POTENTIATION OF LONG-ACTING β_2-AGONIST AND GLUCOCORTICOIDS

MODULATION OF CYCLIC ADENOSINE MONOPHOSPHATE FOR POTENTIATION OF LONG-ACTING β_2-AGONIST AND GLUCOCORTICOIDS IN HUMAN AIRWAY EPITHELIAL CELLS

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Lay Abstract

Asthma is a common chronic lung disease characterized by narrow and inflamed airways that cause breathing difficulties. Current management includes the combination of bronchodilators, to relax the airway, and steroids, to decrease inflammation. Unfortunately, this combination therapy is suboptimal in 35-50% of users, increasing the risk of asthma attacks, hospitalization rate, and health care costs. Recently, there have been studies theorizing that we can improve the therapy's ability to decrease inflammation by increasing cAMP, an important molecule for biological activities. We tested this claim by blocking the breakdown and export of cAMP to increase its levels and measured inflammatory cytokines, molecules that direct the action of immune cells. Our results show that in a model of viral infection, administering the combination therapy while increasing cAMP levels can further decrease inflammatory cytokines prompting further investigation for its potential implication in the clinic.

Abstract

In Canada, asthma is the third most common chronic disease resulting in 250 premature deaths annually and related healthcare expenses exceeding $2.1 billion/year. It is estimated that around 50-80% of asthma exacerbations are due to viral infections. Despite an advanced understanding on how to treat and manage the symptoms of asthma, current therapy is sub-optimal in 35-50% of moderate-severe asthmatics around the world resulting in lung inflammation, persistent impairment of lung function, and increased risk of mortality. Combination of long-acting β_2 agonists (LABA) for bronchodilation and glucocorticoids (GCS) to control lung inflammation represent the dominant strategy for the management of asthma. Increasing intracellular cyclic adenosine monophosphate (cAMP) beyond existing combination LABA/GCS are likely to be beneficial for the management of difficult to control asthmatics that are hypo-responsive to mainstay therapy. In human airway epithelial cells (HAEC), cAMP is either exported by transporters or broken down by enzymes, such as phosphodiesterase 4 (PDE4). We have demonstrated that HAEC express ATP Binding Cassette Transporter C4 (ABCC4), an extracellular cAMP transporter. We also show that ABCC4 and PDE4 inhibition can potentiate LABA/GCS anti-inflammatory responses in a human epithelial cell line in a cAMP-dependent mechanism validating the pursuit of novel ABCC4 inhibitors as a cAMP elevating agent for asthma.

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List of all Abbreviations and Symbols

ABCC4	ATP Binding Cassette Transporter C4
AC	adenylyl cyclase
AKAP	A-kinase anchor proteins
ALI	air liquid interface
ATF-1	activating transcription factor 1
ATP	adenosine triphosphate
β_2AR	β_2-adrenoceptor
cAMP	cyclic adenosine monophosphate
CF-1	ceefourin-1
CF-2	ceefourin-2
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CoA	coactivators
COPD	chronic obstructive pulmonary disease
CRE	cAMP response elements
CREB	cAMP-response element binding protein
DUSP1	dual-specificity phosphatase
FEV_1	forced expiratory volume
FP	Fluticasone
FSK	Forskolin
GEO	Gene Expression Omnibus
GINA	The Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor

GRE	glucocorticoid response element
HAEC	human airway epithelial cells
HBEC-6KT	human bronchial epithelial cell line
IBMX	3-isobutyl-1-methylxanthine
ICS/GCS	inhaled corticosteroid /glucocorticoids
IgE	immunoglobulin E
IHC	immunohistochemical
IL	interleukin
IL-6	interleukin-6
IL-8/CXCL8	interleukin 8
IP-10	IFN-γ induced protein 10
IRF-3	interferon regulatory transcription factor 3
ISH	*in situ* hybridization
JAK 1	Janus kinases 1
JAK2	Janus kinases 2
LABA	long acting β_2 agonist
LRTA	leukotriene receptor antagonist
MAPK	mitogen-activating protein kinases
MRP4	multidrug resistance-associated protein 4
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PDE4	phosphodiesterase 4
PI3K	phosphoinositide 3-kinase
PKA	Protein Kinase A
Poly I:C	Polyinosinic: polycytidylic acid
RGS2	regulator of G-protein signalling
RIG-I	retinoic acid-inducible gene I

RSV	respiratory syncytial virus
SABA	Short-acting β_2 agonist
STAT3	Signal transducer and activator of transcription 3
TBST	Tris-buffered saline with 0.05% Tween 20
TEER	transepithelial electrical resistance
T_h	T helper cell types
T_h2	Type 2 helper T cells
T_h1	Type 1 helper T cells
T_h17	Type 17 helper T cells
TLR3	Toll-like receptor 3

Declaration of Academic Achievement

I, Yechan Kim, declare this thesis to be my own work. I am the sole author of this document. I completed all of the research work with the help of Vincent Hou based at McMaster University, Ryan Huff based at the University of British Columbia, and Jen Aguiar based at University of Waterloo and was supported by Federal and Provincial funding held by my supervisor, Dr. Jeremy Hirota.

Introduction

No one will dispute that breathing is crucial in our life, but at the same time, many people may take breathing for granted. However, it is estimated that there are over 339 million people in the world with asthma in 2018(1). In Canada alone, over 3.8 million people are living with asthma. Asthma is an inflammatory lung disease that can impair a person's ability to breathe, leading to an increase in morbidity, mortality, and hospital care costs(2,3). On a more positive note, although the prevalence of asthma has been on the rise since the early 2000s, the incidence rate has also decreased in Canada(4,5).

Asthma

Definition and risk factors

The Global Initiative for Asthma (GINA) defines asthma as: "a heterogeneous disease, usually characterized by chronic inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that may vary over time and in intensity, together with variable expiratory airflow limitation"(6). Some of the key consequences are bronchoconstriction, airway wall thickening, increased mucus, and inflammation in the airway. It is not entirely clear why some people develop asthma, and others don't, but it is most likely due to a combination of environmental and genetic factors. For example, there is evidence that there was a causal relationship between childhood respiratory syncytial virus (RSV) infection and the development of asthma when infants who had RSV were followed into their teens to see if they developed asthma(7). Additionally, there is evidence supporting the notion that those with allergic rhinitis are at a higher likelihood of developing asthma later on in

life(8). Other risk factors include: having a blood relative with asthma, being overweight, smoking, and exposure to toxic fumes (second-hand smoke, exhaust fumes, pollutions)(9).

Asthma is associated with different T helper cell types (T_h), such as Type 2 helper T cells (T_h2), Type 1 helper T cells (T_h1), and Type 17 helper T cells (T_h17). T_h 2 immune responses lead to the release of cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13 that promote eosinophilic inflammation and immunoglobulin E (IgE) production. IgE triggers the release of inflammatory mediators such as histamine, leukotrienes, and cytokines and causes bronchoconstriction, edema, and hypersecretion of mucous(10). Asthma is also associated with T_h1 immune responses. It is interesting because T_h2 and T_h1 have an opposing effect on each other, where expression of one will tend to suppress the other(11). T_h1 cells are crucial for the development of neutrophilic inflammation in the airways and have also been related to corticosteroid resistance(12). T_h1 inflammation occurs in response to bacterial or viral infection. T_h17 expression has been positively correlated with neutrophil and increase airway responsiveness in asthmatic patients(13). Increased T_h17 mediated inflammation has also been associated with severe asthma(13).

