activates the small GTPases Rap1 and Rap2, members of the Ras superfamily[76].

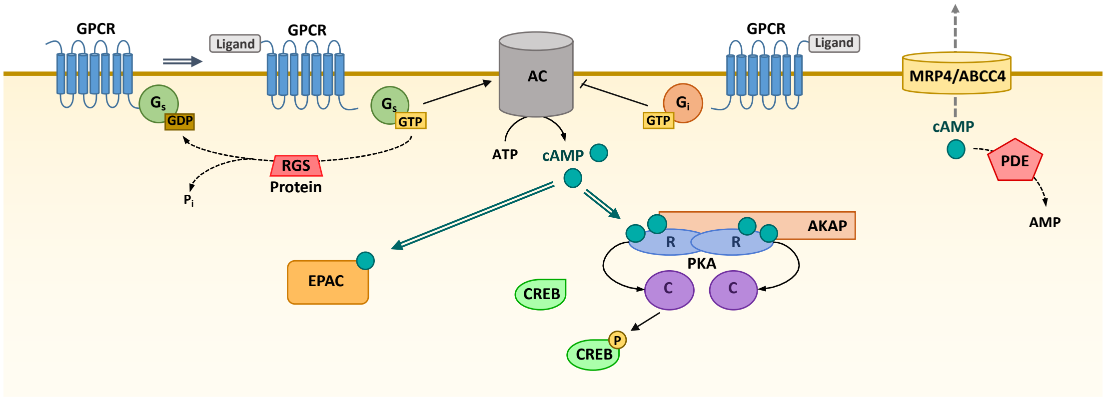

Figure 1.4: The Cyclic AMP Signaling Pathway. Upon a ligand binding to a $G\alpha_s$-coupled GPCR, $G\alpha_s$ (G_s) activates adenylyl cyclase (AC) to catalyze the conversion of ATP to cyclic AMP. $G\alpha_i$ (G_i) inhibits AC, resulting in lower cyclic AMP levels. cAMP mediates its effects through Epac or PKA. PKA phosphorylates CREB which binds to CRE sites in the promoter of CREB-responsive genes. AKAPs localize enzymes to their signaling substrates in various subcellular locations. cAMP is removed through direct degradation by phosphodiesterases (PDEs) or by efflux out of the cell by the transporter MRP4/ABCC4.

A-kinase anchoring proteins (AKAPs) facilitate compartmentalized cAMP signaling by localizing enzymes to their signaling substrates in various subcellular regions[77]. AKAPs also anchor PKA regulatory subunits in the vicinity of other signaling effectors, including GPCRs, protein kinases, phosphatases, phosphodiesterases, and ion channels.

Cells remove cAMP through direct degradation by cyclic nucleotide phosphodiesterases (PDEs) or by the efflux of cAMP out of the cell by the cyclic nucleotide transporter Multidrug resistance protein 4 (MRP4, also known as ATP-binding cassette subfamily C Member 4 or ABCC4). PDEs hydrolyze the 3’ cyclic phosphate bond of cAMP and cGMP and thus regulate the cellular levels of these cyclic nucleotide second messengers as well as their subcellular signaling localization and duration of response[78]. There are 11 families of PDEs of which some selectively degrade only cAMP, cGMP, or degrade both (**Table 1.2**). Cells typically express multiple PDEs which can localize to different subcellular regions of the cell by AKAPs, thereby regulating cAMP and cGMP signal transduction. In this way, PDEs regulate the subcellular localization, amplitude of signaling, and duration of cyclic nucleotide signaling within a cell[78].

MRP4/ABCC4 can efflux a variety of molecules involved in cell communication including cAMP, cGMP, ADP, eicosanoids, and multiple drugs, across the plasma membrane. In this way, MRP4 can regulate cellular cAMP levels in numerous cell types[79–81] (**Figure 1.4**).

Table 1.2: Phosphodiesterases and their Substrate Specificities. Phosphodiesterases can degrade both cAMP and cGMP. Most PDEs preferentially hydrolyze a single cyclic nucleotide, but some hydrolyze both cAMP and cGMP equally well. The PDE4, PDE7, and PDE8 families selectively act only on cAMP and the PDE5 and PDE9 families selectively act only on cGMP[78].

Family	Alternative Name	Isoforms	Substrate Specificity
PDE1	Calcium- and calmodulin-dependent PDE	PDE1A PDE1B PDE1C	cAMP < cGMP cAMP < cGMP cAMP = cGMP
PDE2	cGMP-stimulated PDE	PDE2A	cAMP = cGMP
PDE3	cGMP-inhibited PDE	PDE3A PDE3B	cAMP > cGMP cAMP > cGMP
PDE4	cAMP-specific PDE	PDE4A PDE4B PDE4C PDE4D	cAMP > cGMP cAMP > cGMP cAMP > cGMP cAMP > cGMP
PDE5	cGMP-binding, cGMP-specific PDE	PDE5A	cAMP < cGMP
PDE6	Photoreceptor PDE	PDE6A PDE6B PDE6C	cAMP < cGMP cAMP < cGMP cAMP < cGMP
PDE7	High affinity cAMP-specific PDE	PDE7A PDE7B	cAMP > cGMP cAMP > cGMP
PDE8	High affinity cAMP-specific and IBMX-insensitive PDE	PDE8A PDE8B	cAMP > cGMP cAMP > cGMP
PDE9	High affinity cGMP-specific PDE	PDE9A	cAMP < cGMP
PDE10	cAMP-inhibited PDE	PDE10A	cAMP < cGMP
PDE11	Dual cAMP and cGMP PDE	PDE11A	cAMP = cGMP

cAMP-Mediated Gene Regulation

cAMP-activated PKA can phosphorylate multiple cAMP-responsive transcription factors, including cyclic AMP response element binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF-1)[82]. All of these are members of the CREB family of transcription factors, with CREB being the prototypical transcription factor. Phosphorylated CREB, CREM, and ATF-1 bind to cyclic AMP response element (CRE) sites in the promoter region of CREB-responsive genes. Interaction with the co-activator protein CREB-binding protein (CBP) alters transcription of downstream genes in response to increased cAMP levels[83]. CRE sites exist in 2 forms: the full palindromic sequence TGACGTCA and the less active half-site motif CGTCA/TGACG. Cyclic AMP can thus modulate a diverse range of genes through CREB family members, including ones that regulate proliferation, differentiation, and cell survival in numerous cell types[84].

The Role of Cyclic AMP in Allergic Inflammation

Cyclic AMP regulates both pro- and anti-inflammatory events in both innate and adaptive immune cells[85]. cAMP effects on immune cells are complex with conflicting data in the literature, but in general, increased cAMP levels tend to mediate anti-inflammatory effects and immune suppression (**Table 1.3**).

Table 1.3: Effects of Altered Cyclic AMP Levels on Immune Cell Function. Cyclic AMP regulates both pro- and anti-inflammatory functions in many immune cells, including dendritic cells, macrophages, natural killer cells, T cells, and B cells.

Cell Type	Effect of cAMP on Inflammatory Function	References
Dendritic Cells	▪ Increased cAMP inhibits release of pro-inflammatory cytokines (IL-12 and TNF-α) via PKA ▪ PGE_2 ($G\alpha_s$-linked GPCR agonist) induces DC migration ▪ Increased cAMP promotes Th2 differentiation via PKA ▪ Regulatory T cells (Tregs) suppress DC function by increasing cAMP which decreases costimulatory molecule expression and suppresses actin polymerization at the immunological synapse between DCs and T cells	86–89
Macrophages	▪ Increased cAMP suppresses production of pro-inflammatory cytokines (TNF-α, MIP-1α, and Leukotriene B4) and increased production of anti-inflammatory cytokines (IL-10) via PKA ▪ Increased cAMP inhibits phagocytosis via Epac1 ▪ Increased cAMP inhibits microbial killing ▪ During differentiation, increased cAMP results in increased expression of pro-inflammatory CXCL and CCL chemokines through Epac	90–92
Natural Killer Cells	▪ Increased cAMP inhibits cytotoxic activity via PKA ▪ Decreased cAMP inhibits cytotoxic activity via PKA	93–96
T cells	▪ Increased cAMP inhibits antigen-specific T cell proliferation and cytokine production via PKA ▪ Increased cAMP inhibits T cell chemotaxis ▪ Tregs mediate immune suppression of other T cells and DCs by transferring cAMP through gap junctions and by converting ATP into adenosine which acts on target cells and increases cAMP through $G\alpha_s$ ▪ Tregs suppress T cell responses by nuclear accumulation of ICER/CREM	89,97–101
B cells	▪ Increased cAMP inhibits antigen-stimulated B cell proliferation via PKA	102,103

Cyclic AMP in Asthma

Peripheral blood leukocytes and airway smooth muscle from asthma patients produce less cAMP in response to stimulation with the non-selective β-adrenergic receptor agonist isoproterenol than normal healthy controls do[104–107]. This airway smooth muscle response is mediated by increased expression of the phosphodiesterase PDE4D which selectively degrades cAMP[106].

Treatments for asthma target cyclic AMP in a number of ways. Therapy is largely focused on treating the airway smooth muscle (ASM) which is responsible for excessive, reversible bronchoconstriction. ASM contraction is mediated by Ca^{2+} signaling which is canonically activated by agonists binding to $G\alpha_q$-linked GPCRs; increased Ca^{2+} activates Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) which phosphorylates myosin light chain, thereby triggering constriction[108]. The β_2-AR receptor, a $G\alpha_s$-linked GPCR, is widely targeted in asthma therapy, in particular by LABA and SABA drugs, which increase intracellular cAMP concentrations (**Figure 1.3**). This increase in cAMP results in activation of PKA and Epac and leads to decreased myosin light chain phosphorylation, resulting in reduced ASM contraction[109,110]. In this way, β_2-AR agonists function as bronchodilators and are effective treatments for asthma. The molecular mechanism behind β_2-AR agonist-induced ASM relaxation is multifaceted and includes PKA-mediated decrease in Ca^{2+} mobilization and up-regulation of the $G\alpha_q$-selective RGS2 associated with decreased agonist-induced Ca^{2+} concentrations[111,112]. In addition, cAMP-raising agents inhibit the increased proliferation and migration of ASM cells that contribute to airway remodeling in asthma patients[109,113].

Theophylline (1,3-dimethylxanthine) has been used to treat asthma since the 1930s and is still commonly used in developing countries due to its cost[110]. The mechanism by which theophylline works is not fully understood. Its weak, non-selective inhibition of PDEs in airway smooth muscle cells increases cAMP levels, which mediate bronchodilation, primarily through its effects on PDE3[110,114]. However, theophylline's low potency and efficacy and frequent side effects due to inhibition of other PDEs and antagonism of adenosine receptors has led to a decline in its usage.

The use of selective PDE4 inhibitors has been investigated, especially because PDE4 is highly expressed in the inflammatory cells involved in asthma pathogenesis[115–118]. The four PDE4 family members (encoded by the *Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d* genes) selectively degrade cAMP. Each gene has multiple variants that differ in their N terminus which encodes phosphorylation sites and regulatory domains[78]. PDE4 inhibition is thought to suppress airway inflammation and relax airway smooth muscle cells by raising cAMP levels. The PDE4 inhibitor roflumilast is approved for treatment of chronic obstructive pulmonary disease (COPD), which has airway obstruction. Unlike asthma, COPD symptoms are constant as opposed to episodic. PDE4 inhibitors have many side effects, in particular causing nausea and diarrhea, and these side effects in combination with the efficacy of other agents used for current asthma therapy have shifted the focus of PDE4 inhibitors away from asthma and towards COPD[116,119]. Although PDE4 inhibitors are not currently recommended for asthma patients, there is interest in using such inhibitors for the treatment of asthma[115–117].

Cyclic AMP in Dendritic Cells: Effects on Allergic Inflammation

A novel mouse model of allergic asthma was created by the Eyal Raz lab (University of California San Diego) using the Cre-loxP system to knock out the gene *Gnas* (which encodes $G\alpha_s$) under the control of the CD11c promoter (CD11c$^{\Delta \mathit{Gnas}}$ mice or Δ*Gnas*)[87]. CD11c is a DC marker due to its high expression on DCs. CD11c is also present on other myeloid cells including subsets of macrophages, but since DCs are more efficacious at antigen presentation than macrophages, changes in Δ*Gnas* mice as they relate to helper T cell differentiation can be contributed to the large CD11c$^+$ DC population as opposed to the smaller subset of CD11c$^+$ macrophages[9,120]. Δ*Gnas* mice thus have a DC-selective knockout of $G\alpha_s$ which was predicted to be associated with decreased adenylyl cyclase activation and cAMP concentrations in those cells.

Indeed, isolated Δ*Gnas* bone marrow-derived DCs produce less cAMP in response to the $G\alpha_s$-linked agonists prostaglandin E_2 and isoproterenol. Forskolin (a diterpene originally identified in plants) directly activates adenylyl cyclase and also enhances $G\alpha_s$ coupling[121,122]. Δ*Gnas* DCs have a blunted forskolin response in comparison to fl/fl littermate control cells, consistent with the idea that Δ*Gnas* DCs have a $G\alpha_s$ knockout. Control DCs incubated *ex vivo* with the model antigen ovalbumin (Ova) do not induce naïve CD4$^+$ T cells with a transgenic T cell receptor that recognizes Ova (cells from OT-II mice) to differentiate into Th2 cells, as measured by cytokine secretion of IL-4 and mRNA expression of GATA3. However, ∆*Gnas* DCs preferentially induced naïve CD4$^+$ OT-II T cells to differentiate into Th2 cells, without changes in Th1, Th17, or Treg differentiation. The increased Th2 differentiation could be suppressed by treating DCs with the non-specific cAMP analog 8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic monophosphate (CPT) and the PKA-specific cAMP analog N6-Phenyladenosine-3',5'-cyclic monophosphate (N6) prior to co-culturing the DCs with CD4$^+$ T cells. Treatment with the Epac-specific cAMP analog 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8ME) had no effect on helper T cell differentiation. Treating DCs from fl/fl littermate controls with the PKA inhibitor H89 was able to drive fl/fl DCs to induce Th2 differentiation.

Moreover, ∆*Gnas* mice develop allergic asthma *in vivo* in response to Ova immunization, as demonstrated by increased IgE serum levels, airway hyperresponsiveness, and airway inflammation (as ascertained by histology). ∆*Gnas* mice are in a C57Bl/6 background, which is not predisposed to producing Th2 immune responses (unlike BALB/c mice), so the strong Th2 response in these animals is particularly striking. Unlike other asthma animal models, ∆*Gnas* mice do not require immunization and spontaneously develop asthma at age 6 months, which makes these mice an attractive animal model of allergic asthma as it mimics the spontaneous development of asthma seen in humans.

Summary

Asthma is an airway inflammatory disorder characterized by airway hyperresponsiveness and reduced airflow. Th2 cells secrete the cytokines IL-4, IL-5, and IL-13, which contribute to the allergic asthma disease processes of airway inflammation, increased mucus production, and airway hyperresponsiveness. Asthma is very common, affecting 235 million people globally. Therapies for asthma center on ICS and β_2-AR agonists. These therapies are not able to control asthma symptoms in ~50% of adult patients. As such, there is a great need to find new, effective asthma therapies.

Many existing asthma therapies increase levels of the cyclic nucleotide second messenger cAMP. Cyclic AMP levels regulate pro- and anti-inflammatory effects on immune cells, with increased cAMP concentrations generally associated with reduced inflammation. DCs represent crucial targets for modulating allergic disease due to their role in inducing Th2 differentiation. CD11c$^{\Delta Gnas}$ mice have a DC-selective deletion of $G\alpha_s$ and reduced cAMP accumulation in response to $G\alpha_s$-linked GPCR agonists. Isolated DCs from Δ*Gnas* mice induce Th2 differentiation *ex vivo* and Δ*Gnas* mice develop allergic

asthma *in vivo*, implicating decreased cyclic AMP levels in DCs in the development of allergic inflammation.

In this dissertation, I hypothesize that lower cyclic AMP levels mediate the increased Th2 differentiation in CD11c$^{\Delta Gnas}$ mice. I used transcriptomic analysis of ∆*Gnas* DCs to identify possible mechanisms for this alteration in DC function (**Chapter Two**) and evaluated ∆*Gnas* DCs as a system to study cellular compensation for decreased cAMP levels (**Chapter Three**).

Acknowledgements

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CHAPTER TWO: CHARACTERIZATION OF WT AND CD11C$^{\Delta GNAS}$ DENDRITIC CELLS VIA TRANSCRIPTOMIC ANALYSIS

Abstract

Dendritic cells (DCs) from WT and CD11c$^{\Delta Gnas}$ (Δ*Gnas*) mice have differences in their cyclic AMP signaling and their capacity to induce type 2 helper T cell (Th2) differentiation in co-cultured CD4^{+} T cells. In this study, RNA-Sequencing (RNA-Seq) analysis was performed on isolated CD11c^{+} DCs from WT and Δ*Gnas* mice to investigate potential mechanisms for this change in phenotype. Transcriptomic analysis showed that Δ*Gnas* DCs clustered separately from WT DCs and had many differentially expressed genes, including an increase in genes relating to microtubules. Many GPCRs, Protein Kinase A RIIβ, the transcription factor CREM, and three phosphodiesterases also had altered gene expression. One or more of these gene products may represent targets to modulate DC-induced Th2 inflammation.

Introduction

Isolated CD11c^{+} dendritic cells (DCs) from CD11c$^{\Delta Gnas}$ (Δ*Gnas*) mice produce less cyclic AMP (cAMP) after stimulation by the adenylyl cyclase activator forksolin and the Gα_s agonists isoproterenol and prostaglandin E_2

(PGE_2), indicating that ∆*Gnas* DCs possess changes in their cell signaling as it relates to cAMP[1]. ∆*Gnas* DCs also induce naïve $CD4^+$ helper T cells to differentiate into Th2 cells while WT DCs induce little to no Th2 differentiation, as observed in both *ex vivo* and *in vivo* experiments[1]. *In vivo*, this manifests in the development of allergic asthma in ∆*Gnas* mice. Thus, the aberrant cell signaling in ∆*Gnas* DCs has functional consequences at both the *ex vivo* and *in vivo* level. Here, we investigate changes at the transcriptomic level in WT and ∆*Gnas* DCs to investigate the mechanism behind the altered, pro-allergic phenotype of ∆*Gnas* DCs and to identify potential candidates for further study as potentially novel therapeutics for asthma.

Experimental Procedures

Animals

C57BL/6J mice were purchased from the Jackson Laboratory. $CD11c^{\Delta Gnas}$ mice were a kind gift from Eyal Raz (University of California San Diego, La Jolla, CA).

Bone Marrow-Derived Dendritic Cell Isolation

Bone marrow was collected from murine femurs and tibiae and cultured for 6 days in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10% penicillin-streptomycin, 50μM 2-Mercaptoethanol, and 10ng/mL recombinant mouse GM-CSF (eBioscience). CD11c$^+$ DCs were isolated from floating cells in the culture using CD11c magnetic beads (StemCell Technologies EasySep Mouse CD11c Positive Selection Kit II) per manufacturer's instructions.

RNA-Sequencing Transcriptomic and Statistical Analysis

Libraries for RNA-Sequencing (RNA-Seq) were prepared using the Illumina Truseq stranded mRNA kit at the University of California San Diego Institute for Genomic Medicine Core Facility. Libraries were sequenced using a NovaSeq 6000, with 150 base pair paired-end reads, at the University of California, San Francisco Center for Advanced Technology core facility.

Following sequencing, FASTQ files were analyzed as follows. First, files were inspected for sequencing quality using FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters were trimmed using BBDuk (jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/), yielding cleaned-up FASTQ files. From these files, quantification of gene expression was done via Kallisto using the Ensembl v79 reference transcriptome for mice[2]. Transcript expression from Kallisto was converted to gene-level expression (in transcripts per million (TPM) and estimated counts) via the Tximport package[3]. Estimated counts were used as input to the edgeR package; genes having differential expression (DE) with false discovery rate (FDR) <0.05 were considered statistically significant[4]. Analysis for gene ontology and other similar associations with annotated gene sets was conducted with Enrichr as described below[5]. Sets of significantly up-regulated or down-regulated (FDR<0.05) genes were separately queried (as indicated in the figure legends) to test the likelihood that certain annotated sets of genes were statistically overrepresented among genes that were differentially expressed. We evaluated results from Enrichr for Gene Ontology (GO) annotated sets of genes based on: a) the biological processes they participate in, b) the molecular function gene products play in a cell (e.g., is a gene product a protein kinase or a GPCR etc.), and c) the cellular compartment with which they are associated[6]. In addition, we tested (via Enrichr) associations of DE genes with specific cellular compartments based on their annotations via the Jensen database[7], yielding results that are complementary and consistent with that from GO cellular compartment analysis discussed above. For all analyses via Enrichr, we considered associations to be statistically significant if they had q-values (i.e. p-values adjusted for multiple testing) <0.05.

Visualization of networks of DE genes was done using STRING[8] (https://string-db.org/) with default settings (except for hiding unconnected nodes and disabling structure previews). Genes corresponding to certain pathways or processes were highlighted to provide a visual representation of closely associated sets of genes being simultaneously up- or down-regulated.