Treatment/management of asthma

Currently, there is no cure for asthma, but its symptoms can be controlled. GINA outlines evidence-based strategy focused on symptom control and risk reduction. The most up-to-date guideline set by GINA outlines stepwise management and control of

asthma (Figure 1). Asthma is usually controlled for with a variety of drugs eliciting effects that either decrease inflammation or relieve bronchoconstriction such as:

Long-acting β_2 agonist (LABA): long-acting bronchodilation that lasts for 12-24 hours depending on the type.

Inhaled corticosteroid (ICS, also known as glucocorticoids [GCS]): Steroid that decreases systemic inflammation and can be delivered through the airway.

Short-acting β_2 agonist (SABA): was used as needed as a stand-alone therapy for quick relief through bronchodilation in the event of an asthma attack. Has been shown to have adverse side effects when taken alone.

Leukotriene receptor antagonist (LRTA): Class of oral medication that is non-steroidal but acts as anti-inflammatory bronchoconstriction preventors. It is used primarily when ICS is not a viable option.

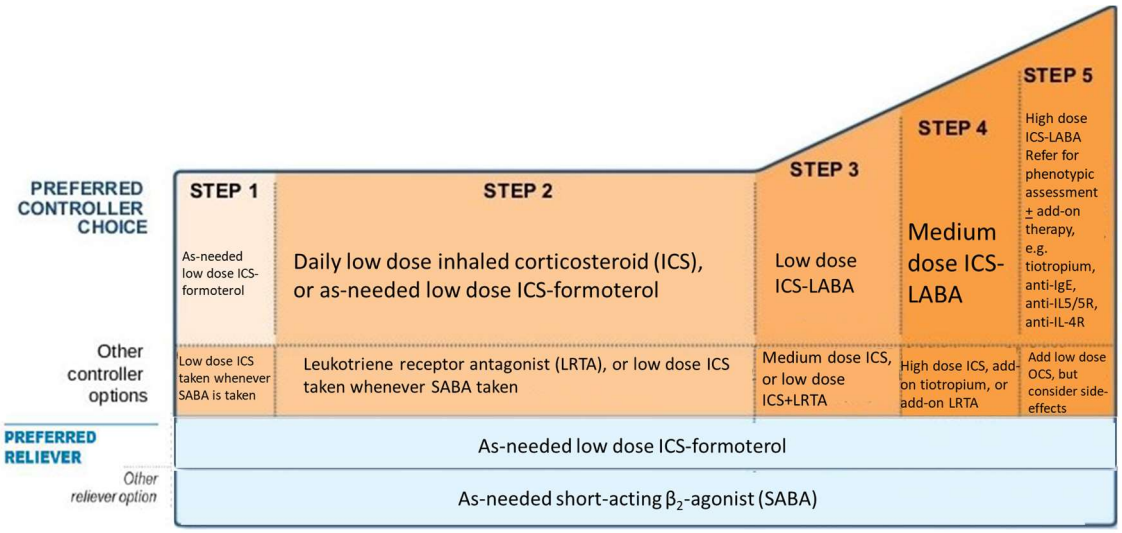

Figure 1 – The 2019 GINA asthma treatment strategy.
An illustrative diagram of GINA's recommended methods for controller choice and reliever choice. Depicted as different steps, each step higher corresponds to higher severity of asthma. Modified from 2019 pocket GINA guideline (6).

In GINA's guideline, management of asthma is broken down into five steps. Mild asthmatics fall under step1 and step 2 category. Step 1 is used when symptoms can be managed through as-needed low dose ICS-formoterol (LABA). Step 2 is when step 1 fails to control asthmatic symptoms adequately and requires a daily low dose of ICS, or as needed low dose ICS-formoterol. Moderate-severe asthmatics fall under the step 3-5 category where doses of LABA/ICS increase with each step depending on success or failure of management. In step 5, patients are referred for phenotypic assessment as increasing LABA/ICS doses is likely not having a significant rescue of lung functions.

In 2019, GINA has made a major change in recommendations for mild asthmatics. They no longer recommend the use of SABA alone treatment as the preferred reliever options as it has been associated with increased exacerbations and lower lung functions(6). Instead, they recommend the use of as-needed LABA/GCS (formoterol/budesonide), which has been suggested to be superior to SABA alone (terbutaline) for asthma control and reducing exacerbation(14). However, if as-needed LABA/GCS is not possible, GINA has also outlined that SABA should only be taken with low dose ICS.

Glucocorticoids

Glucocorticoids (also known as corticosteroids, glucocorticosteroids, or just steroids), are widely used to suppress inflammation in inflammatory and immune diseases. It is most commonly used in the treatment of asthma through the use of ICS(15). The therapeutic effect of GCS lies in its ability to switch off multiple inflammatory genes that encode cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors,

and proteins that are activated during inflammation(15). Inflammatory genes are turned on by transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein-1, by changing the chromatin structure of the gene. GCS has the ability to reverse this change in the chromatin structure of the gene, eliciting anti-inflammatory effects(15). GCS binds onto glucocorticoid receptors (GR) in the cytoplasm, dimerizes, and translocates to the nucleus where they bind on to glucocorticoid response element (GRE), which in turn increase transcription of anti-inflammatory proteins such as lipocortin-1, and interleukin-10(15). The effect mentioned above is called transrepression, where one protein represses the activity of a second protein. However, GCS also can transactivate genes involved in metabolic processes causing adverse side effects. GCS that are clinically used currently such as budesonide, fluticasone, and prednisone to name a few, have a higher selectivity for transrepression over transactivation, minimizing the side effects while maximizing the therapeutic potential(16). Furthermore, it has been noted that GCS seems to preferentially suppress T_h1 cellular immunity axis and cause a shift toward the T_h2 mediated humoral immunity, rather than generalized immunosuppression(17).

Long-acting β_2 agonist

β_2-adrenoceptors (β_2AR) elicit their effects by increasing intracellular cAMP through the activation of adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) into cAMP. SABA and LABA are used as bronchodilators as they can bind to β_2AR(18). Smooth muscle relaxation is believed to be caused through several

mechanisms elicited by cAMP and by non-cAMP mechanisms. Elevation of cAMP has been shown to inhibit calcium ion release from intracellular stores, reduction of membrane calcium ion entry, and sequestration of intracellular calcium ion, leading to relaxation of the airway smooth muscle(18). In addition, although not fully understood, cAMP, through its activation of Protein Kinase A (PKA) phosphorylates key regulatory proteins involved in the control of muscle tone(19). Complementarily, it has also been suggested that β_2AR directly interact with potassium channels, which lead to membrane hyperpolarization and inhibition of calcium influx(20,21). SABAs provide instant symptomatic relief and are used in the event of an acute exacerbation lasting from minutes to 4-6 hours; however, as mentioned before, GINA no longer recommends the use of SABA alone. LABAs, on the other hand, do not elicit as quick of a response but lasts longer from 12 hours to 24 hours(22).

Uncontrolled asthmatics + viral exacerbations

Unfortunately, up to 35-50% of moderate-severe asthmatics do not find LABA/GCS treatment to be optimal, resulting in lung inflammation, persistent impairment of lung function, and increased risk of mortality(3,23–27). Asthma exacerbations can be triggered by various factors such as exercise, exposure to allergens or irritants, changes in the weather, or respiratory infections. Approximately 80% of exacerbations are associated with respiratory tract viral infections including but not limited to: rhinovirus, influenza virus, adenovirus, and respiratory syncytial virus(28). It is interesting to note that severe asthmatic populations in both children and adults have

marked T_h1 inflammation resulting in increased IFN-γ levels(29,30). The mechanisms of virus-induced exacerbations are not fully understood as different viruses may act on different pathways(31). Viral infections lead to inflammation, increasing the levels of neutrophils, eosinophils, $CD4^+$ cells, $CD8^+$ cells, and mast cells through increased expression and translation of IL-6, IL-8, GM-CSF, IP-10, RANTES, and other proinflammatory cytokines(32).

Interleukin-6 (IL-6) is produced by cells from the innate immune system, such as B cells, as well as endothelial cells, fibroblasts, and epithelial cells and has been found to be elevated in several inflammatory diseases such as asthma(33,34). IL-6 binds to a membrane receptor (IL6R) which results in the activation of receptor-associated kinases such as Janus kinases 1(JAK 1) which in turn can activate the Signal transducer and activator of transcription 3 (STAT3 – T_h17 differentiation), mitogen-activating protein kinases (MAPK-regulator of synthesis of inflammation mediators), and Phosphoinositide 3-kinase (PI3K - activation, proliferation, and differentiation of leukocytes) pathway(35). However, the presence of IL-6 in the airway of asthmatic patients may not be due to ongoing systemic inflammation but may be due to the "activated" state of airway epithelial cells(36,37). IL-6 has been correlated with the promotion of IL-13, the primary inducer of mucus by airway epithelial cells, and when IL-6 was knocked out in mice model, there was less mucus production in the IL-6 KO mice compared to wild type mice(38). Also, IL-6 serves a regulatory role of CD4 T cell differentiation by promoting the differentiation of T_h2 cells(39). It has also been shown that IL-6 promotes T_h17 differentiation in the presence of IL-1β(40). As mentioned before, as T_h17 inflammation

is characteristic in those with severe asthma, decreasing IL-6 levels may have therapeutic potential.

Interleukin 8 (IL-8/CXCL8) is a chemokine released by macrophages, epithelial cells, airway smooth muscles cells, and endothelial cells that recruit and activate neutrophils. IL-8 binds to CXCR1/2 and can activate STAT3, MAPK, and the PI3K pathway(41). Both IL-8 and neutrophils are features of difficult-to-treat asthma patients, and virally induced exacerbation in asthmatic patients(42). Neutrophil activation is believed to contribute to airway obstruction, which in turn lead to wheezing and difficulty breathing. In addition, an increase in serum IL-8 levels could potentially be used as a biomarker to identify asthma status, and changes in IL-8 level have been shown to reflect the response to GCS in difficult to control asthma(43). These findings make IL-8 a promising cytokine to target as a way of better controlling asthmatic symptoms.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by human bronchial epithelial cells and have been proposed to have central roles in the pathogenesis of asthma (44). GM-CSF binds to its receptor and activates the JAK2 pathway and can activate STAT3/5 and the MAPK pathway(45). In healthy airways, GM-CSF is expressed at very low or undetectable levels but is significantly increased in asthmatics. This elevated expression can lead to increased recruitment of eosinophils and neutrophils, which may contribute to the enhancement of asthma (44,46,47). Clinical data have demonstrated that GM-CSF neutralization could be an important target for asthma. For example, in asthmatic patients, inhaled glucocorticoids significantly reduced GM-

CSF levels resulting in improved lung function and decreased airway hyperresponsiveness (48,49).

IFN-γ induced protein 10 (IP-10) is a chemokine involved in T-cell recruitment and mast cell activation. IP-10 binds to the chemokine receptor CXCR3 and can activate the PI3K and MAPK pathway; however, the specific signaling pathway has not been well defined yet(50). It is generally induced by a viral infection. However, asthmatic subjects have increased levels of IP-10 in serum, and the levels have been correlated with airway obstruction and reduced bronchodilator response to β-agonists(51). It has also been demonstrated that IP-10 contributes to airway hyperreactivity and T_h2-type inflammation in allergic asthma models(52). IP-10 is more markedly known for its ability to act as a biomarker for viral load, where higher IP-10 levels correspond to higher viral load(51).

RANTES (also known as CCL5) is a chemoattractant for eosinophils, monocytes and T lymphocytes and has been correlated with airway hyperresponsiveness, inflammation, and mucus secretion. RANTES binds to its receptor. CCR5 and can activate phospholipase C (PLC – chemotaxis), PI3K, and MAPK pathway(53). In patients with asthma, the plasma RANTES level is significantly elevated during acute attacks(54). Therapeutic alterations of RANTES level may not be so simple. In a study conducted in mice, they have found that blocking RANTES with Met-RANTES may be challenging to justify in a clinical setting. RANTES plays a vital role in controlling viral replication. If it is blocked before viral infection, it will benefit primary exposure but lead to delayed viral clearance and enhanced disease reinfection(55).

LABA/GCS mechanisms

A clinical study published in 1994 showed a critical finding that asthmatic subjects who were symptomatic despite maintenance therapy with a standard dose of ICS were controlled by the addition of LABA, but not by increasing the dose of ICS(56). LABA/GCS was able to show better symptom score, lung function, use of rescue medication, and exacerbation frequency (57–61). As such, LABA/GCS have become mainstay therapy for the management of asthmatics(62).

Despite these findings, the exact mechanistic basis for the superiority of LABA/GCS remains unclear. At the very fundamental level, LABA is used for bronchodilation, and GCS is used for the suppression of inflammation. However, LABAs alone lack anti-inflammatory activity in asthma, and in fact, in airway smooth muscle cells, and transformed or primary bronchial epithelial cells, LABA alone has been shown to induce and potentiate the expression of certain inflammatory genes such as IL-6, CXCL5, and IL-8. GCS are always given in combination with LABA to suppress LABA's inflammatory effects(63–68). As such, it is difficult to justify the LABA/GCS superiority compared to GCS alone when LABA *should* worsen GCS control of IL-6, CXCL5, and IL8. In fact, it has been shown that when comparing LABA/GCS treatment to GCS alone treatment, they are about equal in controlling exacerbations, but a lower relative risk of exacerbations with LABA/GCS compared to GCS alone(69). It has been shown GCS may have the ability to enhance the β_2AR mRNA levels and β_2AR promoter activity, indicating increased β_2AR gene transcription, can also contribute to the

beneficial effect of GCS in functional response of LABA, however as this is transient, this interaction is likely not the dominant reason for LABA/GCS superiority(70).

Currently, there is growing evidence that LABA/GCS may have additive and synergistic effects on the regulation of anti-inflammatory and bronchoprotective genes. Studies have shown that LABA/GCS, in combination, have shown to additively increase the induction of anti-inflammatory gene dual-specificity phosphatase, DUSP1, an inactivator of MAPK. The MAPK pathway plays a crucial role in the transduction of extracellular signals to cellular response, such as inflammation(71). LABA, when bound to the β_2AR, induces the activation of adenylyl cyclase (AC), which in turn produces a secondary messenger molecule, cyclic adenosine monophosphate (cAMP). cAMP induces the transcription of DUSP1 mRNA(72–74). Similarly, GCS, when bound to GR, transactivates the DUSP1 promoter region, inducing the transcription of DUSP1 (75–77). It has also been observed that LABA and GCS can interact synergistically, where the two in combination can exceed the sum of the two drugs' individual effects. An example of this is regulator of G-protein signalling, RGS2, a bronchoprotective gene (78,79). RGS2 inhibits the Gq protein, a G protein subunit that activates phospholipase C, which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate signal transduction pathway, leading the asthma responses such as airway smooth muscle contraction, mucus secretion, and release of inflammatory cytokines.