Weighted GPCR-G protein linkages analysis was done as previously described[9].

RNA-Sequencing Data

Access to raw and analyzed data and results from DE analysis will be deposited at GEO at time of publication.

Immunofluorescence

Cells were plated overnight on slides coated with poly-D-Lysine. After washing with PBS, cells were fixed with 3.7% PFA for 10 min at room temperature (RT), washed, permeabilized with 0.5% Triton X-100 for 5 min, and washed again. Blocking was done with 10mg/mL Bovine Serum Albumin (BSA) in PBS for 1 hr at RT. After washing, cells were incubated with primary antibody in blocking buffer overnight at 4°C. The following day, cells were washed, incubated with secondary antibody (ThermoFisher Scientific Goat anti-Rabbit IgG (H+L) Cross-Adsorbed ReadyProbes, Alexa Fluor 594) at RT for 1 hr in the dark, washed, and then mounted with Life Technologies ProLong Gold antifade reagent with DAPI. Cells were visualized on a Keyence Fluorescence Microscope BZ-X700 using a 20X objective. Antibody against α-tubulin (Abcam ab18251) was used at 1μg/mL.

Cells treated with PKI (10μM) or CPT (50μM) were first plated on slides coated with poly-D-Lysine and then treated for 24 hrs before washing cells and proceeding with the rest of the immunofluorescence staining protocol.

Real-Time Quantitative PCR (qPCR) and Statistical Analysis

RNA was isolated using RNeasy Mini Kit (Qiagen) and converted to cDNA (Bio-Rad iScript cDNA Synthesis Kit). qPCR was run on Bio-Rad CFX Connect Real-Time PCR Detection System using PerfeCTa SYBR Green SuperMix (Quanta Bio). dCt values were calculated by subtracting the Ct value for the housekeeping gene 18S from the Ct value of the gene being evaluated. Fold-change between 2 genes was calculated by the formula $2^{-(dCT_{Gene\ 1} - dCt_{Gene\ 2})}$.

Statistical significance for *Tubb2b* mRNA expression was determined by unpaired t test. A value of $p<0.05$ was considered statistically significant.

All primers were designed in house except for *Tubb2b*[10]. The primer sequences are listed in **Table 2.1**.

Results

CD11c$^+$ bone marrow-derived DCs were isolated from WT and Δ*Gnas* mice from both genders. RNA-Sequencing (RNA-Seq) was used to define gene expression differences between Δ*Gnas* and WT DCs. RNA-Seq was done on 5 WT and 4 Δ*Gnas* biological replicates (**Table 2.2**).

Table 2.1: Primer Sequences for qPCR. Primers were designed using Primer-BLAST except where indicated.

Primer	**Forward (5'-3')**	**Reverse (5'-3')**
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Tubb2b	TCATCAGACCCACTGACACAG	TTTCCAGTTGCAAATCACTGTC

WT and ∆Gnas DCs express a similar number of genes, but have differences in their gene expression

All WT and ∆*Gnas* samples expressed a similar number of genes, both overall and when stratified according to degree of expression (**Tables 2.3, 2.4**). 0.2 transcripts per million (TPM) was set as the threshold of detection, with higher numbers indicating increased level of expression[9].

Multidimensional scaling (MDS) showed that biological replicates were similar to each other and that WT and ∆*Gnas* DCs clustered separately from one another, indicating that WT and ∆*Gnas* cells have transcriptomic differences. Samples did not cluster according to gender, both within and between genotypes (**Figure 2.1**).

There were 11,861 genes that were highly enough expressed in multiple replicates so as to perform statistical analysis via edgeR[4]. We found that 1,242 genes were significantly (FDR<0.05) differentially expressed in ∆*Gnas* DCs compared to WT DCs (**Table 2.5**). 435 genes were significantly up-regulated >2-fold and 322 genes were significantly down-regulated >2-fold in ∆*Gnas* DCs (**Figure 2.2, 2.3**). Clustering of samples and differentially expressed genes as visualized in a heatmap showed that 1) WT and ∆*Gnas* DCs form distinct, relatively homogenous groups, distinguished by this set of differentially expressed genes, and that 2) groups of genes are co-regulated, i.e., change in a consistent pattern in ∆*Gnas* DCs compared to WT cells (**Figure 2.4**).

Table 2.2: RNA-Sequencing Samples. RNA-Sequencing was done on 5 biological replicates of WT DCs and 4 biological replicates of ∆*Gnas* DCs.

Genotype	Sample	Gender
WT	WT3	Male
	WT4	Male
	WT5	Female
	WT6	Female
	WT7	Female
∆*Gnas*	KO3	Male
	KO4	Male
	KO6	Female
	KO7	Female

Table 2.3: Number of Genes Detected in WT DCs. WT DC biological replicates expressed similar numbers of genes, both overall and when stratified according to level of expression. On average, WT DCs expressed 13,669 genes.

	WT 3	**WT 4**	**WT 5**	**WT 6**	**WT 7**	**Avg**	**Std Dev**
0.2 - 1 TPM	2255	2360	2298	2259	2329	2300	40.4
1 - 10 TPM	4227	4620	4572	4413	4957	4558	242.4
10-50 TPM	4699	4640	4604	4605	4172	4544	189.12
>50 TPM	2671	2222	2282	2238	1920	2267	239.5
Total	13852	13842	13756	13515	13378	13669	189.4

Table 2.4: Number of Genes Detected in ∆*Gnas* DCs. ∆*Gnas* DC biological replicates expressed similar numbers of genes, both overall and when stratified according to level of expression. On average, ∆*Gnas* DC expressed 13,814 genes.

	KO 3	**KO 4**	**KO 6**	**KO 7**	**Avg**	**Std Dev**
0.2 - 1 TPM	2351	2290	2246	2328	2304	39.8
1 - 10 TPM	4581	4774	4563	4743	4665	94.1
10-50 TPM	4722	4496	4671	4514	4601	97.6
>50 TPM	2391	2151	2264	2172	2245	94.7
Total	14045	13711	13744	13757	13814	134.3

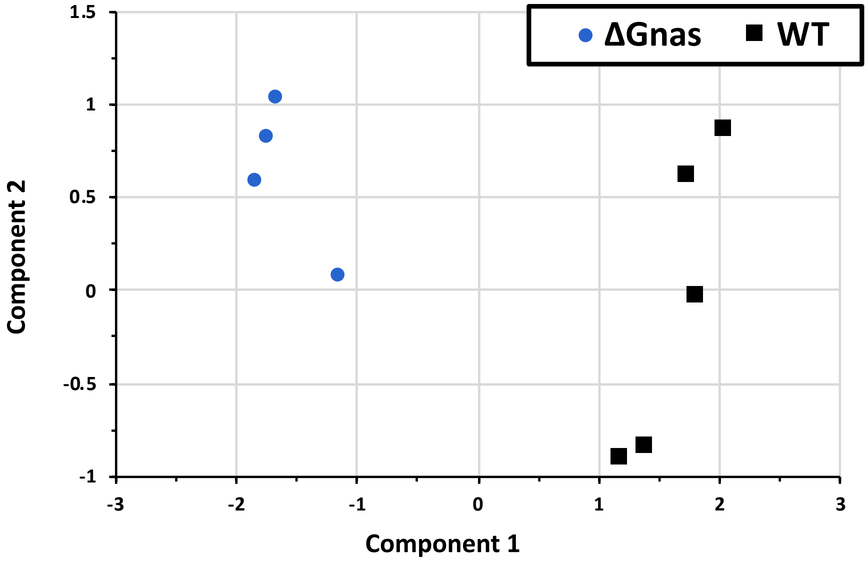

Figure 2.1: Multidimensional Scaling (MDS) Plot of WT and Δ*Gnas* DCs. WT and Δ*Gnas* DCs cluster separately. WT n=5, Δ*Gnas* n=4.

Table 2.5: Number of Differentially Expressed Genes in ∆*Gnas* DCs. The number of genes with changed gene expression in ∆*Gnas* DCs compared to WT DCs varies according to different levels of significance.

FDR	Differentially Expressed Genes
FDR < 0.05	1242
FDR < 0.01	789
FDR <0.001	487

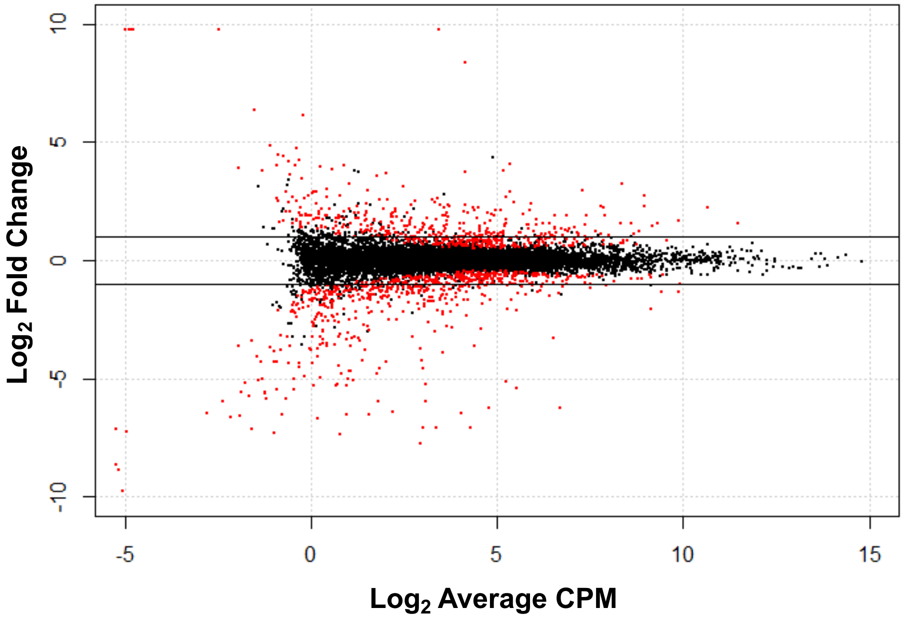

Figure 2.2: Smear Plot of Differentially Expressed Genes in ∆*Gnas* DCs. CPM shows a gene's level of expression. A (+)fold-change value indicates increased expression in ∆*Gnas* DCs, while a (-)fold-change indicates decreased expression compared to WT cells. **Red:** genes with statistically significant expression changes (FDR<0.05). **Black:** non-significant genes (FDR>0.05).

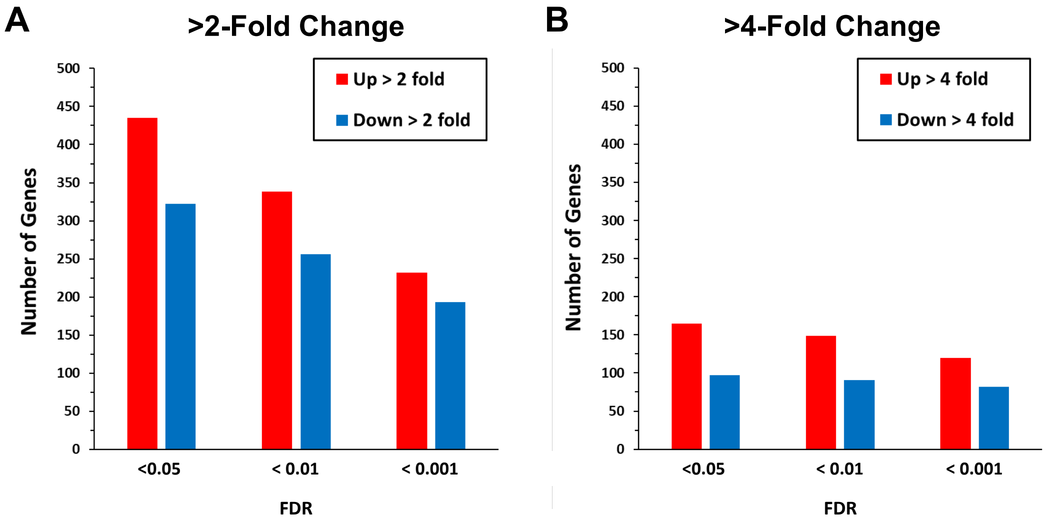

Figure 2.3: Differentially Expressed Genes in Δ*Gnas* DCs According to Level of Significance. The number of genes that had **A)** >2-fold and **B)** >4-fold increased (**Red**) or decreased (**Blue**) expression in Δ*Gnas* DCs compared to WT DCs varies according to fold change thresholds. Most >4-fold gene expression changes have high levels of significance.

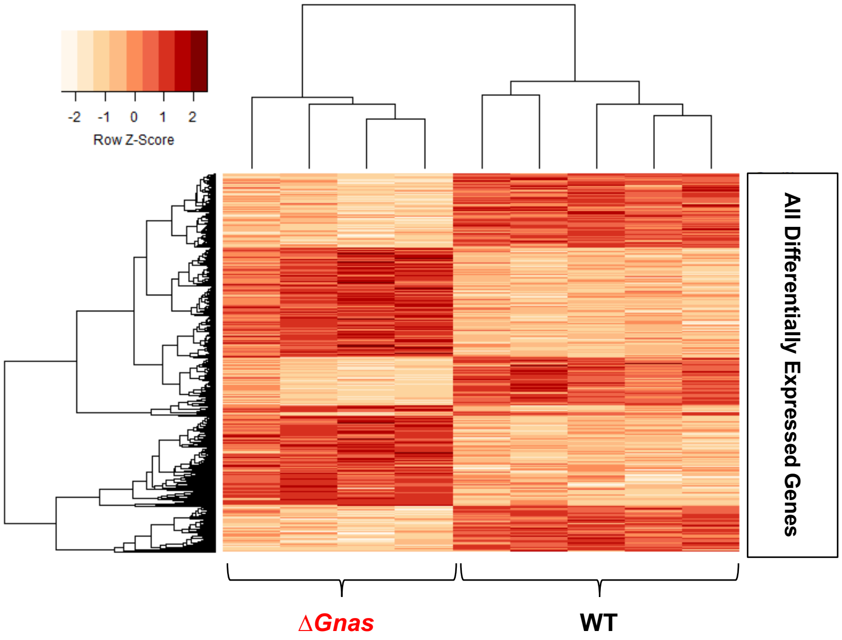

Figure 2.4: Heatmap of Differentially Expressed Genes. WT and Δ*Gnas* DCs have distinct patterns of gene expression. Within each column is the gene expression from a single biological replicate. WT n=5, Δ*Gnas* n=4.

RNA-Seq analysis of down-regulated genes in ∆*Gnas* DCs showed many unique pathways involved and as a result, did not provide a clear direction for further study. Examples of genes with decreased expression in ∆*Gnas* DCs included genes related to NFκB, the Bcl-2 complex, and arginine biosynthesis (data not shown).

Analysis of genes that were increased >2-fold (FDR<0.05) in ∆*Gnas* DCs using STRING showed clusters of up-regulated genes relating to the chromosome and plasma membrane (**Figure 2.5**). As discussed below, the bulk of these chromosome-related genes are associated with organization of microtubules. Thus, up-regulated genes in ∆*Gnas* DCs can be broadly divided into signaling-related genes/proteins on the plasma membrane and structural genes/proteins in the nucleus.

Further analysis via Enrichr of genes up-regulated >2-fold for associations with GO Molecular Function showed an increase in genes relating to motor activity, microtubule binding, and microtubule motor activity (**Figure 2.6**). Many of these genes were present in the cluster of chromosome-related genes in the STRING analysis as well.

Pathway analysis showed that ∆*Gnas* DCs had increased expression of the cell cycle, gastric acid secretion, and hematopoietic cell lineage pathways (**Figure 2.7**). An increase in microtubule-associated genes likely explains the increased cell cycle pathway genes, and increased representation of genes

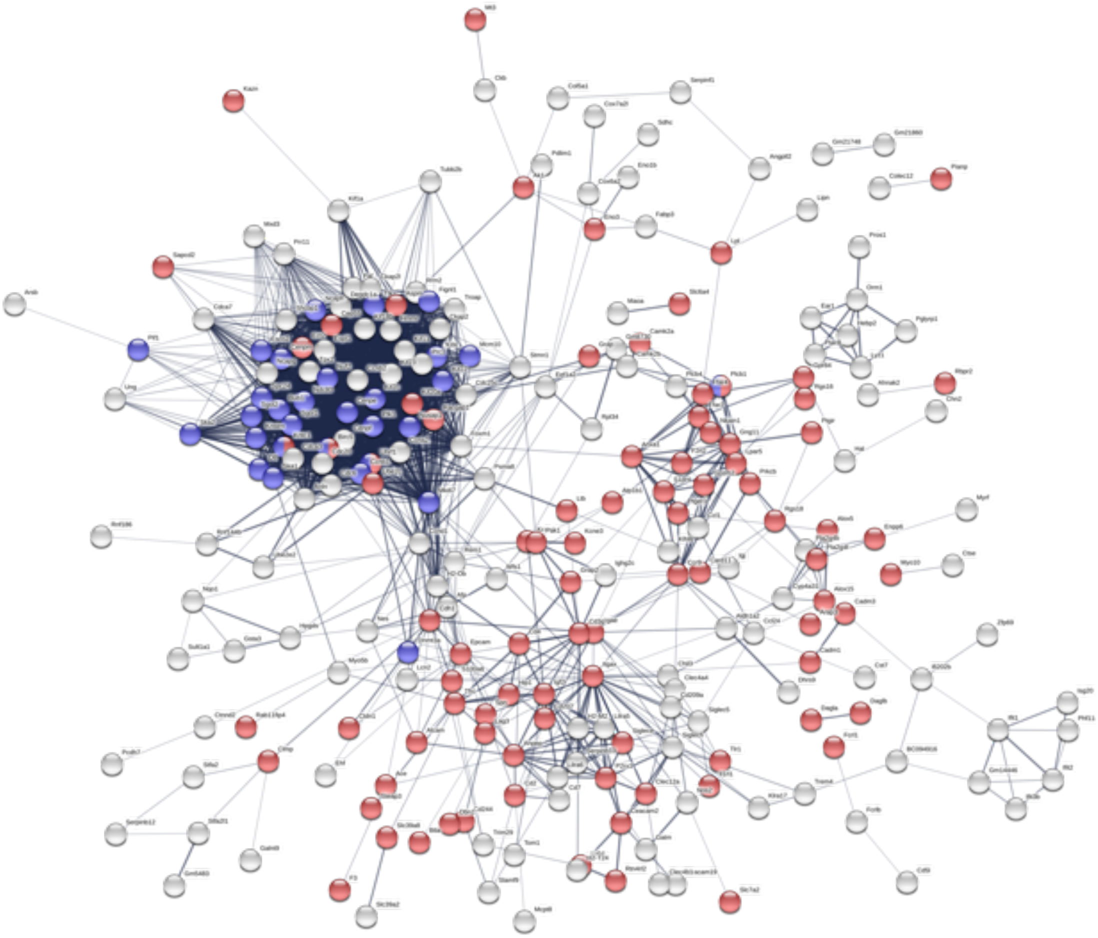

Figure 2.5: STRING Analysis of Up-regulated Genes in Δ*Gnas* DCs. All up-regulated genes >2-fold that were statistically significant (FDR<0.05) were included in the analysis. **Violet:** Chromosome-related genes. **Red:** Plasma membrane-related genes.

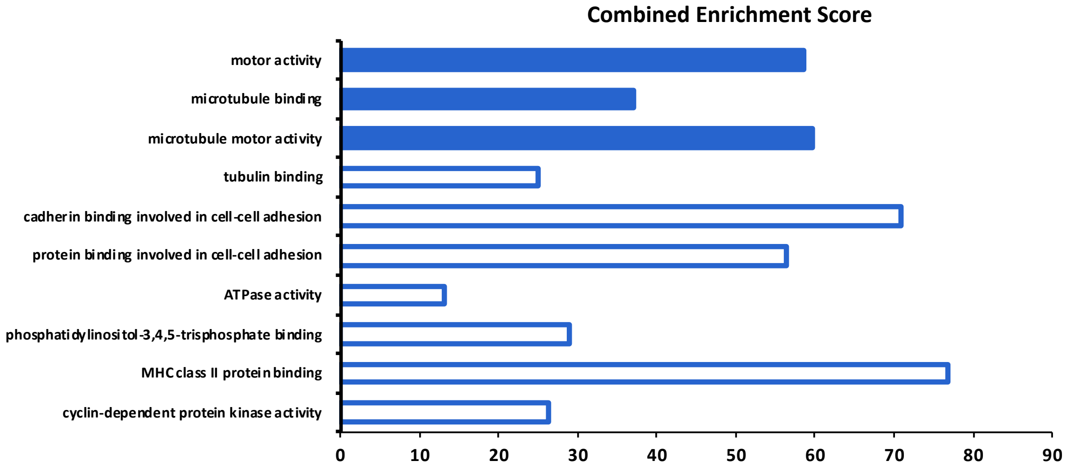

Figure 2.6: Up-regulated Gene Pathways: GO Molecular Function. The enrichment scores for genes up-regulated >2-fold (FDR<0.05) in ∆*Gnas* DCs. Solid bars are pathways that were statistically significant (p-adjusted<0.05).