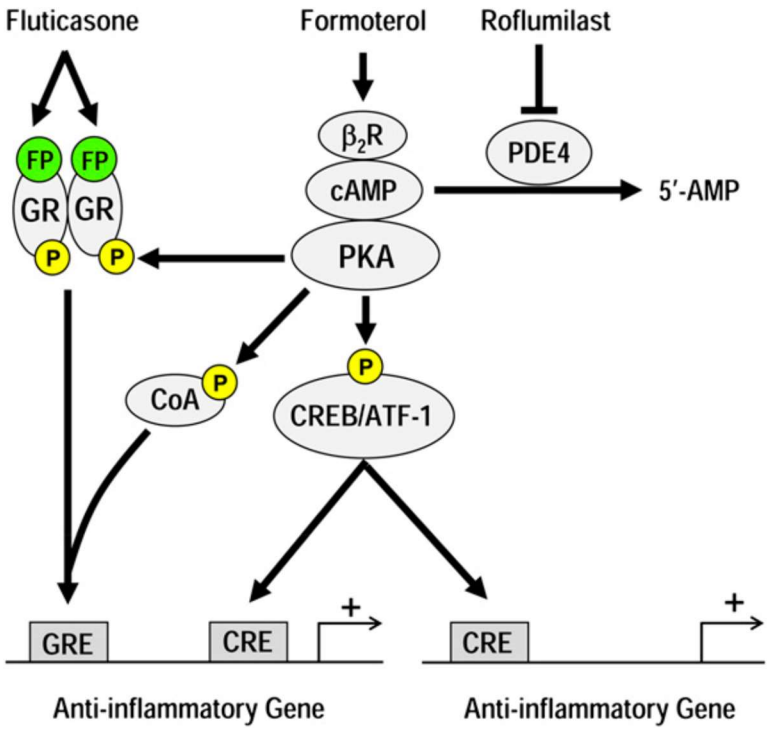

Figure 2 - Enhancement of glucocorticoid-dependent gene expression by cAMP-elevating agents.
Fluticasone (FP), glucocorticoid receptor (GR), glucocorticoid response element (GRE), Protein Kinase A (PKA), β_2 adrenoceptor (β_2AR), coactivators (CoA), cAMP-response element binding protein (CREB), activating transcription factor 1 (ATF-1), cAMP response elements (CRE)(80).

A paper published in collaboration between Dr. Robert Newton, and Dr. Mark A. Giembycz sheds light onto this topic with their proposed pathway (Figure 2). In their pathway, GCS binds onto GR, which in turn translocates to the nucleus and promotes the expression of GREs to promote the expression of anti-inflammatory genes. When LABA binds onto the β_2AR, it activates AC and produces cAMP which activates the PKA signaling cascade. This leads to the phosphorylation of GRs, coactivation of GRE, and the CREB/ATF-1 pathway. The CREB/ATF-1 then activates CRE which may be located in some of the genes upregulated by GR, and genes that are not associated with GRs.

Thereby, the combination of LABA and GCS will result in increased anti-inflammatory gene expression synergistically(80). This forms the basis of our hypothesis that further increasing intracellular cAMP can potentiate LABA/GCS' ability to suppress inflammation.

Regulation of cAMP via ABCC4

ABC transporters make up a superfamily that uses ATP for the transport of substrates across membranes. ABCC4, also known as multidrug resistance-associated protein 4 (MRP4), is expressed in many different tissues such as the prostate, liver, testis, ovary, kidney, and the lungs(81). ABCC4 is unique in that it can localize either to the apical or basolateral membrane in polarized cells(82). ABCC4 can transport cAMP, uric acid, prostaglandins, and leukotrienes (83). ABCC4 expression was first demonstrated in human airway epithelial cells for their ability to transport prostaglandins(84). Our group was able to demonstrate that ABCC4 can also transport cAMP and urate in human airway epithelial cells(85). On top of all this, we were able to show that inhibition of ABCC4 potentiates cystic fibrosis transmembrane conductance regulator (CFTR) activity as well as LABA/GCS anti-inflammatory effect due to the increase in cAMP(86,87).

Regulation of cAMP via PDEs

The PDE superfamily are enzymes that break phosphodiester bonds and are responsible for the hydrolysis of cAMP and cyclic guanosine monophosphate (cGMP) into their respective inactive form, AMP and GMP(88). PDE’s consist of 11 primary

families with each one having differential preferences for cAMP or cGMP. PDE1,2,3,10 and 11 hydrolyze both cAMP and cGMP, while PDE4,7 and 8 are cAMP-specific, and PDE5, 6 and 9 are cGMP-specific. Amongst these, PDE4 is highly relevant for chronic inflammatory airway diseases as it is the major regulator of cAMP levels in inflammatory cells(89,90). PDE4 has 4 different isoforms (A, B, C, D).

Currently, there is only one clinically approved selective PDE4 inhibitor regularly used for the treatment of chronic obstructive pulmonary disease (COPD), roflumilast(91). Roflumilast a long-acting oral PDE4 inhibitor and have been shown to improve postbronchodilator forced expiratory volume (FEV_1), and health-related quality of life in COPD patients. Additionally, in patients with asthma, roflumilast was able to reduce airway hyper-responsiveness, improve bronchial inflammatory cell infiltration, and reduce the levels of IL-6 and tumor necrosis factor-α(92). As effective roflumilast may be, the adverse side effects such as diarrhea, nausea, weight loss, headache, and psychiatric symptoms, the risk/benefit ratio are not impressive. It has been suggested that PDEs have a role in compartmentalized cAMP signaling as well(81). For instance, PDE4B regulates cAMP at the subplasma membrane, whereas PDE4D contributes mainly to cytosolic cAMP regulation which may be necessary in the mechanism proposed by Newton and Giembycz in Figure 2 and could also elucidate the reason why PDE4 inhibitors may be riddled with adverse side effects.

Hypothesis and Objectives

Hypothesis

Overarching

Mechanisms that increase intracellular cAMP will potentiate LABA/GCS ability to suppress viral inflammation

Specific

Inhibition of ABCC4 and PDE4 to further increase intracellular cAMP will potentiate LABA/GCS ability to suppress Poly I:C induced inflammation

Objective 1: Characterizing ABCC4 expression and localization patterns at the gene and protein level and developing novel ABCC4 inhibitors

In this thesis, I characterized the gene and protein expression of ABCC4 in primary and immortalized human airway epithelial cells in both quantitative and qualitative measures. In addition, I validated three ABCC4 inhibitor candidates.

Objective 2: Determine whether the addition of cAMP-elevating agents can improve LABA/GCS control of inflammation for Poly I:C

In this thesis, I validated a model for virally induced inflammation in human airway epithelial cell line with the sub-optimal treatment of LABA/GCS that did not fully suppress inflammatory cytokines. Using this model, I tested whether various cAMP-elevating agents alone and together can rescue the sub-optimal dose of LABA/GCS and further suppress the inflammatory cytokines compared to LABA/GCS alone.

Materials and Methods

Human airway epithelial cell culture

All of the experiments have been performed in vivo under submerged monolayers using a human bronchial epithelial cell line (HBEC-6KT) derived from a healthy non-smoker by expression of human telomerase reverse transcriptase and cyclin-dependent kinase 4 (Ramirez 2004). These cells were cultured in keratinocyte serum-free growth medium supplemented with 0.8ng/mL Epithelial Growth Factor, and 50 µg/mL Bovine Pituitary Extract and 1% Pen/Strep (Thermofisher, USA) at 37 degrees Celsius at 5% CO_2 and a humidified atmosphere.

Reagents

Stimuli – Polyinosinic:polycytidylic acid (Poly I:C), a double-stranded RNA mimic (Invivogen, USA) was used as a viral mimic.

LABA/GCS - Formoterol (Cayman Chemicals, USA), was used as our LABA and Budesonide (Cayman Chemicals, USA) was used as our GCS.

cAMP elevating reagents

- Adenylyl cyclase activator - Forskolin (FSK), (Cedarlane, Canada)
- PDE inhibitor - 3-isobutyl-1-methylxanthine (IBMX), (Peprotech, Canada).
- ABCC4 inhibitors – Ceefourin-1(CF-1) (Abcam, UK), CDRD13 (CDRD, Canada), and CDRD14 (CDRD, Canada).
- PDE4 inhibitors –Roflumilast (Cayman Chemicals, USA), Rolipram (Cayman Chemicals, USA), and Cilomilast (AdooQ Bioscience, USA).

Objective 1: Characterizing ABCC4 expression and localization patterns at the gene and protein level and developing novel ABCC4 inhibitors

Characterization of ABCC4

ABCC4 Gene Expression

With the Hirota Lab collaborators from the University of Waterloo, gene expression of ABCC4 and E-cadherin in healthy human bronchial brushing was analyzed from Gene Expression Omnibus dataset GSE11906(93). To complement this *in situ* data, gene expression of ABCC4 and E-cadherin from HBEC-6KT was analyzed via Nanostring to validate the expression of ABCC4 and the epithelial cell marker, E-cadherin.

Tissue Microarray

Tissue microarray of healthy primary lung tissues (n=10) was performed for immunohistochemical (IHC) staining and *in situ* hybridization (ISH) of ABCC4 in healthy human lung tissue. IHC was performed on Leica Bond Rx autostainer with ABCC4 Ab (M4I-10 ab15602). ISH was performed for ABCC4 mRNA transcript and detected using RNAscope probes (ACD Bio) and chromogen. Cells were counterstained with hematoxylin and eosin stain.

Western Blot

HBEC-6KT cells were grown to confluence and lysed using RIPA Lysis Buffer containing Protease Inhibitor Cocktail powder for 60-90 min at 4°C on a rocker. Lysates were then centrifuged at 16,000xg for 15 min, and the supernatants were collected. Protein quantification was performed using a BCA protein assay. Equal masses (20µg) of

protein were incubated in 1X Laemmli buffer with 0.1 M dithiothreitol at 65°C for 15 min and electrophoresed on 4-15% gradient TGX gels and transferred to PVDF membranes. The membranes were blocked with Tris-buffered saline with 0.05% Tween 20 (TBST), and 5% skim milk powder for 2 hours at 25°C. The membranes were incubated with ABCC4/MRP4 (1:40, Abcam, Ab15602). The membranes were washed in TBST, then incubated with horseradish peroxidase-linked anti-rat secondary antibody (1:3000, Cell Signaling Technology®, 7077S) or anti-mouse secondary antibody (1:3000, Cell Signaling Technology®, 7076S) for 2 hours at 25°C. A chemiluminescence image of the blot was taken, and protein quantification was analyzed in Image Lab software using total protein normalization.

Developing ABCC4 inhibitors

In collaboration with the Centre for Drug Research and Development (CDRD), two small molecules, CDRD13, and CDRD14 (CDRD, Canada) were evaluated for their ability to inhibit ABCC4 by measuring extracellular cAMP level in HBEC-6KT cells. All three of the ABCC4 inhibitor candidates underwent a dose-response analysis (0.01, 0.1, 1, 10, and 100μM) for comparison to known commercially available selective ABCC4 inhibitor, CF-1(Abcam, UK). Cells were incubated with one of the ABCC4 inhibitor candidates and with IBMX (50μM), a non-selective PDE inhibitor to prevent cAMP degradation, for 2 hours. FSK (20μM) was then added as a cAMP elevating agent, and cells were incubated for 6 hours. Supernatants were collected post 6 hours for extracellular cAMP measurement through ELISA.

Objective 2: Determine whether the addition of cAMP-elevating agents can improve LABA/GCS control of Poly I:C induced inflammation

Optimizing stimulus, LABA, and GCS concentrations

Outcome of measurements: GM-CSF, IL-6, and IL-8

Stimuli – We performed a dose-response study on HBEC-6KT, with three concentrations of Poly I:C (0.1, 1, 10μg/mL), to model a viral stimulus.

LABA (Formoterol) – We performed a dose-response study with *fixed GCS (Budesonide)* concentration (10nM) with a five-log concentration of LABA (Formoterol) ranging from 0.001-1nM in the presence of Poly I:C (1 μg/mL).

GCS (Budesonide) – performed a dose-response study with *fixed LABA (Formoterol)* concentration (0.01nM) with five-log concentration of GCS (Budesonide) ranging from 0.1-1000nM in the presence of Poly I:C (1 μg/mL).

The results of these experiments informed downstream experiments where all subsequent methods used 1μg/mL for Poly I:C, 0.01nM for formoterol and 10nM for budesonide.

Drug intervention

All experiments outlined below were performed when cells were 85-95% confluent. Cells were treated with drugs (LABA/GCS, ABCC4 inhibitors, and/or PDE4 inhibitors) and incubated for 2 hours. After incubation Poly I:C was added to the cells. Cell supernatants were collected post-24-hour Poly I:C incubation for the quantification of cytokines GM-CSF, IL-6, IL-8, RANTES, and IP-10 using commercially available enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, USA). Based on the instructions of the kit, a 7-point serial dilution was used as the standard, while the final optical densities

were read at 450 nm with correction at 540 nm in the photospectrometer (SpectraMax i3x, Molecular Devices, USA).

ABCC4 inhibition – We tested to see if the addition of ABCC4 inhibitors (CF-1[10μM], CDRD13[10μM] and CDRD14[10μM]) to LABA/GCS would further decrease cytokine readouts when compared to just LABA/GCS alone in the presence of Poly I:C.

PDE4 inhibition – We tested to see if the addition of PDE4 inhibitors (Rolipram[10μM], Roflumilast[1μM], and Cilomilast[1μM]) to LABA/GCS would further decrease cytokine readouts when compared to just LABA/GCS alone in the presence of Poly I:C.

ABCC4 + PDE4 inhibition – We tested to see if the combination of both ABCC4 inhibitors (CF-1[10μM], CDRD14[10μM] and CDRD14[10μM]) and PDE4 inhibitor (Roflumilast[10μM]) to LABA/GCS would further decrease cytokine readouts when compared to just LABA/GCS alone in the presence of Poly I:C.

Statistical analysis

One-way ANOVAs were performed with a post-hoc Bonferroni correction for multiple comparisons. A p-value <0.05 was accepted to be a statistically significant difference between groups. Data were analyzed using GraphPad Prism Version 6. Data are expressed as either means $\pm$ standard error of the mean (SEM), or mean percentage to control $\pm$ SEM.

Results

Objective 1: Characterizing ABCC4 expression and localization patterns at the gene and protein level and developing novel ABCC4 inhibitors

Characterization of ABCC4

RNAscope (Tissue Microarray)

In situ hybridization of ABCC4 in human lung tissue (representative image of n=10) was performed with ABCC4 mRNA transcripts identified as red punctate dots (Figure 3A). Slide shows that ABCC4 mRNA is present in primary human lung tissues.

ABCC4 gene expression data

The expression level of ABCC4 compared proportionally to E-Cadherin was investigated in primary human airway samples publicly available in gene expression omnibus (GEO) was analyzed and compared with the expression level of ABCC4 compared proportionally to E-Cadherin in nanostring data of HBEC-6KT cells(94). The GSE11906 gene expression data is based on the signal intensity of the probes and is normalized using Mas 5.0. ABCC4 expression had a mean value of (550), and E-Cadherin had a mean value of (10360) with an n=20. ABCC4:E-Cadherin ratio was approximately 1:18.8 (~5.3% ABCC4 relative to both ABCC4 and Cadherin). HBEC-6KT ABCC4 transcript count using Nanostring technology had a mean value of (88.75), and E-Cadherin had a mean value of (1756) with an n=24. ABCC4:E-Cadherin ratio was approximately 1:19.7(~5.1% ABCC4 relative to both ABCC4 and Cadherin).