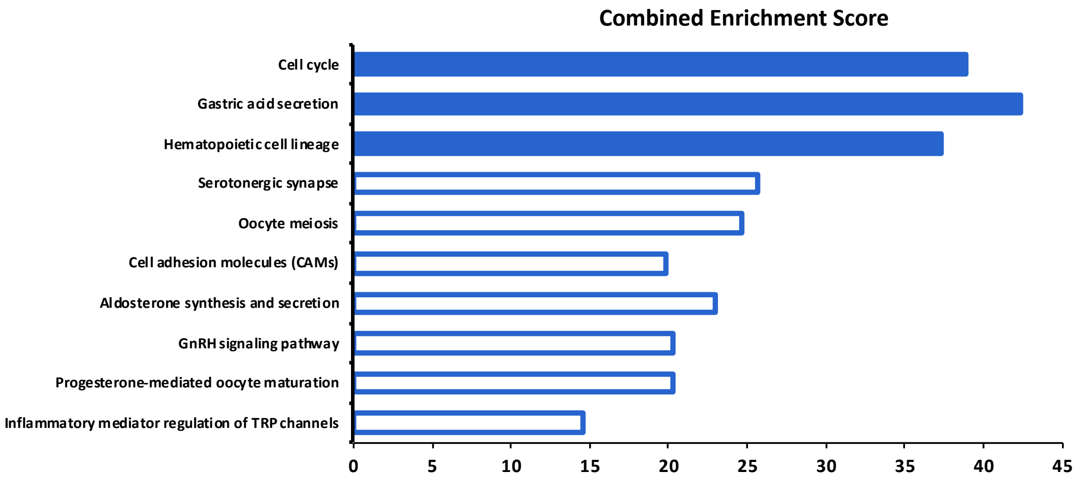

Figure 2.7: Up-regulated Gene Pathways: KEGG Mouse Pathways. The enrichment scores for genes up-regulated >2-fold (FDR<0.05) in ∆*Gnas* DCs. Solid bars are pathways that were statistically significant (p-adjusted<0.05).

relating to hematopoietic cell lineage suggests that Δ*Gnas* DCs could have changes in their maturation, given their origins in hematopoietic progenitor cells.

8 out of 10 of the most enriched GO Biological Processes in Δ*Gnas* DCs related to the cell cycle, microtubules, the kinetochore, or mitosis (**Figure 2.8**). The remaining 2 biological processes were involved in positive regulation of IL-8 secretion. IL-8 is a potent chemoattractant cytokine for neutrophils, but not DCs[11,12]. Interestingly, the Th2 cytokines IL-4 and IL-13 induce airway epithelial cells to produce IL-8[13]. IL-8 induces CXCR1 and CXCR2 expression in DCs which then allows DCs to migrate in response to IL-8[14]. Skin samples from atopic dermatitis patients, a disease associated with Th2 inflammation, reportedly have increased numbers of DCs labeled with the IL-8 receptor as well[14]. Th2 cytokines thus appear to regulate IL-8 secretion which may in turn affect DC migration.

The top 10 cell compartments associated with up-regulated gene expression in Δ*Gnas* DCs were all associated with microtubules or the kinetochore (including the outer kinetochore Ndc80 complex) (**Figure 2.9**).

Leading edge analysis of GO Biological Processes indicates up-regulated genes in Δ*Gnas* DCs that are common across multiple enriched gene sets. The 7 most commonly found genes were all involved in mitosis, the cytoskeleton, or microtubules (**Figure 2.10**). RNA-Seq analysis of up-regulated genes in Δ*Gnas* DCs thus points to an increase in microtubule and kinetochore-associated genes.

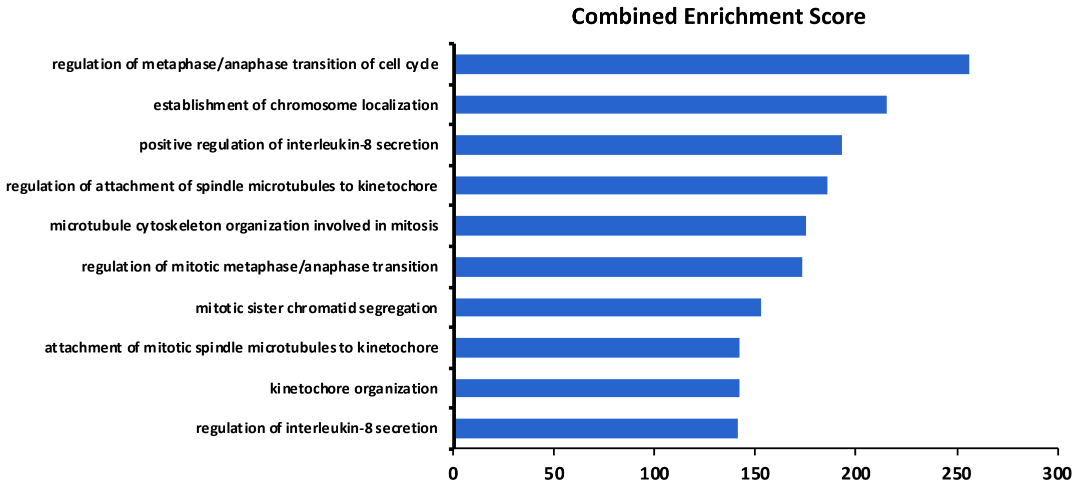

Figure 2.8: Up-regulated Gene Pathways: GO Biological Processes. The enrichment scores for genes up-regulated >2-fold (FDR<0.05) in ∆*Gnas* DCs. Solid bars are pathways that were statistically significant (p-adjusted<0.05).

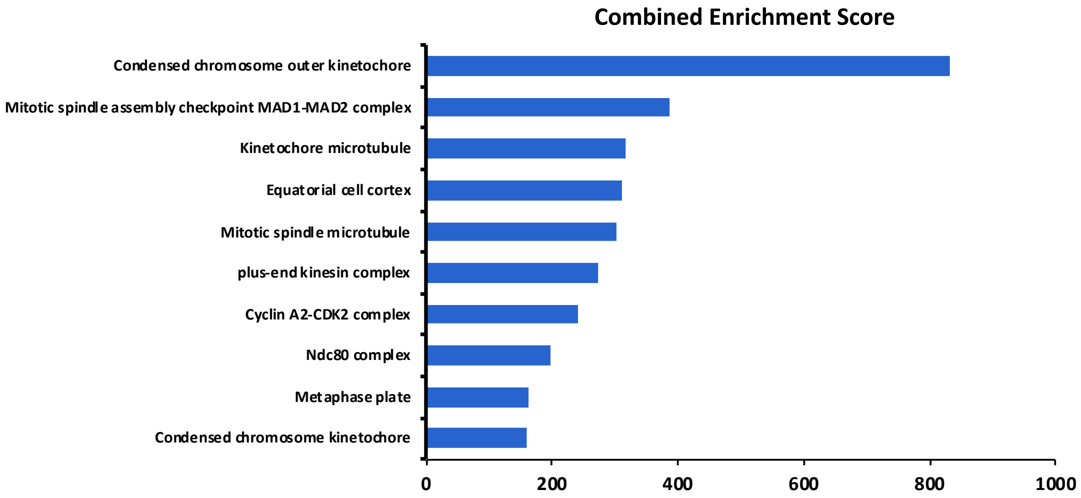

Figure 2.9: Up-regulated Gene Pathways: Jensen Compartments. The enrichment scores for genes up-regulated >2-fold (FDR<0.05) in Δ*Gnas* DCs. Solid bars are pathways that were statistically significant (p-adjusted<0.05).

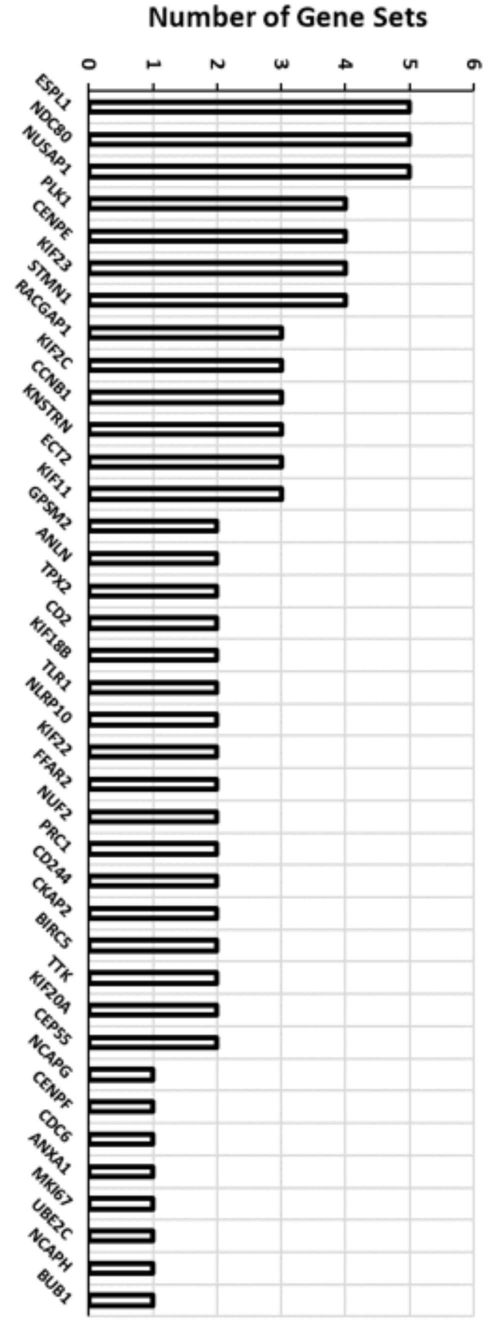

Figure 2.10: Leading Edge Analysis of the Top 10 Enriched GO Biological Processes. These genes are likely to be central to biological processes that are differentially regulated. In total, 38 genes are associated with at least 1 of these processes, of which 7 are associated with 4 or more. These 7 genes all have functions related to the cytoskeleton, microtubules, or mitosis.

Microtubules are hollow tubes comprised of polymerized α- and β-tubulin dimers. RNA-Seq analysis showed that the β-tubulin gene *Tubb2b* was up-regulated >3-fold in Δ*Gnas* DCs (**Figure 2.11A**, FDR<0.0001). We confirmed that *Tubb2b* gene expression was increased in Δ*Gnas* DCs by independent qPCR on multiple biological replicates (**Figure 2.11B**). Immunofluorescence of α-tubulin showed an increase in α-tubulin staining in untreated Δ*Gnas* DCs and in WT DCs treated for 24 hrs with the PKA inhibitor PKI (**Figure 2.11C - E**). Treatment for 24 hrs with the cAMP analog CPT reduced α-tubulin staining in Δ*Gnas* DCs (**Figure 2.11F**). Thus, Δ*Gnas* DCs have increased expression of α- and β-tubulin which form microtubules and this up-regulated expression is mediated by a decrease in cyclic AMP levels and reduced PKA activation. Microtubules are involved in cell migration and intracellular transport, suggesting that changes in microtubule function could contribute to the induction of Th2 differentiation in Δ*Gnas* DCs.

ΔGnas DCs have altered gene expression of cyclic AMP signaling pathway components

Isolated DCs from Δ*Gnas* mice display aberrant cyclic AMP signaling; they produce less cAMP after stimulation by the adenylyl cyclase activator forskolin and $G\alpha_s$ agonists isoproterenol and PGE_2 than WT DCs[1]. To assess if altered expression of cAMP signaling components occurs in DCs that lack $G\alpha_s$ and have lower cAMP, we compared the RNA-Seq data of WT and Δ*Gnas* DCs.

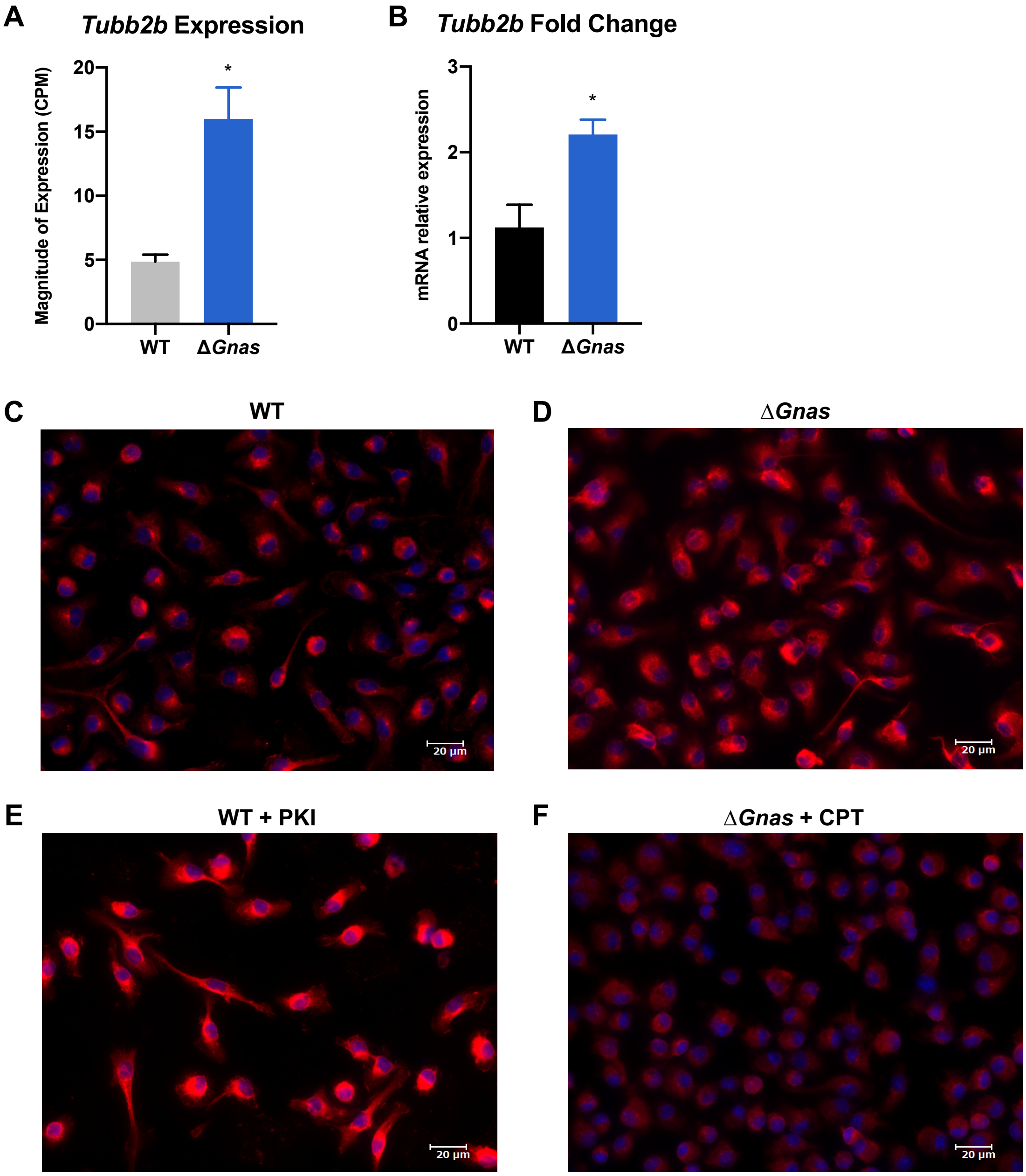

Figure 2.11: Tubulin Expression in DCs. A) Δ*Gnas* DCs had increased gene expression of the β-tubulin gene *Tubb2b* in RNA-Seq data sets. n=4-5, FDR<0.05. **B)** The increase in *Tubb2b* gene expression was reproduced in independent samples, as analyzed by qPCR. n=4, p<0.05. Immunofluorescence showed that **C)** WT DCs had less α-tubulin staining than **D)** Δ*Gnas* DCs and **E)** WT DCs treated for 24 hrs with 10μM PKI. **F)** Treatment with CPT (50μM) for 24 hrs reduced α-tubulin staining in Δ*Gnas* DCs. Images are representative of 3 independent experiments.

Overall, WT and Δ*Gnas* DCs expressed a similar number of GPCRs. *Gnas* DCs have a slightly higher number of lowly expressed GPCRs (0.2-1 TPM) than their WT counterparts (**Tables 2.6, 2.7**). On average, Δ*Gnas* DCs expressed 135 GPCRs and WT cells expressed 128. Both genotypes had similar proportions of GPCRs classified according to their G protein linkages (**Figure 2.12A - B**). In both WT and Δ*Gnas* DCs, $G\alpha_i$-linked receptors form the largest group of GPCRs, followed by $G\alpha_q/G\alpha_{11}$, $G\alpha_s$, and $G\alpha_{12}/G\alpha_{13}$. When GPCRs were weighted according to expression, such that highly expressed receptors are given more weight than lower expressed GPCRs, Δ*Gnas* cells had decreased representation of $G\alpha_i/G\alpha_o$-linked GPCRs, but unchanged gene expression of all the $G\alpha_i$ isoforms *Gnai1*, *Gnai2*, and *Gnai3* (which encode $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, respectively) (**Figure 2.12C - D, Figure 2.13**)[9].

The 3 highest expressed GPCRs in both WT and Δ*Gnas* DCs were the chemokine receptors CCR7, CCR2, and CXCR4 (**Figure 2.14**). Other chemokine receptors had high levels of expression as well, including CCR5 and CCR1. Multiple adhesion GPCRs (e.g., ADGRE1 and ADGRE5), prostaglandin receptors (e.g., PTGER4 and PTGIR) and proton-sensing GPCRs (e.g., GPR132, GPR65, and GPR68) also had high levels of expression in DCs. 24 GPCRs had statistically significant changes in gene expression in Δ*Gnas* cells, of which 11 were increased and 13 were decreased (**Figure 2.15**).

Table 2.6: Number of GPCRs in WT DCs. Biological replicates of WT DCs expressed similar numbers of GPCRs, both overall and when stratified according to level of expression. On average, WT DCs expressed 128 GPCRs.

	WT 3	**WT 4**	**WT 5**	**WT 6**	**WT 7**	**Avg**	**Std Dev**
0.2 - 1 TPM	41	44	34	34	36	38	4.0
1 - 10 TPM	51	52	52	48	44	49	3.1
10-50 TPM	25	26	25	22	26	25	1.5
>50 TPM	15	15	14	20	14	16	2.2
Total	132	137	125	124	120	128	6.1

Table 2.7: Number of GPCRs in Δ*Gnas* DCs. Biological replicates of Δ*Gnas* DCs expressed similar numbers of GPCRs, both overall and when stratified according to level of expression. Δ*Gnas* DC express more GPCRs than WT cells do, with an average of 135.

	KO 3	**KO 4**	**KO 6**	**KO 7**	**Avg**	**Std Dev**
0.2 - 1 TPM	54	39	51	42	47	6.2
1 - 10 TPM	44	51	45	51	48	3.3
10-50 TPM	27	31	26	25	27	2.3
>50 TPM	16	11	13	14	14	1.8
Total	141	132	135	132	135	3.7

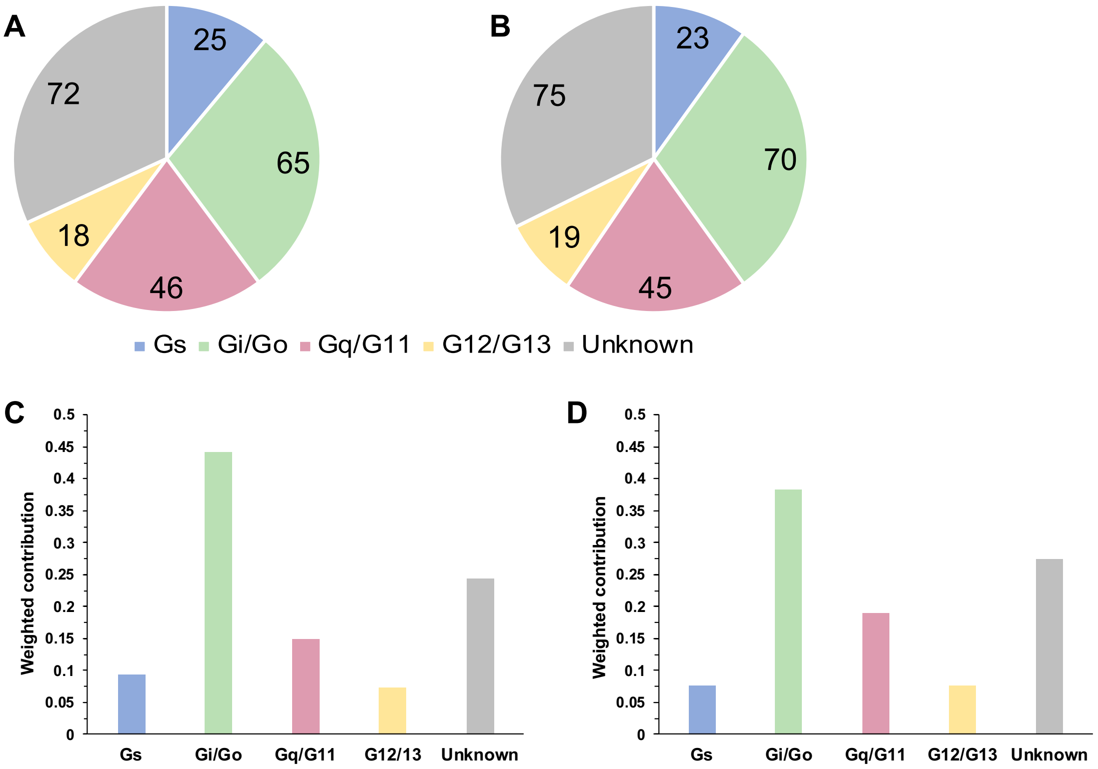

Figure 2.12: GPCR-G protein Linkages in WT and Δ*Gnas* DCs. A) WT and **B)** Δ*Gnas* DCs display similar ratios of GPCRs in regard to their G protein-linkages. **C)** WT and **D)** Δ*Gnas* DCs had similar weighted GPCR-G protein linkages, with Δ*Gnas* DCs having a small decrease in GPCR-Gα_i tone. Ratios of weighted GPCR-G protein linkages were calculated such that higher expressed genes were given more weight than lower expressed genes[9]. WT n=5, Δ*Gnas* n=4.