Immunohistochemical Staining (Tissue Microarray)

We were able to show that ABCC4 protein is expressed in healthy human lung tissues (representative image of n=10) (Figure 3B). ABCC4 protein expression is localized to airway epithelial cells in the basal regions of the pseudostratified epithelium.

Western blot

To complement our findings in our primary tissue samples, we ran a western bot for the expression of ABCC4 in our HBEC-6KT cells. A band for ABCC4 was seen in HBEC-6KT cells, indicating the presence of ABCC4 (Figure 3C).

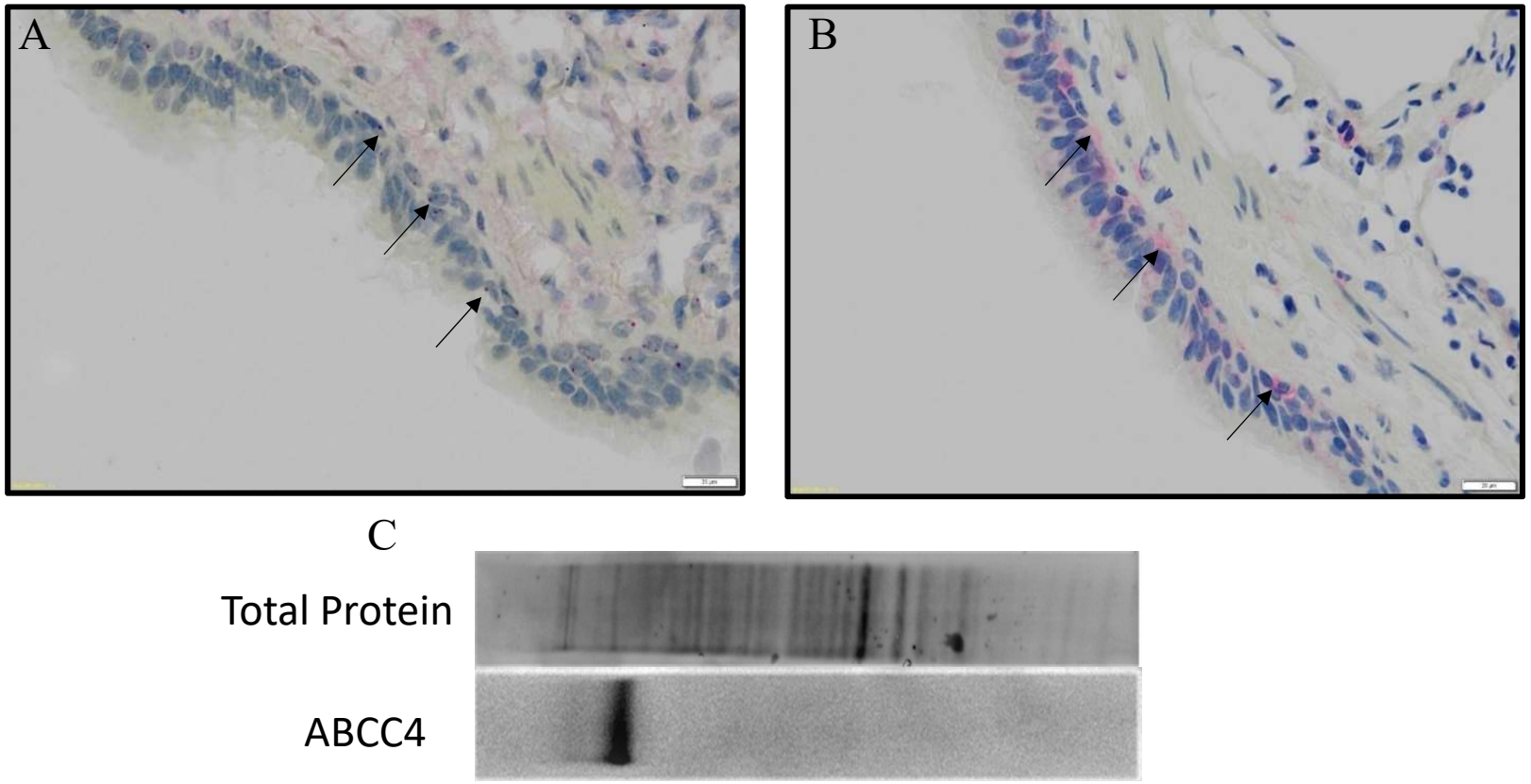

Figure 3 - Characterization of ABCC4 expression and localization patterns at the gene and protein level.
(A*) In situ* hybridization of ABCC4 in human lung tissue. mRNA transcripts are identified by red punctate dots pointed out by arrows. Cells were stained with hematoxylin and eosin stain where the blue represents the nuclei. (B) Immunohistochemical staining of ABCC4 [shown in pink/red] in healthy human lung tissue pointed out by arrows. (C) Western blot of ABCC4 in HBEC-6KT cells accompanied by the total protein.

Developing ABCC4 inhibitors

We were able to demonstrate that all three of our candidate small molecules have the capability to inhibit ABCC4 through the measurement of extracellular cAMP levels (Figure 4). CF-1 had an IC50 value of 4.76 µM and was able to inhibit 47.3% of cAMP at 10 µM. CDRD13 and CDRD14 displayed better potency compared to CF-1, with CDRD13 having an IC50 value of 1.57µM and being able to suppress 66.3% of cAMP at 10 µM, and CDRD14 having an IC50 value of 0.82 µM and being able to suppress 63.8% of cAMP at 10 µM.

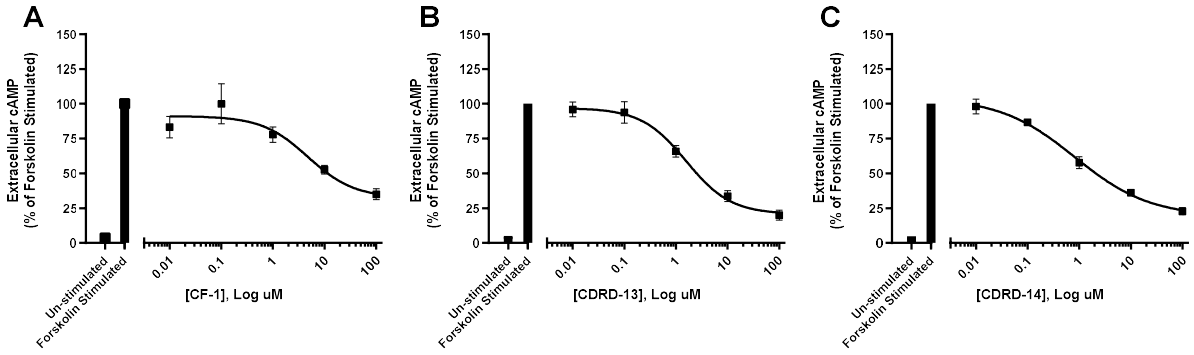

Figure 4 – ABCC4 inhibitor candidate dose response.
(A) CF-1, (B) CDRD13, and (C) CDRD14 were tested for their ability to inhibit transport of cAMP from the intracellular compartment to the extracellular compartment. HAECs were treated with 20µM of forskolin to stimulate cAMP production and 50µM IBMX, a non-selective PDE inhibitor, to prevent degradation of cAMP and treated with CF-1, CDRD13, and CDRD14 in increasing log concentrations (0.01, 0.1, 1, 10, 100µM). Extracellular cAMP was measured using ELISA assays. Each result was normalized to forskolin stimulation. All of the studies were n=5. The standard error of means is represented by the error bars attached to each point.

Objective 2: Determine whether the addition of cAMP-elevating agents can improve LABA/GCS control of Poly I:C induced inflammation

Optimizing stimulus, LABA, and GCS concentrations

Stimulus Dose Response

The inflammatory response of cells to viral mimic, Poly I:C was evaluated by measuring inflammatory cytokines, GM-CSF, IL-6, and IL-8 (Figure 5). Downstream Poly I:C concentration has been decided to be 1µg/mL as it released a satisfactory signal.

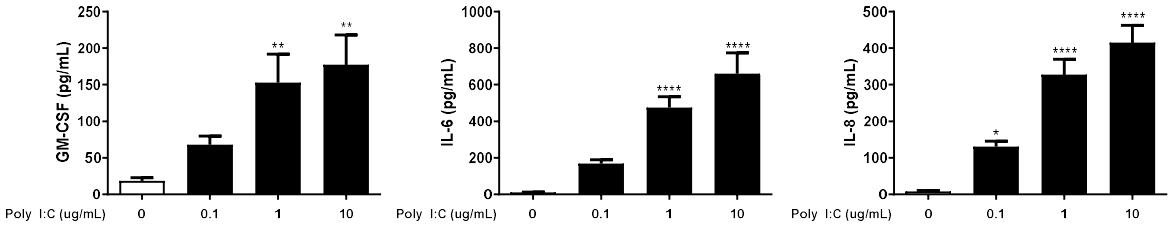

Figure 5 - Stimulus dose response.
Cells were exposed to 3 doses of Poly I:C (0.1, 1, 10µg/mL), and GM-CSF, IL-6, and IL-8 inflammatory cytokines were measured using ELISA assays. Results are shown as mean ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$*, $P < 0.001$**, $P < 0.0001$**** compared with negative control to varying doses of Poly I:C). n=8.

LABA Dose Response

A dose-response of formoterol (0.0001, 0.001, 0.01, 0.1, and 1nM) at a fixed concentration of budesonide (10nM) was performed to determine a sub-optimal concentration of formoterol in the presence of Poly I:C by measuring inflammatory cytokines, GM-CSF, IL-6, and IL-8 (Figure 6). A sub-optimal concentration of formoterol that did not completely suppress cytokine release has been determined to be 0.01nM. This concentration of formoterol will be used in downstream experiments.

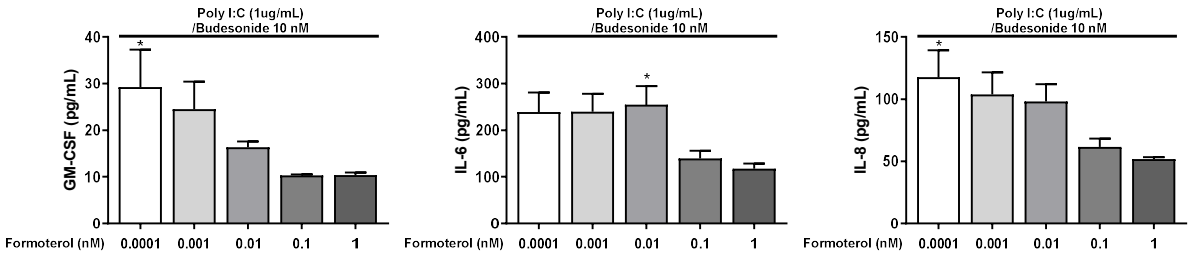

Figure 6 - LABA dose response.

A dose-response analysis measuring GM-CSF, IL-6, and IL-8 was conducted for cells exposed to Poly I:C (1µg/mL) treated with a fixed concentration of budesonide (10nM) with varying doses of formoterol (0.0001, 0.001, 0.01, 0.1, and 1nM). Results are shown as mean $\pm$ SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$*, for comparisons against 1nM). n=4

GCS Dose Response

A dose-response of budesonide (0.1, 1, 10, 100, and 1000nM) at a fixed concentration of formoterol (0.01nM) was performed to determine a sub-optimal concentration of budesonide in the presence of Poly I:C by measuring inflammatory cytokines, GM-CSF, IL-6, and IL-8 (Figure 7). A sub-optimal concentration of budesonide that did not completely suppress cytokine release has been determined to be 10nM. This concentration of budesonide will be used in downstream experiments.

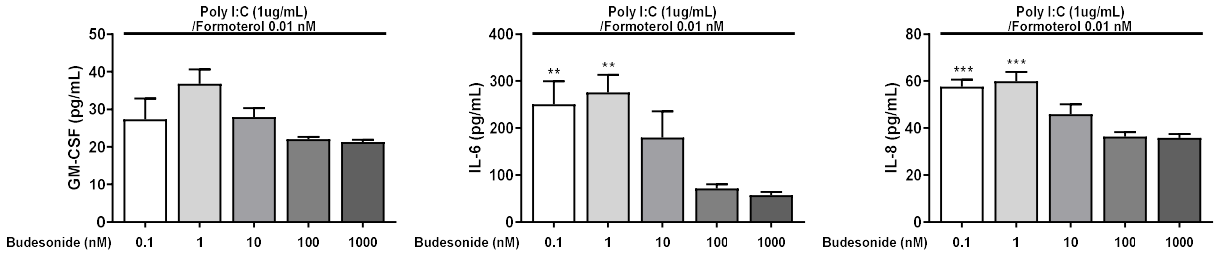

Figure 7 - GCS dose response.

A dose-response analysis measuring GM-CSF, IL-6 and IL-8 was conducted for cells exposed to Poly I:C (1µg/mL) treated with a fixed concentration of formoterol (0.01nM) and varying doses of budesonide (0.1, 1, 10, 100, and 1000nM). Results are shown as mean ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.01$**, $P < 0.001$*** for comparisons against 1000nM). n=4

Drug intervention

ABCC4 inhibition

GM-CSF: LABA/GCS suppressed Poly I:C induced GM-CSF by 87.2%, and the further addition of ABCC4 inhibitors did not significantly impact GM-CSF release (Figure 8).

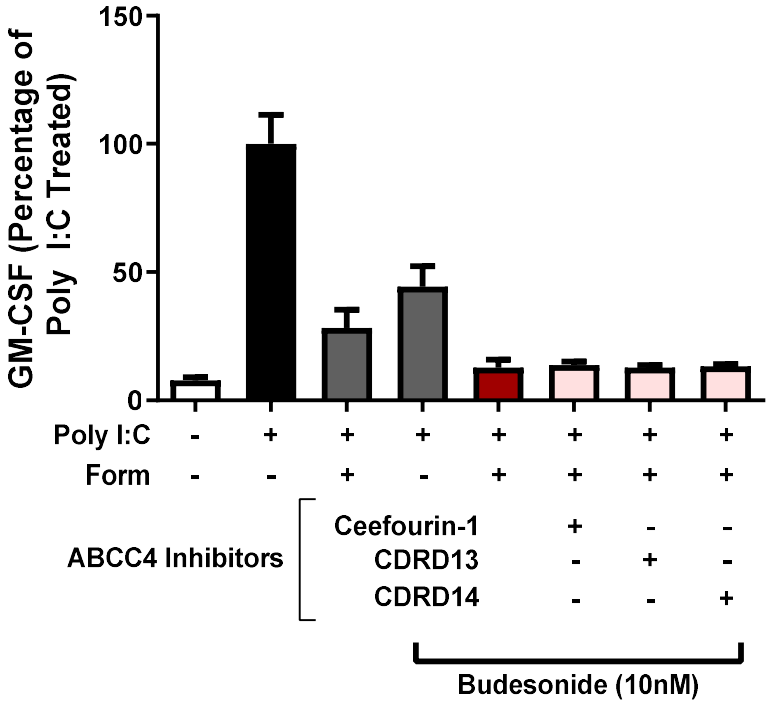

Figure 8 - ABCC4 inhibition intervention study – GM-CSF.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of GM-CSF was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (no significance was found comparing LABA/GCS to LABA/GCS with ABCC4 inhibitors). n=5

IL-6: LABA/GCS suppressed Poly I:C induced IL-6 by 25.0% compared to the positive control. Addition of CF-1 was able to suppress Poly I:C induced IL-6 by 46.8% compared to positive control (21.8% further suppression, $p<0.01$ compared to LABA/GCS), Addition of CDRD13 was able to suppress Poly I:C induced IL-6 by 59.4% compared to positive control (34.4% further suppression, $p<0.0001$ compared to LABA/GCS) (Figure 9).