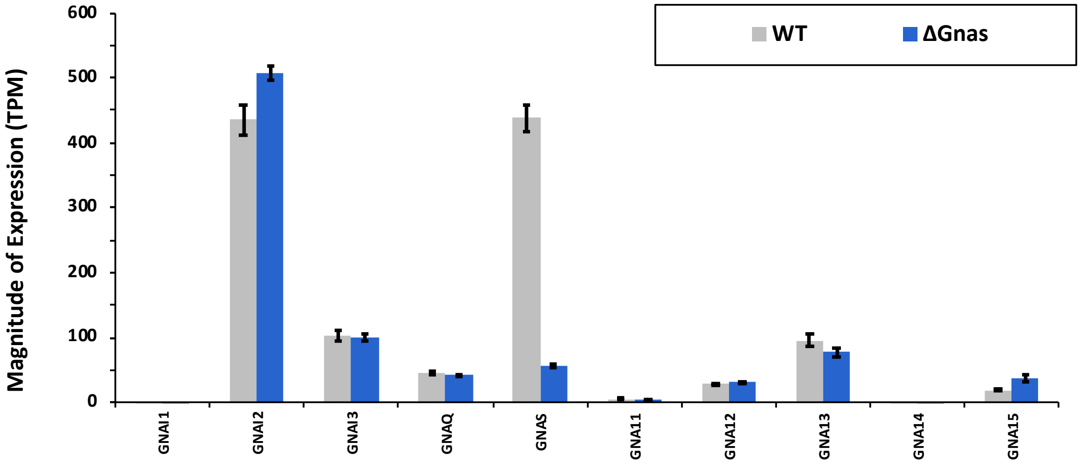

Figure 2.13: Gene Expression of Gα Proteins. Δ*Gnas* DCs had a difference in the gene expression of *Gnas* (FDR<0.05). No other Gα genes were differentially expressed. WT n=5, Δ*Gnas* n=4.

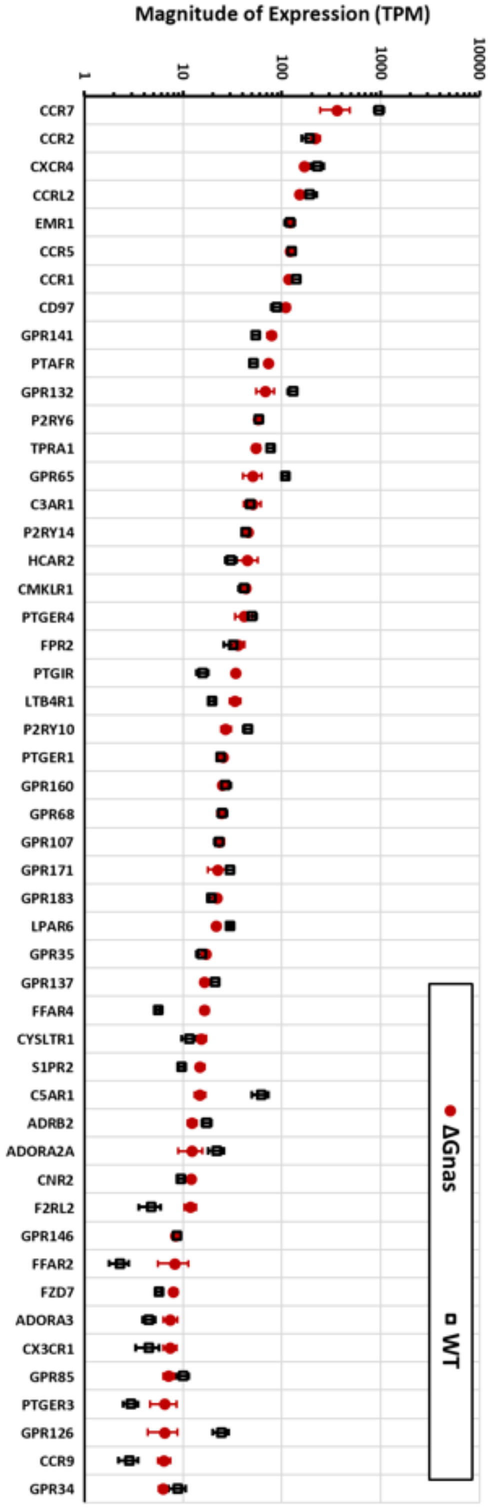

Figure 2.14: Highest Expressed GPCRs. The 50 highest expressed GPCRs and their expression in WT (**Black**) and Δ*Gnas* (**Red**) DCs.

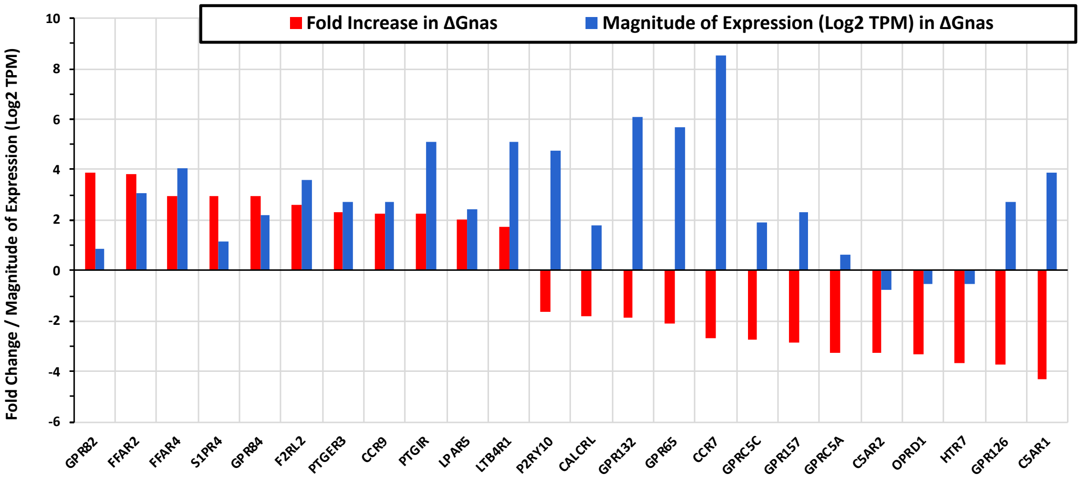

Figure 2.15: Differentially Expressed GPCRs in Δ*Gnas* DCs. 24 GPCRs had statistically significant changes in gene expression in Δ*Gnas* DCs compared to WT cells and are graphed with their fold change (**Red**) and level of expression (**Blue**) in Δ*Gnas* DCs. n=4-5, FDR<0.05.

We assessed the gene expression of cAMP pathway signaling components downstream of GPCRs: $G\alpha$ proteins, adenylyl cyclases, Protein Kinase A (PKA) subunits, Epac, A-kinase anchoring proteins (AKAPs), the cyclic nucleotide transporter MRP4, PDEs, and cAMP-responsive transcription factors. The genes *Gnas* which encodes $G\alpha_s$, *Crem* which encodes the transcription factor CREM, *Prkar2b* which encodes PKA regulatory subunit RIIβ, and the 3 phosphodiesterase genes *Pde3b*, *Pde4b*, and *Pde4d*, had decreased expression in Δ*Gnas* DCs (**Table 2.8**, **Figures 2.16, 2.17, 2.18**). The expression of other components of the cyclic AMP pathway was unchanged.

Table 2.8: Differentially Expressed Genes Related to the cAMP Pathway. In addition to a decrease in Gα_s expression, ∆*Gnas* DCs also had decreased expression of other genes relating to the cAMP pathway. No cAMP signaling pathway components downstream of GPCRs had increased expression in ∆*Gnas* DCs compared to WT DCs. n=4-5.

Gene	Fold Change	FDR	CPM (Expression)
Gnas	-7.78	2.4E-33	268.2
Pde4d	-5.72	7.05E-13	10.6
Crem	-3.70	1.15E-09	39.7
Pde3b	-2.85	1.15E-06	7.6
Prkar2b	-2.38	1.62E-06	19.6
Pde4b	-2.58	6.36E-06	110.0

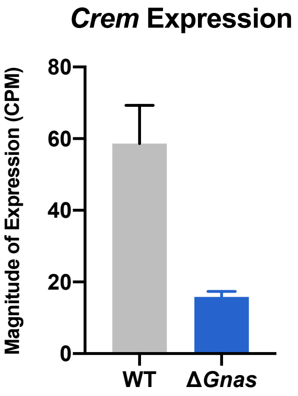

Figure 2.16: *Crem* Expression in WT and Δ*Gnas* DCs. Δ*Gnas* DCs had a 3.7-fold decrease in *Crem* expression compared to WT cells. CPMs are graphed. WT n=5, Δ*Gnas* n=4. FDR<0.05.

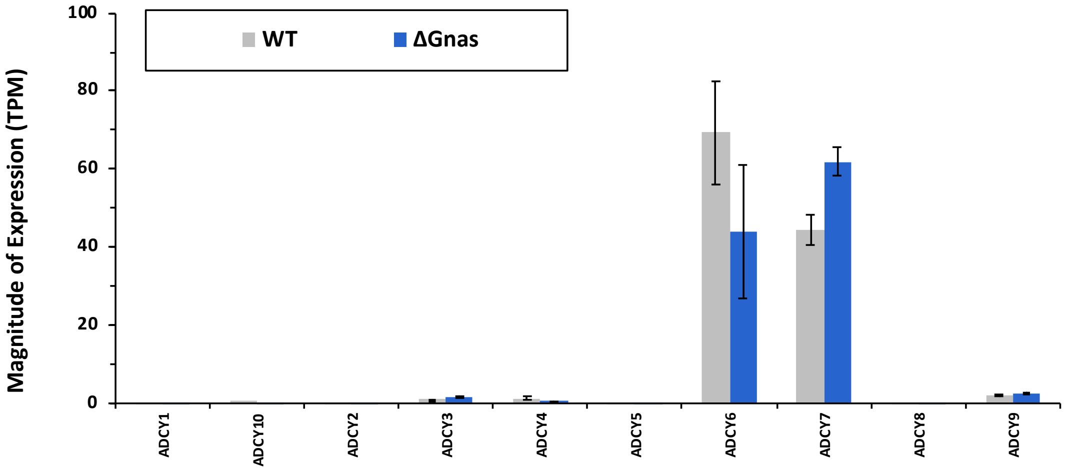

Figure 2.17: Adenylyl Cyclase Gene Expression in WT and Δ*Gnas* DCs. Δ*Gnas* DCs had no statistically significant changes (FDR<0.05) in the expression of any of the 10 adenylyl cyclase genes (9 membrane bound, 1 soluble). WT n=5, Δ*Gnas* n=4.

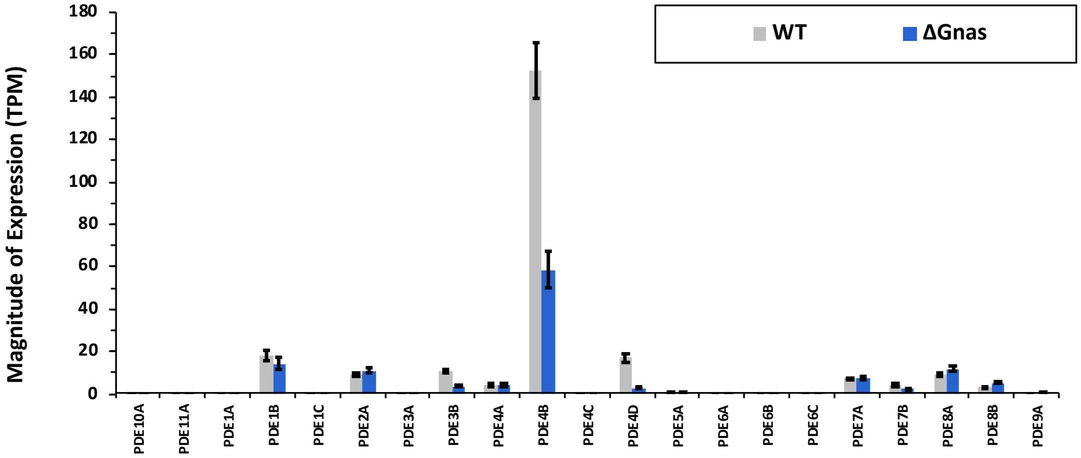

Figure 2.18: Phosphodiesterase Gene Expression in WT and Δ*Gnas* DCs. Δ*Gnas* DCs had significantly decreased expression of the PDEs *Pde3b*, *Pde4b*, and *Pde4d*. *Pde4b* is the predominant PDE isoform in both WT and Δ*Gnas* DCs. WT n=5, Δ*Gnas* n=4. FDR<0.05.

Discussion

In this study, we sought to uncover the mechanism behind the increased Th2 induction of ∆*Gnas* DCs and identify potential candidates for further study. Transcriptomic analysis of WT and ∆*Gnas* DCs identified an increase in microtubule-associated genes which needs further study, but might be utilized as a potentially novel therapeutic for asthma.

∆*Gnas* DCs had 1,242 genes with statistically significant gene expression differences in comparison to WT cells. Most notably, ∆*Gnas* DCs had an increase in genes relating to microtubules and the kinetochore. DCs are terminally differentiated cells, so the increased expression of kinetochore- and mitotic spindle-associated genes was surprising[15,16]. Further investigation showed that many of these genes are also associated with microtubules, suggesting that changes in microtubule function perhaps have a role in the pro-allergic/Th2 phenotype of ∆*Gnas* DCs.

Certain prior studies have implicated a role for microtubule-associated genes in DCs. Gene array analysis of monocyte-derived DCs from young and aged human donors found that DCs from aged donors had reduced expression of genes involved in cell cycle arrest, DNA replication and repair, and microtubule-associated proteins[17]. Such results support the notion that the increase in microtubule-related genes in ∆*Gnas* DCs is a real effect and may be of physiological consequence. Of note, in aged mice, DCs are less effective in stimulating $CD4^+$ T cell proliferation[18]. DCs from aged humans have decreased phagocytosis of antigens, secrete more of the pro-inflammatory cytokines TNF-α and IL-6 in response to LPS stimulation, and have reduced capacity to migrate in response to MIP-3β and SDF-1[19–21].

Limited data exist regarding the function of microtubules in DCs. Certain data implicate their role in DC stimulation of $CD8^+$ T cells. Cross-presentation is the process in which DCs uptake extracellular antigens and present them on MHC class I molecules to activate cytotoxic $CD8^+$ T cells instead of loading them on MHC class II molecules to present them to helper $CD4^+$ T cells as they usually do[22]. This action contributes to host defense against tumors and many viruses, but is unlikely to be important in asthma where pathology is influenced by the cytokines secreted by $CD4^+$ T cells and not the effects of $CD8^+$ cells. Treatment with colchicine, a microtubule depolymerizing agent, allowed for antigen internalization but ablated localization in the trans-Golgi area, indicating a functional microtubule-dependent transport system is required for cross-presentation in DCs[23]. Microtubule depolymerizing agents also increase cytotoxic $CD8^+$ T cell activity in mice and increase the efficacy of DC-based cancer vaccines[24]. While these data are not directly related to $CD4^+$ T cell differentiation, they do support the premise that microtubules affect DC function.

The microtubule depolymerizing agent ansamitocin P3 induced expression of the maturation markers CD80, CD86, CD40, and MHC-II on murine and human DCs[25]. Ansamitocin P3 treatment also induced proliferation of co-cultured $CD4^+$ and $CD8^+$ T cells at a comparable level to the positive control lipopolysaccharide (LPS) and increased lymph node homing and migration in tumor-resident DCs in mice *in vivo*[25]. The mechanism for Δ*Gnas* DCs' increased ability to induce CD4$^+$ Th2 differentiation has not yet been defined, but changes in microtubule function may contribute to it, perhaps by affecting their CD4$^+$ T cell stimulatory ability and/or migratory capacity.

Cyclic AMP is involved in the regulation of microtubule dynamics, although there is conflicting data related to this regulation. In PC12 cells (rat pheochromocytoma cells), activated GTP-bound Gα_s bound to tubulin, increased tubulin's GTP hydrolytic activity, and reduced microtubule stability[26]. PKA phosphorylation of microtubule-associated protein 2 (MAP2) reduces its microtubule-nucleating activity[27–29]. In contrast, PKA phosphorylation decreases the microtubule-destabilizing activity of Oncoprotein18 (Op18), which results in an increase in tubulin polymerization in human myelogenous leukemia K562 cells[30].

Increased cAMP levels also affect cell migration, with data indicating that increased cyclic AMP levels and PKA activation can have both a negative (inhibitory) and positive effects on cytoskeletal organization and cell migration[31]. These experiments have been done in different cell types (i.e. granulosa cells, keratinocytes, and pancreatic cancer cells, among others) which may contribute to the conflicting results[32–35]. In DCs, treatment with the PKA-specific cAMP analog N6-Benzoyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP), but not the Epac-specific cAMP analog 8ME, increased expression of the co-stimulatory molecules CD80 and CD86 and induced chemotaxis in response to CXCL12[36]. PGE_2 also increases expression of matrix metalloproteinase-9 which induces DC migration to sites of inflammation[37]. Decreased cAMP levels in DCs alter DC function[1]. Our data suggest that microtubules may contribute to it and may be a potential novel target to reduce Th2 inflammation in asthma.

GPCRs are attractive drug targets due to their cell-specific expression and accessibility from the extracellular space[38,39]. We identified 24 GPCRs with altered gene expression in Δ*Gnas* DCs. These GPCRs represent potential targets for reducing Th2 bias in DCs by increasing cAMP levels. We identified 3 $G\alpha_i$-linked GPCRs with increased expression that could be targeted with antagonists to raise cAMP (e.g., GPR84, PTGER3, and CCR9). In addition, 2 Gαs-linked GPCRs (e.g., GPR65 and HTR7) have decreased expression in Δ*Gnas* DCs. Agonists of these might raise cAMP levels in DCs and reduce DC-induced Th2 inflammation.

Crem encodes the cAMP-responsive element modulator (CREM), a CREB family transcription factor, which had decreased gene expression in Δ*Gnas* DCs. PKA activation leads to CREM phosphorylation; phosphorylated CREM can bind to CRE sites and initiate transcription of cAMP-responsive genes. However, some CREM variants function as suppressors of CRE-mediated transcription. Bone marrow-derived DCs from *Crem*$^{-/-}$ mice had increased CD86 expression and induced more antigen-dependent T cell proliferation[40]. Moreover, *Crem*$^{-/-}$ mice had a stronger inflammatory response in a mouse model of contact dermatitis than Crem$^{+/+}$ mice; thus, decreased *Crem* expression could also play a role in ∆*Gnas* mice's increased Th2 inflammation[40].

∆*Gnas* DCs also had decreased expression of the genes encoding PKA RIIβ (*Prkar2b*) and 3 cAMP-degrading phosphodiesterases (PDE3b, PDE4B, and PDE4D*)*. These changes are explored further in **Chapter Three**.

Acknowledgements

Chapter Two, in part, is currently being prepared for submission for publication of the material. The authors are Chinn, A.M., Sriram, K., Salmerón, C., Lee, J., Raz, E. & Insel, P.A. The dissertation author was the primary investigator and author of this material.

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CHAPTER THREE: THE PHOSPHODIESTERASE PDE4B IS A REGULATOR OF CELLULAR COMPENSATION FOR CHRONICALLY DECREASED CYCLIC AMP LEVELS

Abstract

Dendritic cells (DCs) from CD11c$^{\Delta Gnas}$ mice have a Gα_s knockout and reduced cyclic AMP (cAMP) responses after treatment with various agonists. We hypothesized that Δ*Gnas* DCs have chronically reduced cAMP levels and that (akin to what occurs in cells with increased cAMP levels), cells may compensate for decreased cAMP concentrations by altering the expression of components of the cAMP signaling pathway. Multiple G protein-coupled receptors (GPCRs), Protein Kinase A (PKA) RIIβ, and the phosphodiesterases PDE4B and PDE4D had altered mRNA expression in Δ*Gnas* DCs compared to WT DCs which may contribute to cellular compensation for decreased cAMP levels. We found that PDE4B expression is decreased in Δ*Gnas* DCs which have lower cyclic AMP concentrations. By contrast, expression of *Pde4b* and *Pde4d* increases in DCs if cAMP levels are increased, a response that is PKA-dependent. PDE4B thus acts as a regulator of cyclic AMP concentration and its expression rises and falls with changing cAMP levels in DCs to buffer changes in cAMP concentration and return the cell to cyclic AMP homeostasis. Unlike WT cells, Δ*Gnas* DCs co-cultured with naïve CD4+ T cells induce Th2

differentiation. Even with Δ*Gnas* DC's decreased PDE4B expression, PDE4B is still highly expressed in both WT and Δ*Gnas* DCs. Treatment with the PDE4B-specific inhibitor A33 reduced Δ*Gnas* DC-induced Th2 differentiation, suggesting that PDE4B inhibition may provide a novel means to increase cAMP levels and reduce Th2-associated inflammation.

Introduction

Cellular levels of cyclic AMP can have a profound impact on a cell's phenotype. As such, maintaining cAMP homeostasis is essential[1]. In immune cells, increased cAMP levels mediate anti-inflammatory effects, while decreased cAMP levels promote pro-inflammatory effects[2-19] (**Table 1.3**). When cAMP concentrations increase (e.g., by cAMP-elevating drugs), the expression of PDE4B and PDE4D increases[20–22]. This increase in expression of cAMP-hydrolyzing PDEs is likely an attempt to restore homeostasis by reducing the increased cAMP levels.

Treatment with antagonists of $G\alpha_s$-linked GPCRs and agonists of $G\alpha_i$-linked GPCRs decreases cellular cAMP concentrations, an approach used to treat a variety of diseases. Examples of this include the $G\alpha_s$-linked β_1-adrenergic receptor antagonist metoprolol to treat hypertension and congestive heart failure and the $G\alpha_i$-linked μ-opioid receptor agonist morphine as an analgesic[23–25]. Surprisingly, prior work has not defined how cells compensate for decreased cAMP levels. We hypothesized that since cells compensate for increased cAMP concentrations by altering the expression of cAMP pathway components to reduce cAMP levels, changes in these or other signaling components may occur in cells with decreased cAMP levels as a means to restore cellular homeostasis.

Experimental Procedures

Animals

C57BL/6J and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice were purchased from the Jackson Laboratory. CD11c$^{\Delta Gnas}$ mice were a kind gift from Eyal Raz (University of California San Diego, La Jolla, CA).

BMDC Isolation

Bone marrow was collected from murine femurs and tibiae and cultured for 6 days in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10% penicillin-streptomycin, 50μM 2-Mercaptoethanol, and 10ng/mL recombinant mouse GM-CSF (eBioscience). CD11c^{+} DCs were isolated from floating cells in the culture using CD11c magnetic beads (StemCell Technologies EasySep Mouse CD11c Positive Selection Kit II) per manufacturer's instructions.

DC2.4 Cells

DC2.4 cells were cultured at 5% CO_2 in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10% penicillin-streptomycin, 1X NEAA, 10mM HEPES, and 55μM 2-Mercaptoethanol.

S49 Cells

S49 cells were cultured at 10% CO_2 in DMEM with 4.5g/L glucose, 2mM L-glutamine, 10% heat-inactivated horse serum, 1mM sodium pyruvate, and 10mM HEPES. Cells were continuously maintained in logarithmic growth.

Real-Time Quantitative PCR (qPCR)

RNA was isolated using RNeasy Mini Kit (Qiagen) and converted to cDNA (Bio-Rad iScript cDNA Synthesis Kit). qPCR was run on Bio-Rad CFX Connect Real-Time PCR Detection System using PerfeCTa SYBR Green SuperMix (Quanta Bio). dCt values were calculated by subtracting the Ct value of the housekeeping gene 18S from the Ct value of the gene being evaluated. Fold change between 2 genes was calculated by the formula $2^{-(dCT_{\text{Gene 1}} - dCt_{\text{Gene 2}})}$.

All primers were designed with Primer-BLAST using thermodynamic oligo alignment and analyzed with Integrated DNA Technologies (IDT) OligoAnalyzer Tool to minimize self- and hetero-dimer tendencies for each primer set[26,27]. Primer sequences are listed in **Table 3.1**.

Table 3.1: Primer Sequences for qPCR. Primers were designed using Primer-BLAST.

Primer	Forward (5’-3’)	Reverse (5’-3’)
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Adcy1	TCCACATCACAAAGACGACC	CATCCTCTTGGCTGGTTTGA
Adcy2	CCAGTCCTACGATTGTGTCT	CACTGAACTTCGGCTTGGAAA
Adcy3	TCCATGACCAGAAAGAACGT	GGAAGCGTAGACACTCGATG
Adcy4	TTCTTCACACTCCTGGTCCT	TACGACTGGTGGTAGAGGTC
Adcy5	GGGGATTGTTACTACTGCGT	CGTCGAATTGCCACTTTCTG
Adcy6	GACCTTCCTCATACTTGGCG	TCAGGGTTCAGAGCATCTTG
Adcy7	CAGCATCGCCTTCAGTCAT	CTCGACATACACCAGCACAT
Adcy8	AGCATCACTGTCTTCGCATT	ACACCACATAGCACAGAACC
Adcy9	TTCCATTGTGATGTCCCCCT	GATCTTGGTGCGGTGTAGG
Adcy10	GGTATGGCATATCTCATCTGC	TAGTCGATCTCCCTAACACGT
Akap1	ATGAGGTGGAGATTCGCTAC	GTGCTGTATTGCCTGTCATC
Akap2	GCTGAAGGAGAAAGAGCCAA	TCAGTGTGGTGAGTGTGAAC
Akap3	TTGTACTTTGCTGGTGACGA	CGTGACATTTCCCACTGCTT
Akap4	GAAGAGCAATGCCAAGACAAT	TTCATCACGGATTCTACCAGC
Akap5	AGAACCCAGTAATGGCATCC	TCTTCAGCAGCAGTCTTTCT
Akap6	CAGCGAGCAGTATACCAGTG	TGTCAATCAGCCAATCCCAG
Akap7	CACTGTGAGTCTTCCATCGT	GCTTTTTGTTCTGCGTCTCT
Akap8	GAGAGCCCTAAACCCAAACC	AGGAATCAGCATGTCACAGG
Akap9	GGAGTTGGAAGAGATGAGGG	TGAGTTATGATGCCGTCTCG
Akap10	ACCAGATTGAAGTGCTGACC	TGGCTTGGAGGGAAAAGTAC
Akap11	GCAGAGAGAGAGTTGAGCAA	CTGTCTTCACCAACACTGAGA
Akap12	CCAGTCACTCAAACAACCGA	TGTCCTCTCCATGCTGAAGA
Akap13	GAGGATAGGGGATGTGCTTG	AGTTGACAGACTGGTTGTGC
Akap14	ACCAGAAAAGGGGCGAAAA	CAGCATAATACACCCAGCG
Epac1	CCTCCTGTCATCCCCTTCA	TCGGCTTCTGAGTGGTGAT
Epac2	CTTCTGGCTCCTCCCTATGG	AAGGTCGCTGTGCTTATGTTT
Gnai1	GATTCGGCAGCGTACTATC	AGGTCATAGTCACTCAGGGC
Gnai2	CTTTGGCCGCTCACGAGAAT	GATCCACTTCTTGCGCTCAG
Gnai3	ATGGGACGGTTGAAGATTGA	CACGCCTGCTAGTTCTGAAG
Gnas	GCAGAAGGACAAGCAGGTCT	CCCTCTCCGTTAAACCCATT
Mrp4	CGGACACATGGATGATTTGC	AGAACCAGGAAGACGACTGA
Pde1a	TGCTTGAGAATCACCACGTC	TTGCTGAAAATGCCCTGACA
Pde1b	GACATCAGCCACCCAACTAA	CTGGACACTCTTTTCTGCCA
Pde1c	GAGAAAAGGCAGGCGAAAAG	CGGTGTTGGAGTGATCCTTC
Pde2a	GACCGATGGAGATGATGGAC	CCACACGTTCATACAGCTCT
Pde3a	GTACGCCGAGCAGATCCT	CCTCTCTTGTGGTCCCATTC
Pde3b	GAAGAGGCACAGCAACCAAA	TCCTTCCTGATTTTTCTCCCA
Pde4a	CTCTCTGCTACCTCCACAAC	GATGAAGAGGATTCATTGGA
Pde4b	TGTCGATCATCCTGGAGTCT	AAGATGTCGCAGTGTTCCTC
Pde4c	CAACTACTCTGACCGCATCC	TAGTCAATGAATCCCACCTGG
Pde4d	CATCCTGGGGTGTCAAATCA	GTTCTCTAGGACCGAGGAGT
Pde5a	CCCTGTTCCTTGTCTGTGAG	CAACTTCTGCATTGAACCGG

Table 3.1: Primer Sequences for qPCR, Continued.

Primer	Forward (5’-3’)	Reverse (5’-3’)
Pde6a	GTACCTCAACTTTGTGAACCTGA	CTGCCTCTCTATATCCGTGA
Pde6b	TGGAGCTAGTCAAATGTGGC	TCGATAGGCTTTGCTGACAG
Pde6c	CCACAGAGGCACCAATAACT	TTCCAGGTGATGTCGCTCTA
Pde7a	ACTTACACCTTGACGATGGC	TTTCACTCCACTGCTTGCTT
Pde7b	CAAGAAAAAGGTGAAACGAC	GTTGTGACCGTGGTAATCTT
Pde8a	CAAAGCGGTTTCCTCCAGAA	CTGAGCATTAAACTGTGGCG
Pde8b	TCAGTCCTTCCTCTTCTTCA	CCGGTTCTTGTCACTCTTAG
Pde9a	TTGACTACAGCAACGAGGAG	TCCAGTAAACAGTCCACCCA
Pde10a	TGAGAAAGGGATTGCTGGTC	TACCGCTGATCTTGTTCACC
Pde11a	ATCAAGTGAAGAAGTCCTGGG	CAGCTCCATGAACATCCGTA
Prkaca	CCCGAGATTATCCTGAGCAA	CACCTTCCCAGAGACGATTTT
Prkacb	CCTCCATTCTTTGCTGACCA	TTTATGTCACTCACGCCGTT
Prkar1a	TTGTTGAAGTGGGACGACTG	CGGTCCAACTTAACGCACT
Prkar1b	CTCGGCAGAAGTCAAACTCC	CGTCCAGGTGAGAAAAGAGC
Prkar2a	CTGGGGAAGTGAGCATCTTG	GGGCAAGTTCTCCGAAGTAC
Prkar2b	TAACAAACCAAGAGCAGCGT	TTCATAGGTGGCGATGTTCC

Cyclic AMP Assay

DCs were cultured at 350,000 cells/well in a white bottom, white walled 96-well plate and cultured overnight. Cells were stimulated with various drugs and then the media was removed so as to only measure intracellular cAMP and not excreted cAMP. Intracellular cAMP levels were then assayed by HitHunter cAMP Assay for Biologics (DiscoverX) per the manufacturer's instructions.

Western Blot

Isolated CD11c$^+$ DCs were washed twice with PBS and re-suspended in RIPA buffer, 1X protease inhibitor cocktail, and 1X phosphatase inhibitor cocktail. Samples were sonicated 3X and then centrifuged at max speed for 15 min at 4^{o}C. Supernatants were stored at -70^{o}C. Protein was quantified by Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were mixed with Laemmli Buffer and boiled at 95^{o}C for 10 min and run using Criterion Vertical Electrophoresis Cell (Bio-Rad) and transferred with Criterion Blotter (Bio-Rad). Membranes were blocked in 5% milk/1X TBST at room temp and incubated with primary antibody (anti-Gnas: Abcam ab83735; anti-PDE4B: LifeSpan Biosciences LS-B11018) at 4^{o}C overnight on a shaker. Blots were then washed, incubated with secondary antibody for 1 hr at room temp on a shaker, and visualized. Afterwards, they were stripped, washed, blocked, and re-incubated with anti- β-tubulin antibody (Abcam ab21057) as a loading control.

Phosphodiesterase Activity Assay

PDE activity was measured using Cyclic Nucleotide Phosphodiesterase Assay Kit (Enzo Life Sciences). Isolated CD11c$^+$ DCs were resuspended in PDE Assay Buffer and 1X protease inhibitor and sonicated. Samples were then centrifuged at 10,000 rpm for 10 min at 4^{o}C. Lysates were removed and desalted per manufacturer's instructions before being stored at -70^{o}C. Protein was quantified by Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Afterwards, 2.5μg of protein/sample were assayed according to the manufacturer's instructions.

BMDC-T Cell Co-culture

Bone marrow was collected from mouse femurs and cultured for 7 days in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10% penicillin-streptomycin, 50μM 2-Mercaptoethanol, and 10ng/mL recombinant mouse GM-CSF. CD11c$^+$ DCs were isolated from floating cells using CD11c magnetic beads (StemCell Technologies EasySep Mouse CD11c Positive Selection Kit II) per the manufacturer's instructions.

Splenocytes were isolated from the spleens of OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice and then naïve CD4$^+$ T cells were isolated from this splenocyte population using EasySep Mouse CD4$^+$ T cell Isolation Kit (StemCell Technologies).

Isolated DCs were treated with Ova (100μg/mL) for 24hrs and then co-cultured with isolated naïve OT-II CD4$^+$ T cells in a 1:1 ratio for 3 days in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10% penicillin-streptomycin, 50μM 2-Mercaptoethanol, and 10ng/mL recombinant mouse GM-CSF. CD4$^+$ OT-II T cells were then removed from the culture and stimulated with plate-bound anti-CD3 antibody (10μg/ml) and anti-CD28 antibody (1μg/ml) for 24hrs. Cytokine levels in the supernatant were assayed by ELISA kits (eBioscience) per manufacturer's instructions.

For inhibition of Th2 response by PDE4 inhibitors, WT or CD11c$^{\Delta Gnas}$ DCs were isolated as above and then incubated with A33, Ro 20-1724, or DMSO control for 24 hrs, washed, and then co-cultured with OT-II T cells.

Statistical Analysis

Statistical analysis was done using two-way ANOVA with Sidak's multiple comparisons test to correct for multiple comparisons. An unpaired t test was used to determine statistical significance when comparing two groups. A value of $p<0.05$ was considered statistically significant.

Results

Dendritic cells from CD11c$^{\Delta Gnas}$ (Δ*Gnas*) mice have expression of the gene *Gnas* which encodes Gα_s prominently inhibited under the control of the CD11c promoter. We used these Δ*Gnas* DCs as a system to study how cells compensate for chronically decreased cAMP levels. Isolated CD11c$^+$ Δ*Gnas* DCs have reduced cAMP accumulation in response to the adenylyl cyclase activator forskolin and the Gα_s-linked GPCR agonists isoproterenol and Prostaglandin E_2 (PGE_2)[2]. WT DCs that were incubated with Ova and then co-cultured with naïve CD4$^+$ helper T cells from OT-II mice (which have a transgenic T cell receptor that recognizes Ova) do not induce Th2 differentiation, with little to no IL-4 secreted by co-cultured CD4$^+$ T cells[2]. In contrast, CD4$^+$ T cells co-cultured with Δ*Gnas* DCs secrete IL-4 and have increased mRNA expression of the Th2 master transcriptional regulator GATA3; Δ*Gnas* DCs thus increase Th2 differentiation[2]. This effect can be ablated by treating Δ*Gnas* DCs with the cAMP analog 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT) prior to co-culturing them with CD4$^+$ T cells, implicating the role of aberrant cyclic AMP levels in the pro-Th2 phenotype of Δ*Gnas* DCs[2].

DCs from CD11c$^{\Delta Gnas}$ mice have chronically decreased cyclic AMP levels

DCs from ∆*Gnas* mice had a 45% reduction in their basal cAMP levels (**Figure 3.1A**), indicating they experience chronic, abnormally low cAMP concentrations as a result of reduced of reduced *Gnas* expression (**Figure 3.1B**). We then assessed the expression of cAMP signaling pathway components in WT and ∆*Gnas* DCs to examine if cells compensate for decreased cyclic AMP levels (**Figure 1.4**).

∆Gnas DCs have changes in their GPCR mRNA expression

To determine if GPCR expression is altered in cells with decreased cAMP levels, we performed RNA-sequencing (RNA-Seq) on WT and ∆*Gnas* DCs to determine their GPCR mRNA profiles. Data were validated by qPCR on selected genes (data not shown). ∆*Gnas* cells expressed more GPCRs than WT cells did (**Tables 2.6, 2.7**). Classifying GPCRs by their G protein-linkage revealed that $G\alpha_i$-linked GPCRs were the largest group of receptors (**Figure 2.12A - B**). Twenty GPCRs had changed (either increased or decreased) gene expression >2-fold in ∆*Gnas* DCs (FDR<0.05) (**Figure 2.15**). Seven of these GPCRs linked to $G\alpha_i$, four of which had increased expression >2-fold (*S1pr4*, *Gpr84*, *Ptger3*, and *Ccr9*) and three of which had decreased expression >2-fold (*Ccr7*, *Oprd1*, and *C5ar1*). Weighting GPCR expression for the expression of each receptor (higher expressed receptors given more weight) revealed that

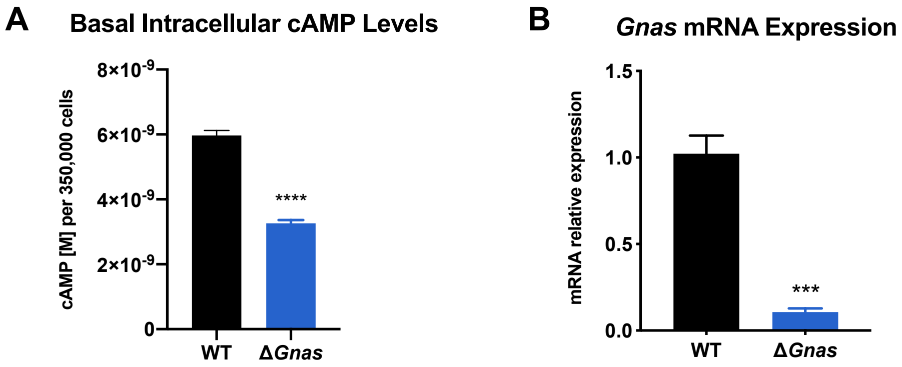


Figure 3.1: Basal Cyclic AMP Levels and *Gnas* mRNA Expression in WT and Δ*Gnas* DCs. A) Δ*Gnas* DCs have a 45% reduction in their basal cyclic AMP levels. n=5-6, $p<0.0001$. **B)** Δ*Gnas* DCs have reduced *Gnas* gene expression. n=6, $p<0.001$.

Δ*Gnas* cells had a small decrease in the overall GPCR-Gα_i tone when compared to WT DCs (**Figure 2.12C - D**). Cells with decreased basal cAMP levels thus have changes in their GPCR mRNA expression which may contribute to cellular compensation for decreased cAMP levels.

ΔGnas DCs have unaltered Gα_i and adenylyl cyclase mRNA expression

In contrast with the altered gene expression of multiple G_i-linked GPCRs, mRNA expression of the 3 Gα_i isoforms Gα_{i1}, Gα_{i2}, and Gα_{i3} (encoded by the genes *Gnai1*, *Gnai2*, and *Gnai3*, respectively) were unchanged in Δ*Gnas* DCs as assessed by qPCR (**Figure 3.2B**). RNA-Seq also showed that there were no significant differences in the mRNA expression of any of the Gα proteins except for Gα_s (encoded by *Gnas*) (FDR<0.05) (**Figure 2.13**). *Gnai2* was the highest expressed Gα in bone marrow-derived DCs, followed by *Gnas* (**Figure 3.2A**).

Mice express 9 membrane-bound adenylyl cyclase and 1 soluble adenylyl cyclase. We used qPCR to determine the mRNA expression of all 10 adenylyl cyclases. *Adcy6* (which encodes the membrane-bound adenylyl cyclase 6) was the highest expressed adenylyl cyclase and there were no changes in adenylyl cyclase expression of Δ*Gnas* DCs (**Figure 3.3A - B**). Results were confirmed by RNA-Seq as well (**Figure 2.17**). Thus, DCs do not compensate for decreased cAMP levels by changing the mRNA expression of the adenylyl cyclases which produce cAMP nor Gα_i which inhibits adenylyl cyclase.

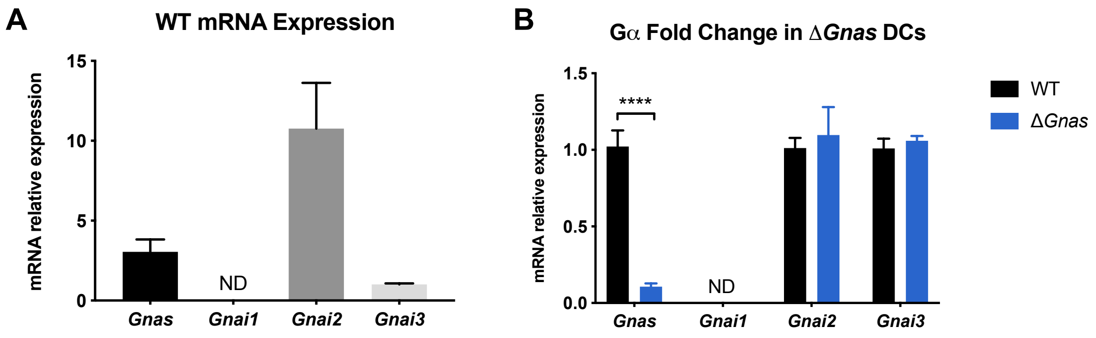


Figure 3.2: Gα mRNA Expression in WT and Δ*Gnas* DCs. A) *Gnai2* is the highest expressed Gα in WT DCs, followed by *Gnas*. n=6. **B)** Δ*Gnas* DCs have reduced *Gnas* gene expression. n=6, p<0.001.

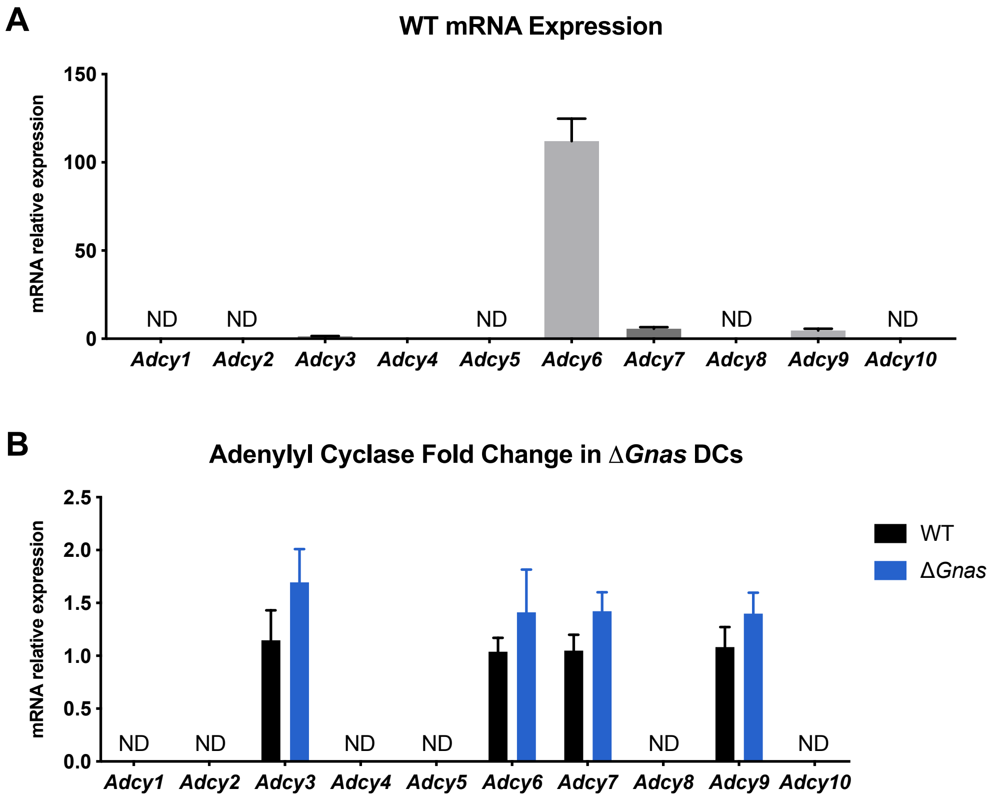


Figure 3.3: Adenylyl Cyclase Gene Expression in WT and Δ*Gnas* DCs.
A) *Adcy6* is the highest expressed adenylyl cyclase in WT DCs. n=3.
B) Δ*Gnas* DCs have no changes in their mRNA expression of the 9 membrane-bound (*Adcy1*-*9*) and soluble (*Adcy10*) adenylyl cyclase. n=6.

∆Gnas DCs have increased mRNA expression of Akap6

A-kinase anchoring proteins (AKAPs) are scaffold proteins that tether protein complexes, including PKA, to different subcellular regions of the cell. We used qPCR to define the AKAP mRNA profile of DCs by qPCR and found that *Akap13* (AKAP-Lbc) is the highest expressed AKAP in these cells (**Figure 3.4A**). Of the 14 different AKAPs, only *Akap6* (mAKAP) had altered expression in ∆*Gnas* DCs, increasing <2-fold (**Figure 3.4B**). However, *Akap6* is very lowly expressed and so it is dubious if this increase in mRNA expression impacts cell physiology.

DCs with low cyclic AMP levels have decreased gene expression of the cAMP effector Protein Kinase A RIIβ

cAMP mediates its effects primarily through protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). There are 2 Epac genes (*Epac1* and *Epac2*) and neither had detectable expression in CD11c$^+$ DCs as assayed by qPCR (threshold of detection set at dCt<25) or RNA-Seq (threshold of detection set at TPM>0.2) (data not shown).

The genes for the 4 regulatory PKA subunits RIα, RIβ, RIIα, RIIβ are *Prkar1a*, *Prkar1b*, *Prkar2a*, and *Prkar2b*, respectively, of which PKA RIα has the highest gene expression (**Figure 3.5A**). There are 2 PKA catalytic subunits Cα and Cβ in murine cells which are encoded by *Prkaca* and *Prkacb*; PKA Cγ subunit is not expressed in the mouse[28].

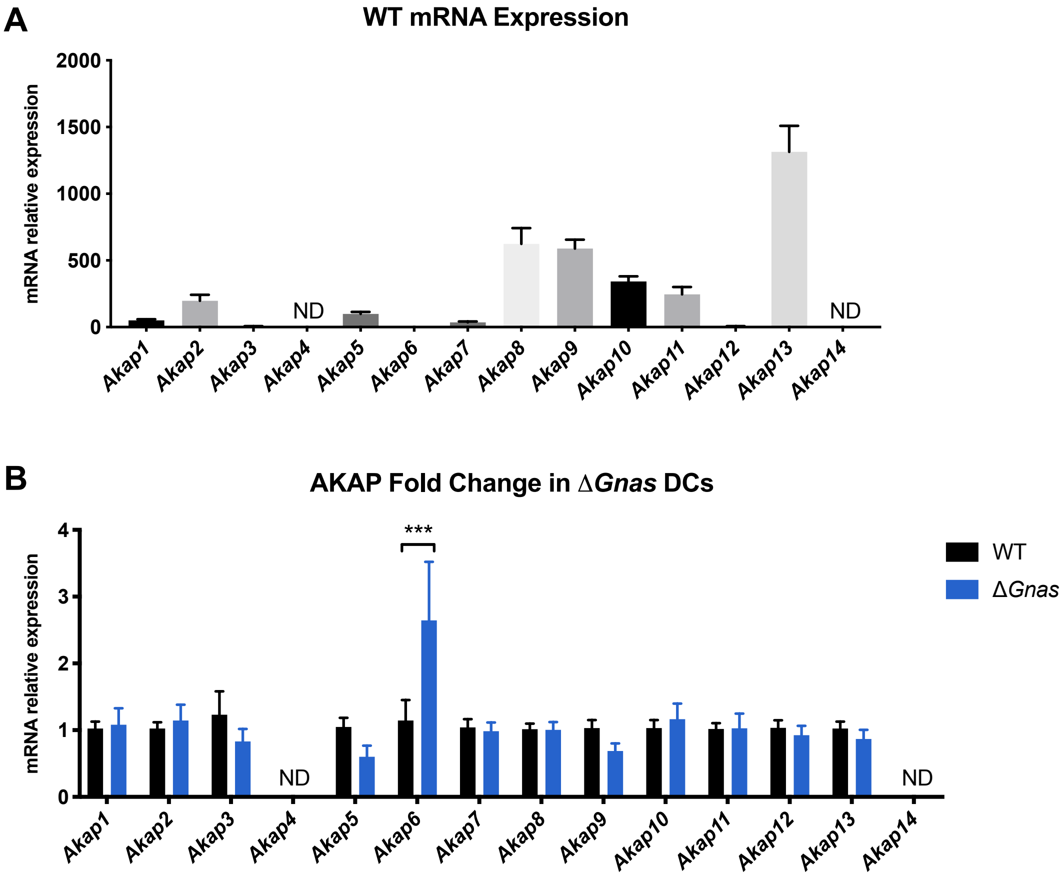


Figure 3.4: AKAP Gene Expression in WT and Δ*Gnas* DCs. A) *Akap13* is the highest expressed AKAP in WT DCs. n=6. **B)** Δ*Gnas* DCs have increased expression of the lowly expressed *Akap6* gene. n=6, p<0.001.

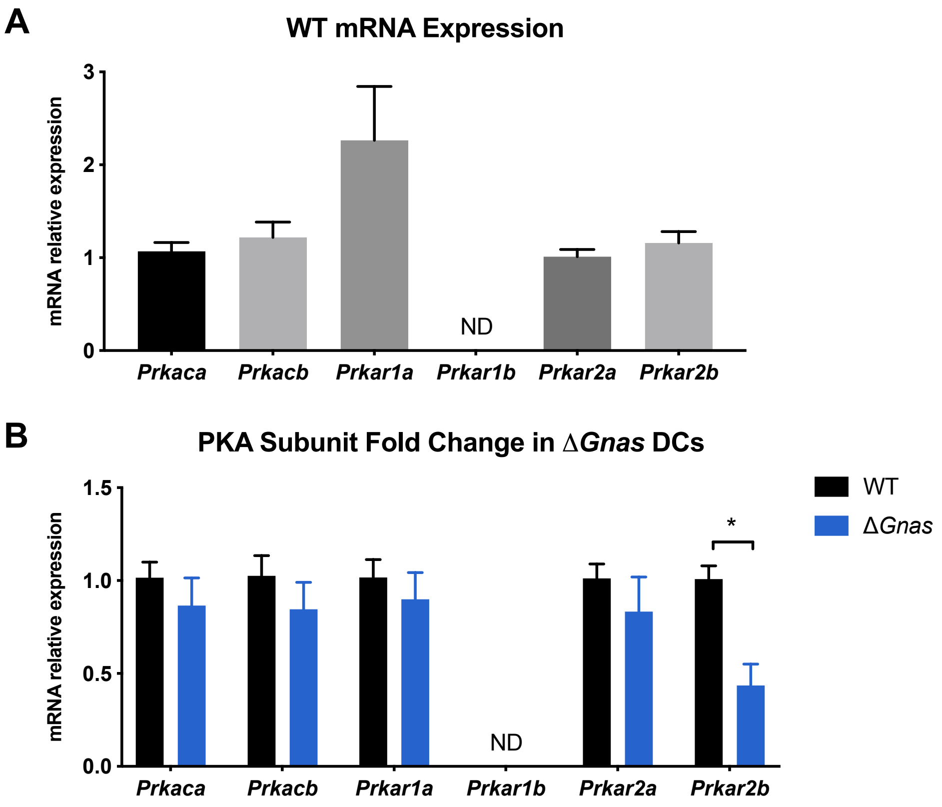


Figure 3.5: PKA Subunit mRNA Expression in WT and Δ*Gnas* DCs.
A) *Prkar1a* is the highest expressed regulatory subunit in WT DCs. n=5.
B) Δ*Gnas* DCs have a >55% decrease in *Prkar2b* expression. n=5, $p<0.05$.

We found that Δ*Gnas* DCs had a >55% reduction (or >2-fold decrease) in *Prkar2b* mRNA which encodes the PKA regulatory subunit RIIβ, a result confirmed by RNA-Seq (**Figure 3.5B, Table 2.8**). Thus, there appears to be a feedback loop between cyclic AMP levels and PKA RIIβ gene expression.

Since *Prkar2b* expression was decreased in Δ*Gnas* DCs which have reduced basal cAMP levels, we tested if its expression would increase in response to increased cAMP levels. To determine this, WT and Δ*Gnas* DCs were treated with PGE_2, a $G\alpha_s$-linked GPCR agonist which raises endogenous cAMP levels (**Figure 3.6A**). PGE_2 treatment for 24 hrs did not increase *Prkar2b* expression in either WT or Δ*Gnas* DCs (**Figure 3.6B**). Treatment with the cell permeable cyclic AMP analogs 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT, non-specific), N6-Monobutyryladenosine 3′,5′-cyclic monophosphate (6MB, cAMP-specific), and 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8ME, Epac-specific) for 24 hrs also did not alter *Prkar2b* expression (**Figure 3.6C**). Thus, while *Prkar2b* expression is decreased in response to chronically reduced cAMP concentrations, *Prkar2b* expression does not appear to change when cells experience increased cAMP levels for 24 hrs.

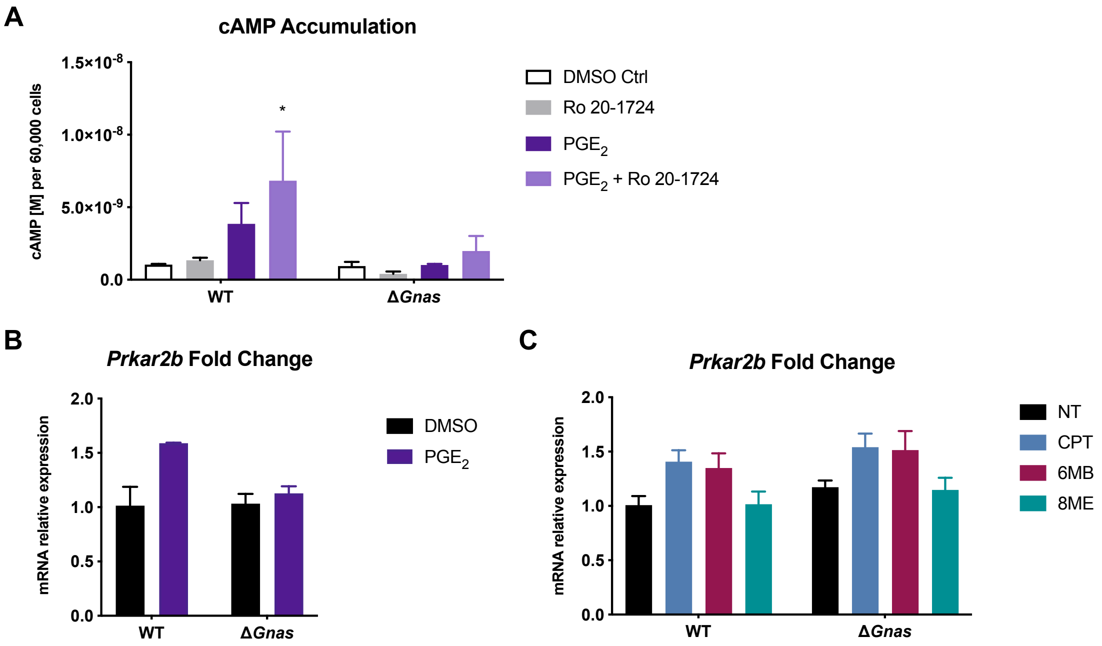


Figure 3.6: *Prkar2b* Gene Expression is Not Altered to Compensate for Increased Cyclic AMP Levels. A) Prostaglandin E_2 (PGE_2) treatment (10μM) for 30 min increased cAMP accumulation in DCs. The PDE4 inhibitor Ro 20-1724 (100μM for 1hr treatment) was used to boost cyclic AMP levels to enable detection in the assay. n=2, $p<0.05$. **B)** PGE_2 treatment (10μM) for 24 hrs did not affect *Prkar2b* expression in WT and Δ*Gnas* DCs. n=2-4. **C)** Treatment with the cAMP analogs CPT (non-specific, 50μM), 6MB (PKA-specific, 50μM), and 8ME (Epac-specific, 50μM) for 24 hrs did not affect *Prkar2b* expression in WT and Δ*Gnas* DCs. n=3.

PDE4B expression is decreased in ∆Gnas DCs to compensate for reduced cyclic AMP levels

cAMP is removed from the cell by being effluxed by the transporter multidrug resistance-associated protein 4 (MRP4 or ABCC4) and by hydrolysis by phosphodiesterases (PDEs). The mRNA expression of *Mrp4* was not changed in ∆*Gnas* DCs as determined by qPCR, indicating that ∆*Gnas* DCs do not alter their *Mrp4* gene expression in response to decreased cAMP levels (**Figure 3.7**).

Phosphodiesterases (PDEs) hydrolyze cyclic AMP, an important mechanism by which cells control their cAMP levels (**Table 1.2**). We determined the PDE mRNA profile of WT and ∆*Gnas* DCs by qPCR (**Figure 3.8A - B**) and confirmed our results with RNA-Seq data (**Figure 2.18**). Of the 21 PDEs in the mouse, WT DCs expressed the mRNA of 10 PDEs (*Pde1b*, *Pde2a*, *Pde3b*, *Pde4A*, *Pde4b*, *Pde4d*, *Pde5a*, *Pde7a*, *Pde7b*, and *Pde8a*; threshold of detection set at dCt>25), of which *Pde4b* is the predominant PDE in DCs. Only 2 PDEs had altered expression in ∆*Gnas* DCs compared to WT cells: *Pde4b* had a 50% reduction and *Pde4d* had a 60% reduction in mRNA levels (**Figure 3.8C**). *Pde4b* is highly expressed with a dCt = 6.8 in WT cells as determined by qPCR, while *Pde4d* is lower expressed with a dCt = 11.2. As dCt is on a Log_2 scale, *Pde4b* is thus expressed 20-fold higher than *Pde4d*. As such, we chose to focus on PDE4B for the remainder of our experiments.

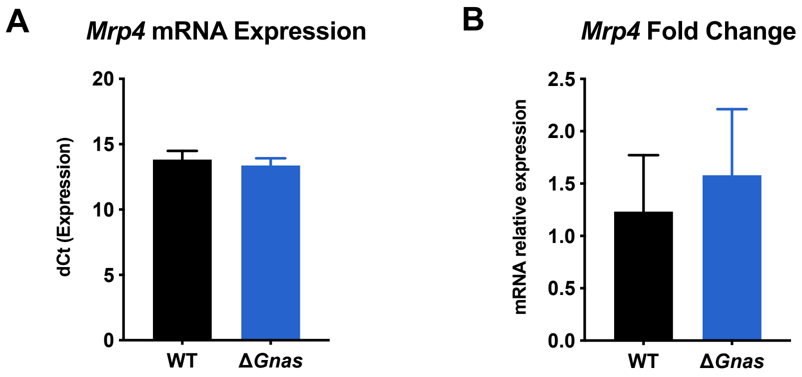


Figure 3.7: *Mrp4* mRNA Expression is Unchanged in WT and Δ*Gnas* DCs. **A)** dCt values relative to the housekeeping gene 18S on WT and Δ*Gnas* DCs. n=3. **B)** Δ*Gnas* DCs have unchanged *Mrp4* expression in comparison to WT cells. n=3.

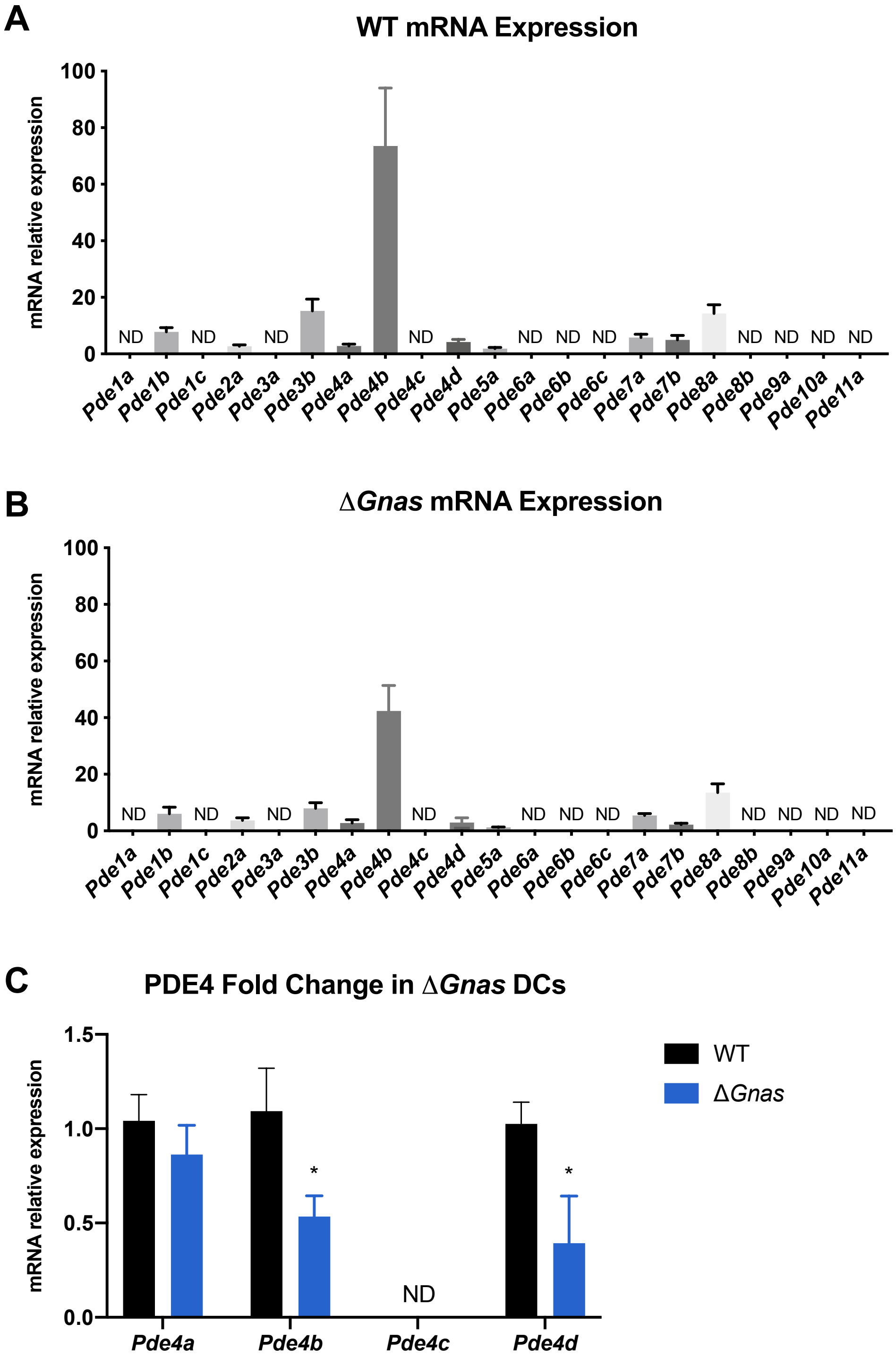


Figure 3.8: Phosphodiesterase Expression in WT and Δ*Gnas* DCs.
A) PDE mRNA profile of WT DCs. n=7. **B)** PDE mRNA profile of Δ*Gnas* DCs. n=6. **C)** PDE4 isoform fold changes in Δ*Gnas* DCs. Δ*Gnas* DCs have >50% reduction of *Pde4b* and *Pde4d* expression. n=5-6, $p<0.05$.

We verified that Δ*Gnas* DCs had decreased PDE4B protein expression through Western blotting (**Figure 3.9A - B**). Furthermore, Δ*Gnas* DCs had reduced overall phosphodiesterase activity as measured using a modified malachite green assay (**Figure 3.9C**). Treatment of WT DCs with the adenylyl cyclase inhibitor MDL-12,330A decreased *Pde4b* mRNA expression, confirming that the reduced *Pde4b* mRNA expression in Δ*Gnas* DCs is due to decreased cAMP levels and not the loss of $G\alpha_s$ (**Figure 3.10A**). Treatment of WT DCs with the endogenous protein kinase inhibitor (PKI) for 24 hrs reduced *Pde4b* mRNA in WT cells, suggesting that the change in gene expression is mediated by PKA (**Figure 3.10B**).

Both PDE4B and PDE4D degrade cAMP and have cAMP response elements (CRE sites) in their promoters, suggesting that when cells experience decreased cAMP levels, *Pde4b* and *Pde4d* expression decreases through CREB in an attempt to increase cAMP levels back toward ambient levels[20,29].

To test if the results obtained for *Pde4b* in DCs were observed in other cell types, we assessed its expression in wildtype (WT) and Kin^- T lymphoma S49 cells. Kin^- S49 cells have functional $G\alpha_s$, but no detectable PKA activity[30]. We found that Kin^- cells had an 80% decrease in their *Pde4b* mRNA levels compared to WT S49 cells, thus supporting the conclusion that decreased PKA activation is the likely mechanism for the decrease in *Pde4b* expression in Δ*Gnas* DCs as a compensation for decreased cyclic AMP concentrations (**Figure 3.10C**).

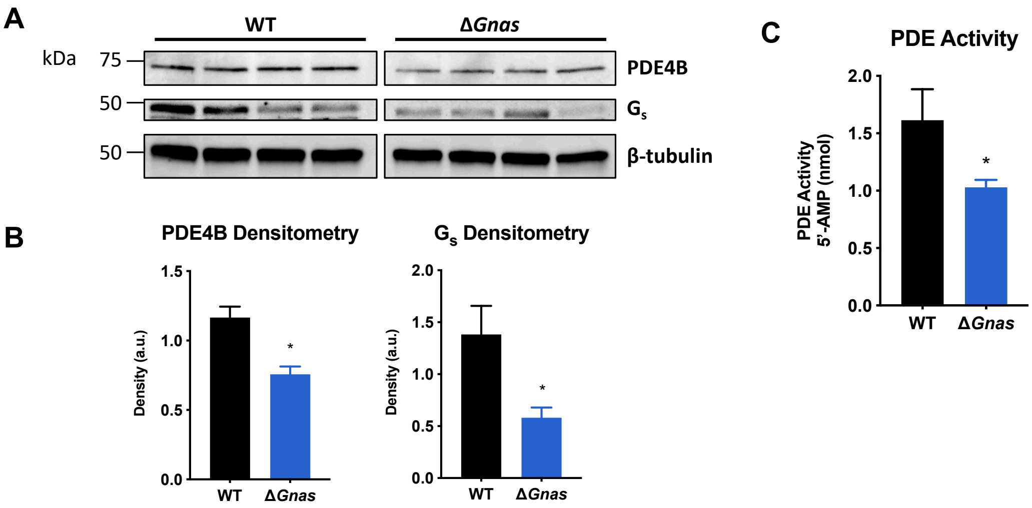


Figure 3.9: Δ*Gnas* DCs Have Decreased PDE4B Expression and Phosphodiesterase Activity. A-B) Western blotting of WT and Δ*Gnas* DCs showed that Δ*Gnas* DCs have decreased PDE4B and $G\alpha_s$ protein. n=4, $p<0.05$. **C)** Δ*Gnas* DCs have decreased overall PDE activity. n=5-6, $p<0.05$.

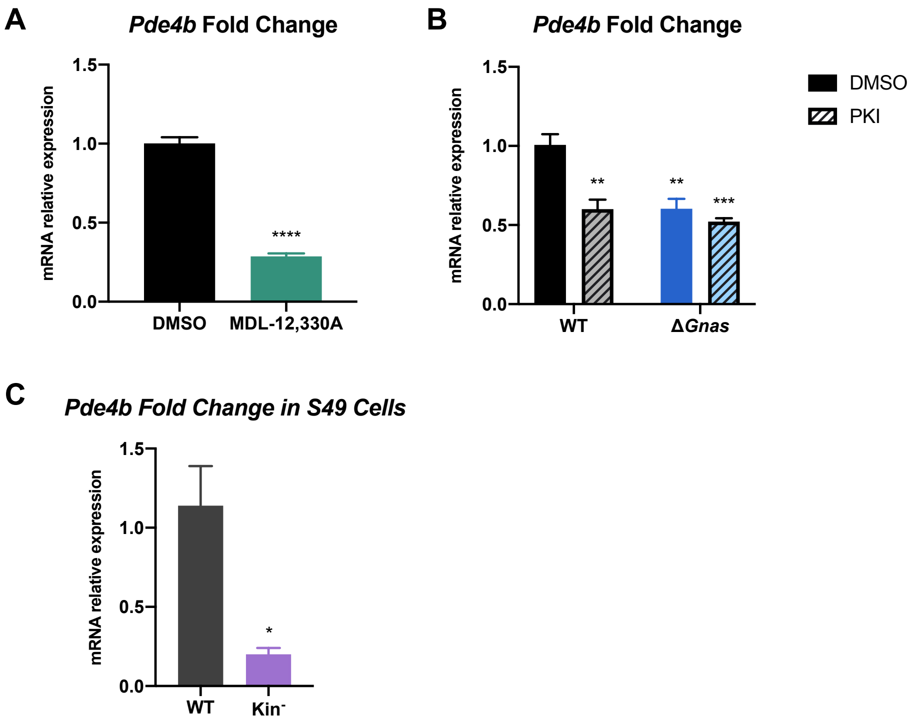


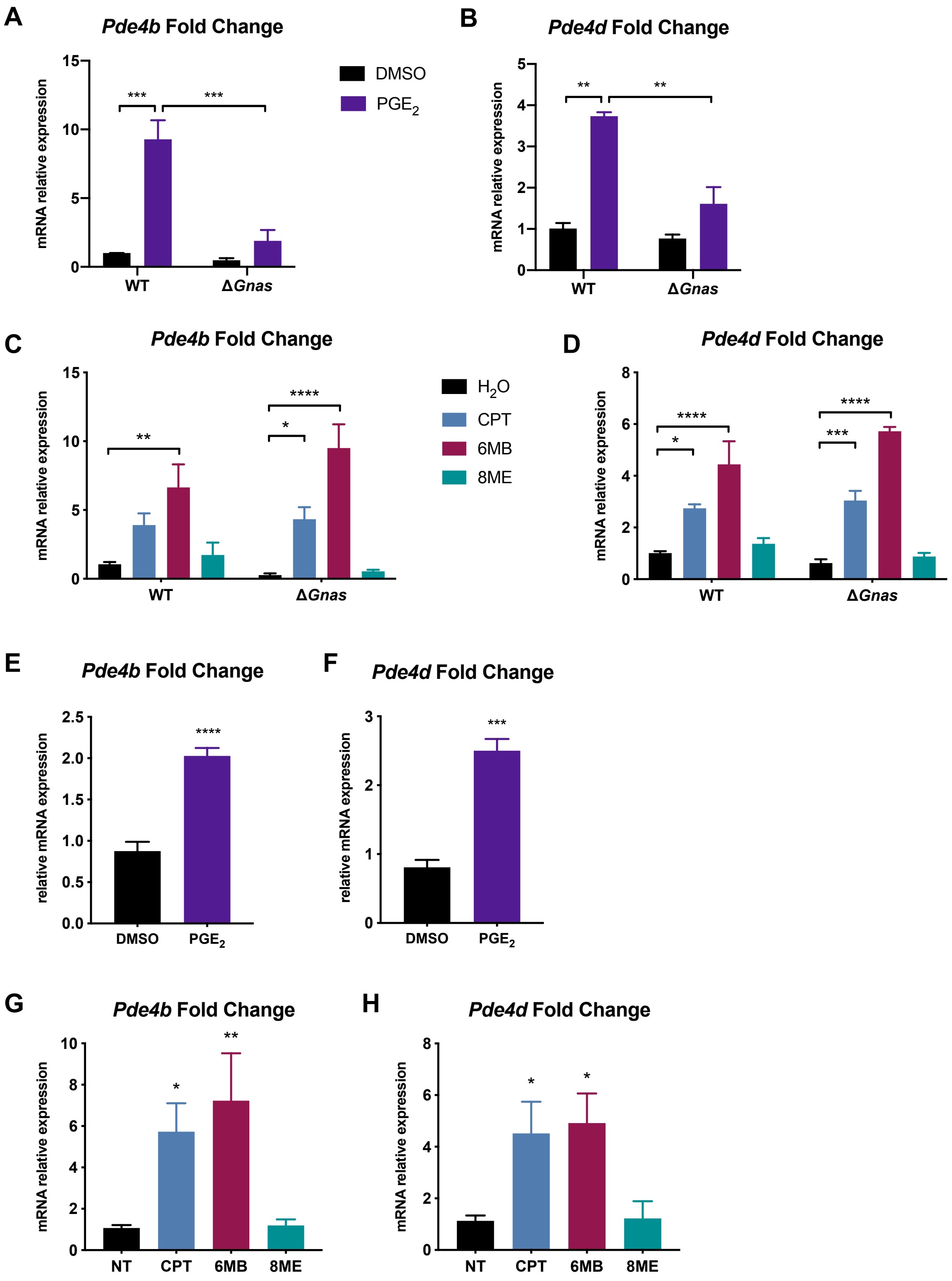
Figure 3.10: Decreased Cyclic AMP Levels and Decreased PKA Activation Mediates Reduced *Pde4b* Expression. A) WT DCs treated with the adenylyl cyclase inhibitor MDL-12,330A (10μM) for 16 hrs have decreased *Pde4b* expression. n=3. **B)** Treatment with the endogenous PKA inhibitor PKI (10μM) for 24 hrs reduced *Pde4b* expression in WT DCs. n=4. **C)** Kin$^-$ S49 cells which lack PKA activity have decreased basal *Pde4b* expression compared to WT S49 cells with functional PKA activity. n=3-5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The phosphodiesterase PDE4B is a regulator of compensation for both increased and decreased cAMP levels

When cells have decreased cAMP levels, the expression of *Pde4b* and *Pde4d* is reduced. To test if their expression is also responsive to increased cAMP levels, WT and ∆*Gnas* DCs were treated with PGE_2 for 24 hrs to raise endogenous cAMP levels (**Figure 3.6A, 3.11A - B**). PGE_2 treatment increased *Pde4b* and *Pde4d* expression in DCs, but ∆*Gnas* DCs had a blunted increase in *Pde4b* and *Pde4d* expression compared to WT cells.

To investigate the mechanism of this increase in gene expression, WT and ∆*Gnas* DCs were treated for 24hrs with the non-specific cAMP analog CPT, the PKA-specific cAMP analog 6MB, and the Epac-specific cAMP analog 8ME. Both CPT and 6MB increased the expression of *Pde4b* and *Pde4d* in WT and ∆*Gnas* DCs, but treatment with 8ME did not alter their gene expression, indicating the mechanism for the increase in PDE expression is via PKA (**Figure 3.11C - D**). Even though ∆*Gnas* DCs had lower basal *Pde4b* and *Pde4d* expression, they had a higher fold-increase than WT cells in *Pde4b* and *Pde4d* mRNA expression. We treated DC2.4 cells, a mouse dendritic cell line, with PGE_2, CPT, and 6MB, as an alternative DC to confirm the results with WT DCs regarding the increase in *Pde4b* and *Pde4d* (**Figure 3.11E - H**). Thus, the expression of *Pde4b* and *Pde4d* is dynamic and regulated by cAMP/PKA, with decreased expression in cells with lower cAMP levels and increased expression in cells with higher cAMP levels.

Figure 3.11: *Pde4b* and *Pde4d* Expression is Increased to Compensate for Increased Cyclic AMP Levels Via PKA. WT and Δ*Gnas* DCs treated with PGE_2 (10μM) for 24 hrs had increased **A)** *Pde4b* and **B)** *Pde4d* expression. n=2-4. Treatment with the cell-permeable cAMP analogs CPT (non-specific, 50μM) and 6MB (PKA-specific, 50μM) for 24 hrs increased **C)** *Pde4b* and **D)** *Pde4d* expression. 8ME treatment (Epac-specific, 50μM) did not increase *Pde4b* and *Pde4d* expression. n=3-5. PGE_2 (10μM) treatment of DC2.4 cells for 24 hrs increased **E)** *Pde4b* and **F)** *Pde4d* expression. n=4-5. In DC2.4 cells, treatment with CPT and 6MB increased **G)** *Pde4b* and **H)** *Pde4d* expression, but 8ME treatment did not. n=5-11. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.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Pde4b Fold Change
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WT
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PDE4B is a novel dendritic cell target to raise cyclic AMP levels in dendritic cells and reduce dendritic cell-induced Th2 inflammation

Based on its high expression in DCs, both with respect to mRNA levels (dCt = 6.8) and its expression relative to other PDEs, PDE4B is a novel DC target to increase cyclic AMP in these cells. Increased cAMP levels have an anti-inflammatory effect on DCs, so antagonism of PDE4B could potentially reduce DC-induced inflammation[2,3,5] (**Table 1.3**). Even though ∆*Gnas* DCs have reduced PDE4B expression compared to WT cells, ∆*Gnas* DCs still express high levels of PDE4B, which is the predominant PDE isoform in these cells (**Figure 3.8B**).

To validate PDE4B's effectiveness as a drug target *ex vivo*, WT and ∆*Gnas* DCs were treated with Ova and either vehicle control, the PDE4B-specific inhibitor A33, or Ro 20-1724 which inhibits PDE4A, PDE4B, PDE4C, and PDE4D. DCs were then co-cultured with naïve CD4$^+$ T cells from OT-II mice. Treatment of WT and ∆*Gnas* DCs with Ro 20-1724 and A33 increased Th17 differentiation in co-cultured CD4$^+$ T cells, as assessed by an increase in T cell-secreted IL-17A (**Figure 3.12A**). This result is consistent with recent data showing that increased cAMP levels in DCs promote Th17 differentiation[22,31]. Both Ro 20-1724 and A33 treatment of WT and ∆*Gnas* DCs increased Th17 differentiation, indicating that PDE4B can be targeted even in ∆*Gnas* DCs whose expression is decreased to compensate for lower cAMP concentrations.

∆*Gnas* DCs preferentially induce naïve CD4$^+$ T cells to differentiate into Th2 cells[2] (**Figure 3.12B**). We found that treatment of DCs with Ro 20-1724 and A33 decreased the amount of IL-4 secreted by co-cultured CD4+ T cells equally well, indicating that PDE4B accounts for the majority of the PDE4 activity in ∆*Gnas* DCs and that both PDE4- and PDE4B-specific inhibition in DCs reduces Th2 differentiation (**Figure 3.12B**). These experiments demonstrate the utility of targeting PDE4B to reduce dendritic cell-mediated Th2 inflammation.

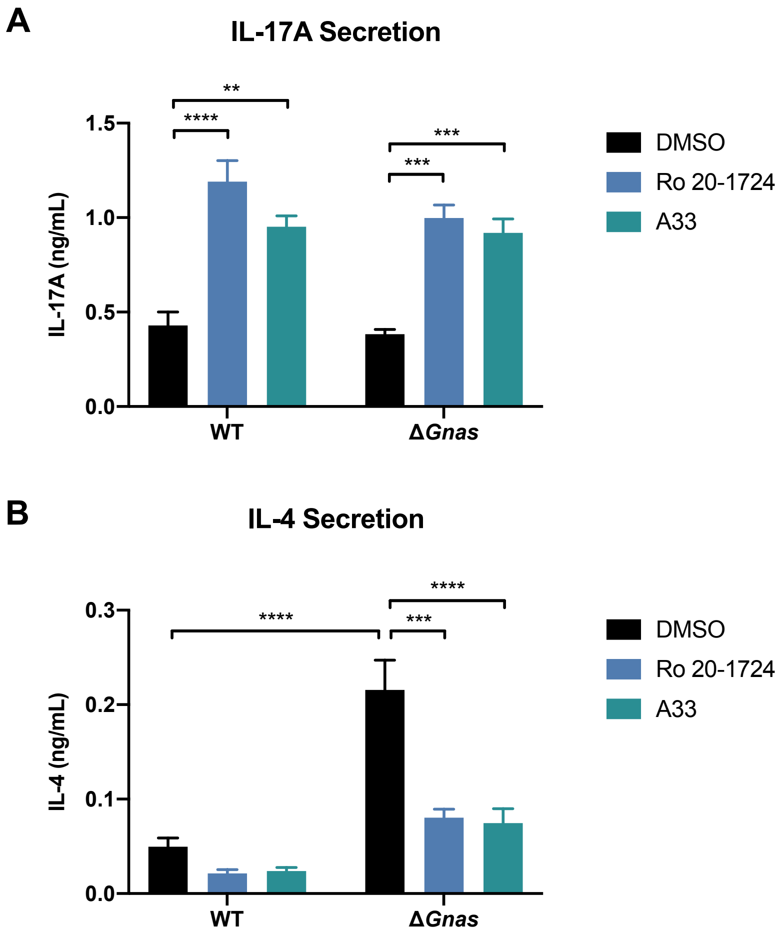

Figure 3.12: PDE4 and PDE4B-Specific Inhibition in DCs Increases Th17 Differentiation and Decreases Th2 Differentiation. WT and Δ*Gnas* DCs were treated with DMSO vehicle control, the PDE4 inhibitor Ro 20-1724 (10μM), or the PDE4B-specific inhibitor A33 (10μM) for 24 hrs before DCs were washed and incubated with co-cultured naïve CD4$^+$ OT-II T cells. **A)** Both Ro 20-1724 and A33 treatment increased Th17 differentiation in co-cultured CD4$^+$ T cells. n=4. **B)** Ro 20-1724 and A33 treatment both decreased Δ*Gnas* DC-induced Th2 differentiation. n=4. **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

It is critical that cells maintain cAMP homeostasis because different cellular levels of cAMP can induce different functional responses and phenotypes. We hypothesized that cells would attempt to compensate for chronically decreased cAMP levels by altering the expression of components in the cAMP signaling pathway. We found that CD11c$^{\Delta Gnas}$ DCs, which have reduced cAMP levels, had altered mRNA expression of multiple GPCRs including $G\alpha_i$-linked GPCRs, but unchanged mRNA expression of all other $G\alpha$ proteins (besides $G\alpha_s$) and the adenylyl cyclases. *Akap6* had increased mRNA expression >2-fold, but its low basal expression suggests that this increase in mRNA expression may have little effect on cell physiology.

Δ*Gnas* DCs, which had a 45% decrease in basal cAMP levels, had a >50% reduction in *Prkar2b* mRNA, suggesting there may be a feedback loop between cAMP levels and PKA RIIβ expression. To our knowledge, this is the first time such a connection has been reported. *Prkar2b* levels were insensitive to raised cAMP levels over 24 hrs, as induced by treatment with cAMP analogs and PGE_2. Thus, PKA RIIβ may be involved in cellular compensation for decreased cAMP levels, but it does not appear to be involved in relatively rapid compensation for increased cAMP levels.

While there were no changes in the mRNA expression of the cyclic nucleotide transporter *Mrp4*, Δ*Gnas* DCs had a >50% decrease in mRNA expression in both *Pde4b* and *Pde4d* levels. PDE4B is the predominant PDE isoform in mouse dendritic cells. This, in combination with *Pde4b*'s high mRNA expression and *Pde4d*'s low expression suggested that PDE4B is primarily responsible for Δ*Gnas* DCs' reduced phosphodiesterase activity. We verified that decreased *Pde4b* expression occurs in response to decreased cAMP levels by reproducing the results in WT DCs treated with the adenylyl cyclase inhibitor MDL-12,330A to lower cAMP levels. Decreased *Pde4b* expression in WT DCs was also reproduced by treatment with the endogenous PKA inhibitor PKI and in mutant Kin$^-$ S49 T lymphoma cells which lack PKA activity, indicating that cells compensate for decreased cyclic AMP levels by decreasing expression of the cAMP-degrading PDE4B via PKA, which would serve to increase cAMP levels.

In both WT and in Δ*Gnas* DCs that have reduced cellular cAMP concentration, *Pde4b* and *Pde4d* mRNA levels increased in response to treatment with the nonspecific cAMP analog CPT, the PKA-specific cAMP analog 6MB, and the $G\alpha_s$-linked GPCR agonist PGE_2, but not in response to treatment with 8ME, an Epac-specific cAMP analog. Thus, *Pde4b* and *Pde4d* mRNA increases to compensate for increased cAMP levels via PKA. In this way, PDE4B acts as a "thermostat" or homeostatic regulator of cellular cAMP concentration, rising with increased cAMP levels and falling with decreased cAMP levels. Changes in PDE4B expression are not sufficient to completely restore cAMP levels back to "normal", as Δ*Gnas* DCs have lower cAMP levels than WT DCs do and $G\alpha_s$-linked GPCR agonists increase cAMP levels in WT

DCs. However, altering the expression of the highly expressed cAMP-degrading PDE4B helps ameliorate the lower cAMP levels in Δ*Gnas* DCs.

Both *Pde4b* and *Pde4d* have CRE sites in their promoter regions, suggesting CREB or another CREB family member mediates their gene expression changes. It is known that increased cyclic AMP concentrations as a result of forskolin (adenylyl cyclase activator), isoproterenol (β-adrenergic receptor agonist), and 3-isobutyl-1-methylxanthine (IBMX; nonspecific PDE inhibitor) all induce increases in the mRNA expression of multiple PDEs, including *Pde3a*, *Pde3b*, *Pde4a*, *Pde4b*, *Pde4d*, and *Pde7b*[20–22,32–38] (**Table 3.2**). A corresponding increase in PDE4B and PDE4D protein expression in response to increased cyclic AMP levels has been verified in other cell types[20–22]. The cyclic nucleotide transporter MRP4 also has increased mRNA and protein expression in response to treatment by IBMX and CPT[38]. Our data regarding compensation for increased cAMP levels is consistent with the established pattern of increased cAMP-degrading PDE expression in response to raised cAMP levels.

Cellular compensation for decreased cAMP levels has not been previously studied and the current work thus extends prior evidence for a role for PDEs in compensation for increased cAMP concentration. Using prior studies regarding PDEs as a guide, we found that *Pde3b* expression was decreased in Δ*Gnas* DCs by RNA-Seq, but this decrease was not statistically significant in independent qPCR (**Table 2.8**, **Figure 3.8B**). We did not see

Table 3.2: Effects of Increased Cyclic AMP on Components of the Cyclic AMP Pathway. Cells compensate for increased cAMP levels by altering the expression of signaling components in the cAMP pathway. Entities (targets) whose expression can be altered by increased cAMP and/or CREB along with the cell type in which the experiments were conducted. References for each example are noted by superscripts.

Target	Effects of Increased cyclic AMP	Cell Type
PDE3A	▪ Increased PDE3 activity with forskolin, cholera toxin, and 8-Br-cAMP (cAMP analog) treatment[32] ▪ Decreased PDE3A protein with isoproterenol (β-adrenergic receptor agonist) stimulation through inducible cAMP early repressor (ICER, an endogenous repressor of CRE-mediated transcription)[33,34].	▪ Human T lymphocyte Jurkat cell line ▪ Rat cardiomyocytes
PDE3B	▪ Increased PDE3 activity with forskolin, cholera toxin, and 8-Br-cAMP (cAMP analog) treatment[32] ▪ Increased PDE3B mRNA and protein with 3-isobutyl-1-methylxanthine (IBMX, a nonspecific PDE inhibitor) treatment through CREB[33,34].	▪ Human T lymphocyte Jurkat cell line ▪ Mouse 3T3-L1 preadipocytes
PDE4A	▪ Increased mRNA expression by treatment with the β_2-adrenergic receptor agonist salbutamol and the PDE4 inhibitor rolipram[35] ▪ Decreased PDE4A mRNA expression with forskolin treatment[32]	▪ Human monocyte cell line U937 ▪ Human T lymphocyte Jurkat cell line

Table 3.2: Effects of Increased Cyclic AMP on Components of the Cyclic AMP Pathway, Continued.

Target	Effects of Increased cyclic AMP	Cell Type
PDE4B	▪ Increased mRNA expression by treatment with salbutamol and the PDE4 inhibitor rolipram[35]	▪ Human monocyte cell line U937
	▪ Increased mRNA and protein expression of PDE4B2 by treatment with dibutyryl-cAMP (db-cAMP, a cyclic AMP analog and PDE inhibitor)[20]	▪ Rat cortical neurons
	▪ PDE4B2 promoter activity was suppressed by dominant negative CREB mutants and stimulated by a constitutively active mutant[20].	▪ Rat cortical neurons
PDE4D	▪ Increased mRNA and protein expression of PDE4D1/PDE4D2 by treatment with dibutyryl-cAMP[20]	▪ Rat cortical neurons
	▪ Increased PDE4D1 and PDE4D2 mRNA expression with forskolin treatment[32]	▪ Human T lymphocyte Jurkat cell line
	▪ Increased PDE4D1 and PDE4D2 protein expression by forskolin and 8-Br-cAMP treatment[21]	▪ Rat aortic vascular smooth muscle cells
	▪ Increased mRNA expression of PDE4D5 by treatment with forskolin, isoproterenol, 8-Br-cAMP, or IBMX treatment[36]	▪ Human airway smooth muscle cells
	▪ Increased PDE4D7 protein expression with IBMX and theophylline treatment[22]	▪ Human B cells

Table 3.2: Effects of Increased Cyclic AMP on Components of the Cyclic AMP Pathway, Continued.

Target	Effects of Increased cyclic AMP	Cell Type
PDE7B	▪ Increased PDE7B mRNA expression by treatment with the Gα_s-linked D1 dopamine agonist SKF82958 but not treatment with the Gα_i-linked D2 dopamine antagonist quinpirole[37]	▪ Rat striatal neurons
	▪ Forskolin-stimulated PDE7B1 promoter activity occurs through CREB[37]	▪ Rat striatal neurons
MRP4	▪ Increased mRNA, protein, and promoter activity by treatment with db-cAMP and IBMX[38]	▪ HeLa cells
	▪ Increased mRNA expression by treatment with CPT and db-cAMP + IBMX[38]	▪ Human coronary artery
	▪ Increased mRNA expression by treatment with CPT and db-cAMP + IBMX which was ablated by transfection with Epac siRNA[38]	▪ Human megakaryoblastic leukemia M07e cells

decreased expression of *Pde3a*, *Pde4a*, *Pde7b*, and *Mrp4* in Δ*Gnas* DCs; however, DCs do not appear to express Epac, thus conceivably changes in Epac expression and actions might occur in other cell types. As such, all changes we observed in gene expression in DCs appear to occur solely through the actions of PKA. Indeed, increased *Mrp4* mRNA in response to raised cAMP levels by CPT treatment occurs through the actions of Epac[38]. It may be that changes in PDE3A, PDE3B, PDE4A, PDE4D, PDE7B, and MRP4 expression are also decreased to compensate for chronically low cAMP levels in other cell types, but this is not seen in our system which does not examine the effects of Epac.

Non-specific PDE4 inhibitors that inhibit all PDE4 isoforms (PDE4A, PDE4B, PDE4C, and PDE4D) are effective at reducing airway inflammation, both in studies of mice and in humans[39–45]. Their effects on DCs have not been previously assessed. In a murine model of allergic asthma, the PDE4 inhibitors rolipram, piclamilast, and ciclamilast reduced airway hyperresponsiveness, mucus secretion, eosinophil infiltration, and IL-4 levels in bronchoalveolar lavage (BAL) fluid; these responses correlated with reduced PDE activity[39–41]. In humans, roflumilast increased forced expiratory volume (FEV_1) and reduced allergen-induced sputum eosinophilia and airway hyperresponsiveness[42–45].

Unfortunately, PDE4 inhibitors are also associated with multiple adverse side effects, including nausea and emesis. One study found that 59% of patients discontinued roflumilast usage due to adverse side effects[46]. Roflumilast is thus approved for the more difficult to treat COPD, but not for asthma whose mainstays of treatment have good tolerability (inhaled corticosteroids and LABA)[47]. PDE4D has been proposed to be responsible for the major side effect of emesis[48,49]. Mice do not have an emetic reflex, but in a surrogate test of emetic potential (measuring duration of ketamine/xylazine-induced anesthesia), the PDE4B-specific inhibitor A33 had minimal emetic potential, in contrast to the non-specific PDE4 inhibitor rolipram[50].

Based on its high expression, PDE4B may be a useful drug target to increase cellular cAMP levels in DCs via use of an inhibitor. We found that both the PDE4 inhibitor Ro 20-1724 and the PDE4B-specific inhibitor A33 blunted Δ*Gnas* DC-induced Th2 differentiation in co-cultured naïve CD4$^+$ T cells, demonstrating the utility of PDE4B as a DC drug target *ex vivo* and suggesting its potential as a DC-specific therapy for other diseases mediated by DC-induced inflammation. Although no PDE4B-specific inhibitors are currently on the market, several have been reported in the literature[47,50–52]. Furthermore, PDE4B$^{-/-}$ mice do not develop airway hyperresponsiveness after Ova sensitization, have reduced numbers of eosinophils and levels of the Th2 cytokines IL-5 and IL-13 in BAL fluid, and have reduced DC migration to the draining bronchial lymph nodes[53]. Thus, a PDE4B-specific inhibitor may not only be efficacious as an asthma therapy (especially for those with Th2-driven asthma), but may also limit the side effects associated with nonspecific PDE4 inhibition.

Many drugs on the market raise cAMP levels to treat a wide variety of diseases (e.g., salmeterol for asthma, metoprolol for hypertension and congestive heart failure, and morphine for analgesia)[23–25,54]. The current findings suggest a more specific strategy to raise cAMP levels for treatment: targeting the PDEs that particular cells utilize to compensate for altered cAMP levels. Determining which PDEs different cell types express and whose expression is altered to compensate for changes in cAMP concentrations may identify other PDE isoforms that can be targeted to treat diseases in which decreased cellular cAMP concentrations contribute to disease pathophysiology. Moreover, for diseases that are treated by raising cAMP levels via $G\alpha_s$-linked GPCR agonists or $G\alpha_i$-linked GPCR antagonists, using these drugs in combination with a PDE isoform-specific inhibitor to prevent cells from compensating for artificially raised cAMP concentrations may prove useful.

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CHAPTER FOUR: CONCLUSIONS AND DISCUSSION

Asthma is an inflammatory disorder of the airways characterized by chronic airway inflammation and airway hyperresponsiveness which results in variable limitation of expiratory airflow[1]. The prevalence of asthma increased suddenly in the 1970s, more than doubling by the late 1990s, and currently rests at 4.5%, affecting an estimated 235 million people[1–5]. The rise in asthma prevalence has imposed a significant public health challenge, exceeding the economic burden of HIV and tuberculosis combined[6,7]. In addition, it is estimated that the asthma of 50% of adult patients and 38% of children is uncontrolled by current asthma treatments, necessitating the need for new, effective asthma therapies[8]. Asthma cases can be divided into either type 2 (type 2 helper T cell (Th2)-high) or non-type 2 (Th2-low) asthma[9–11]. Type 2 asthma is strongly associated with allergic asthma, increased eosinophilic inflammation, and markers of type 2 helper T cell (Th2)-mediated inflammation.

Dendritic cells (DCs) are the key antigen-presenting cells in asthma and initiate immune responses to various allergens and induce naïve helper T cells to differentiate into helper T cell subsets, one of which includes type 2 helper T (Th2) cells. Th2 cells are a central mediator of inflammation due to their secretion of the inflammatory cytokines IL-4, IL-5, and IL-13. Due to DCs' role in inducing Th2 differentiation, DCs are a crucial target for modulating Th2 inflammation in asthma and represent a potential new cell type to target in asthma therapy.

CD11c$^{\Delta Gnas}$ (Δ*Gnas*) mice have a DC-specific knockout of *Gnas*, the gene that encodes Gα_s which activates adenylyl cyclase to produce cAMP. These mice develop allergic asthma both spontaneously and in response to antigen sensitization *in vivo*[12]. Isolated CD11c$^+$ DCs from Δ*Gnas* mice induce co-cultured naïve CD4$^+$ helper T cells to differentiate into Th2 cells, unlike their WT counterpart which does not[12]. This effect is reversed *ex vivo* and *in vivo* by treatment with the cAMP analog CPT[12]. CD11c$^{\Delta Gnas}$ mice thus point to aberrant cAMP signaling as a mediator of DC-induced Th2 inflammation.

RNA-Sequencing of WT and Δ*Gnas* DCs showed that both expressed a similar number of genes, but they had several key differences in their pattern of expression. While down-regulated genes in Δ*Gnas* DCs did not provide a clear direction to suggest the mechanism behind the increased Th2 differentiation of Δ*Gnas* DCs, there was a striking up-regulation in genes relating to the chromosome and cytoskeleton in Δ*Gnas* DCs. Further analysis revealed that a large portion of these up-regulated genes were associated with microtubules and the kinetochore. Microtubules are composed of dimers of α- and β-tubulin. We confirmed by qPCR that Δ*Gnas* DCs had increased expression >2-fold of the β-tubulin gene *Tubb2b* and found that WT DCs treated with the endogenous PKA inhibitor PKI had an increase in α-tubulin staining, indicating that increased tubulin expression in Δ*Gnas* DCs is mediated by decreased PKA activation. The microtubule depolymerizing agent ansamitocin P3 also induces expression of the DC maturation markers CD80, CD86, CD40, and MHC-II; induces proliferation of co-cultured $CD4^+$ and $CD8^+$ T cells; and increases the lymph node homing and migration of tumor-resident DCs in mice[13]. Treatment with PGE_2 and the PKA-specific cAMP analog N6-Benzoyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP) also induces DC migration[14,15]. These results suggest that cAMP levels in DCs regulate microtubules which may in turn affect their capacity to induce $CD4^+$ T cell proliferation and affect DC migration, both of which would be expected to affect Th2 inflammation. Further studies are needed to define the precise role of microtubules in DC function.

RNA-Seq analysis revealed Δ*Gnas* DCs had a decrease in the gene expression of cAMP-responsive element modulator (CREM). As a CREB family transcription factor, CREM may contribute to the cAMP-mediated changes in DCs. More data is needed to determine if and how CREM affects DC-mediated Th2 inflammation.

Δ*Gnas* DCs had a 45% reduction in their basal cAMP concentration. This decrease in cAMP levels coincided with altered gene expression of 24 GPCRs. Some of these GPCRs may represent targets to raise cyclic AMP levels and for drugs to treat asthma, particularly with antagonists of up-regulated $G\alpha_i$-linked GPCRs or agonists of down-regulated $G\alpha_s$-linked GPCRs to raise intracellular cAMP concentrations and reduce DC-induced Th2 inflammation. Δ*Gnas* DCs had no changes in the rest of their $G\alpha$ and adenylyl cyclase genes and likely insignificant changes in *Akap6* expression. Thus, cells with chronic, decreased cyclic AMP levels do not compensate for lower cAMP levels by changing the expression of Gα, adenylyl cyclase, or AKAP genes.

Gene expression of *Prkar2b*, which encodes PKA RIIβ, was decreased >55% in Δ*Gnas* DCs in both RNA-Seq and independent qPCR data. To our knowledge, this is the first time a feedback loop between cAMP levels and PKA RIIβ subunit expression has been reported. Cells may attempt to compensate for decreased cAMP levels by reducing the expression of *Prkar2b*, but PKA RIIβ is not involved in compensating for short-term increases in cAMP levels as treatment with PGE_2 and cAMP analogs did not change its expression.

The gene expression of the cyclic nucleotide transporter MRP4 is unchanged in Δ*Gnas* DCs, but *Pde4b* and *Pde4d* mRNA expression is decreased >50% in Δ*Gnas* DCs by both qPCR and RNA-Seq. *Pde4b* is highly expressed in both WT and Δ*Gnas* DCs and so we chose to focus on it as its high expression makes PDE4B a likely contributor to intracellular cAMP concentrations. WT DCs treated with the adenylyl cyclase inhibitor MDL-12,330A and PKI also had decreases in their *Pde4b* expression, showing that decreased cAMP levels causes downregulation of *Pde4b* mRNA through PKA. PDE4B expression is thus decreased in an attempt to compensate for chronic, decreased cAMP levels. Furthermore, treatment with PGE_2 and the cAMP analogs CPT, 6MB, and 8ME increased *Pde4b* and *Pde4d* gene expression. Thus, the cAMP-degrading phosphodiesterase PDE4B acts as a regulator of intracellular cAMP concentrations, with its expression rising and falling to buffer changes in cellular cAMP levels.

Even though ∆*Gnas* DCs have decreased PDE4B expression, PDE4B is still highly expressed in these cells and remains the predominant PDE isoform. We found that treatment of WT DCs with both the pan-PDE4 inhibitor Ro 20-1724 and the PDE4B-specific inhibitor A33 increased Th17 differentiation which is associated with increased cAMP levels in DCs[16,17]. Treatment of ∆*Gnas* DCs with Ro 20-1724 and A33 reduced Th2 differentiation in co-cultured CD4+ T cells equally well, demonstrating that PDE4B is a novel DC target that can be targeted *ex vivo* to reduce DC-induced Th2 differentiation and suggesting a more precise way of raising cAMP in cells for the treatment of diseases that may benefit from increases in cAMP levels. Specifically targeting the cyclic AMP signaling components whose expression is altered to compensate for changed cAMP levels in those cell types may be more effective than current therapies that target other entities in the cAMP signaling pathway.

Summary

Isolated dendritic cells from CD11c$^{\Delta Gnas}$ mice have decreased basal cyclic AMP concentrations that results in increased DC-induced Th2 inflammation *ex vivo* and *in vivo*[12]. Transcriptomic analysis of ∆*Gnas* DCs revealed an increase in microtubule-associated genes that is mediated by decreased PKA activation, suggesting that changes in microtubule function contribute to ∆*Gnas* DCs' change in phenotype, perhaps through effects on migration or stimulation of T cell proliferation. To compensate for chronically decreased cAMP concentrations, ∆*Gnas* DCs have altered gene expression of multiple GPCRs, PKA RIIβ, CREM, PDE4B, and PDE4D (**Figure 4.1**). Expression of the cAMP-hydrolyzing PDE4B is dynamic, rising and falling to compensate for changes in intracellular cyclic AMP concentrations. PDE4B is a novel dendritic cell target to increase cAMP levels and reduce DC-induced Th2 inflammation. Further studies are needed to determine its effectiveness *in vivo*.

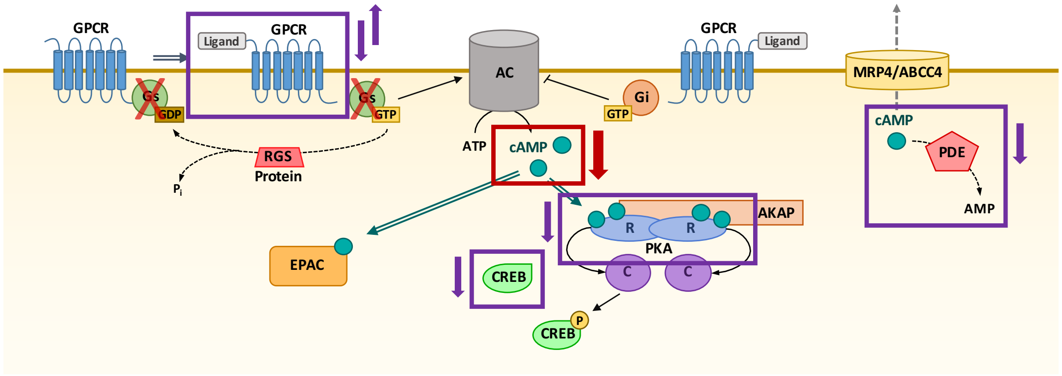

Figure 4.1: Cellular Compensation for Decreased Cyclic AMP Levels. Cells compensate for chronically decreased cAMP levels (Red) by altering the gene expression of multiple GPCRs (some are up-regulated and some are down-regulated) and down-regulating the gene expression of PKA RIIβ, the CREB family transcription factor CREM, PDE4B, and PDE4D. PDE4B protein expression and PDE activity was confirmed to be changed in response to lower cyclic AMP concentrations, and PDE4B expression also increases to compensate for increased cyclic AMP levels as well.

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