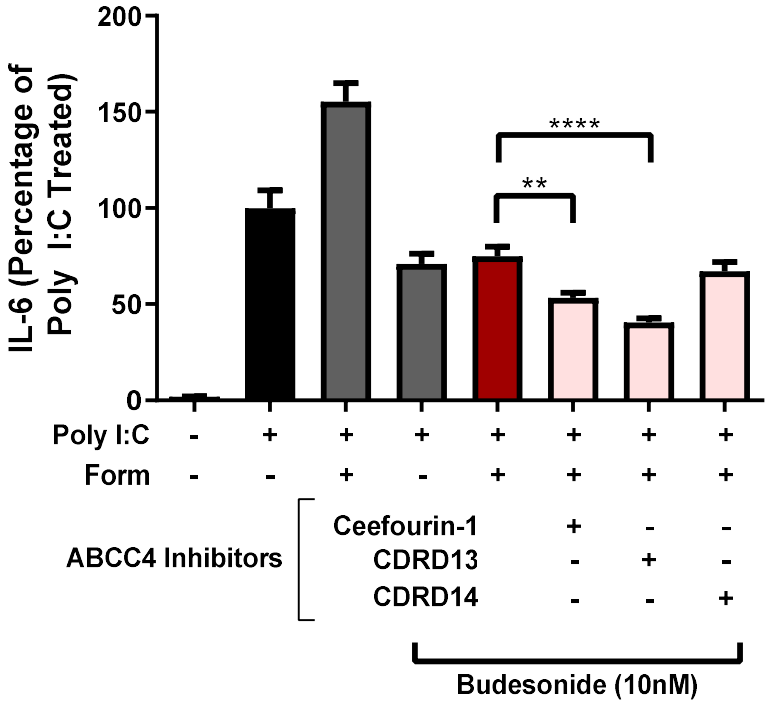

Figure 9 - ABCC4 inhibition intervention study – IL-6.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IL-6 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.01$**, $P < 0.0001$**** compared with LABA/GCS to LABA/GCS with ABCC4 inhibitors). n=5

IL-8: LABA/GCS suppressed Poly I:C induced IL-8 by 32.4% compared to the positive control. Addition of CDRD13 was able to suppress Poly I:C induced IL-8 by 55.0% compared to positive control (22.6% further suppression, $p<0.05$ compared to LABA/GCS)(Figure 10).

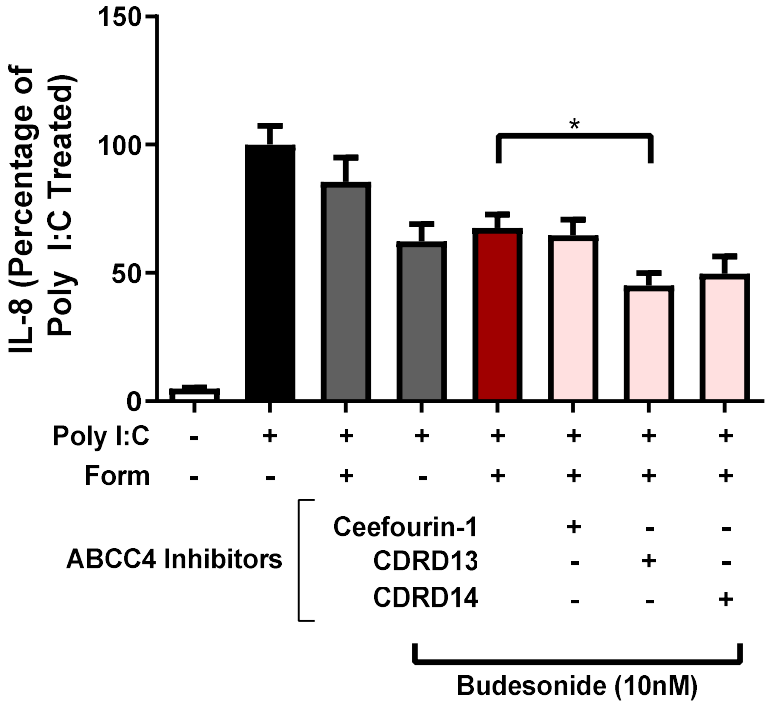

Figure 10 - ABCC4 inhibition intervention study – IL-8.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IL-8 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$* compared with LABA/GCS to LABA/GCS with ABCC4 inhibitors). n=5

IP-10: LABA/GCS suppressed Poly I:C induced IP-10 by 32.1% compared to the positive control. Addition of CDRD13 was able to suppress Poly I:C induced IP-10 by 64.2% compared to positive control (32.1% further suppression, $p<0.01$ compared to LABA/GCS)(Figure 11).

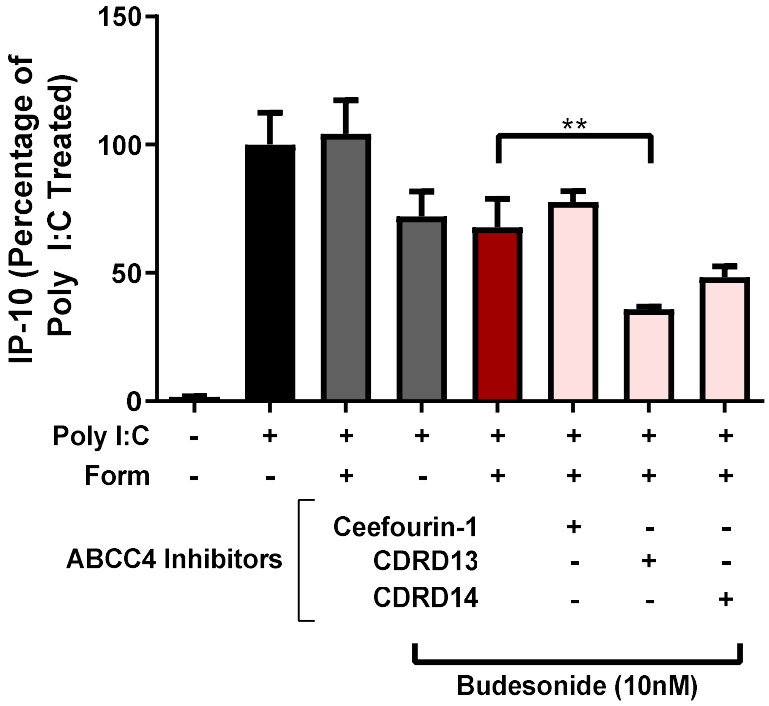

Figure 11 - ABCC4 inhibition intervention study – IP-10.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IP-10 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (P < 0.01** compared with LABA/GCS to LABA/GCS with ABCC4 inhibitors). n=5

RANTES: LABA/GCS suppressed Poly I:C induced RANTES by 26.8% compared to the positive control. Addition of CF-1 was able to suppress Poly I:C induced RANTES by 11.6% (15.2% lesser suppression, $p<0.05$ compared to LABA/GCS). Addition of CDRD13 was able to suppress Poly I:C induced RANTES by 8.7% (18.0% lesser suppression, $p<0.01$ compared to LABA/GCS)(Figure 12).

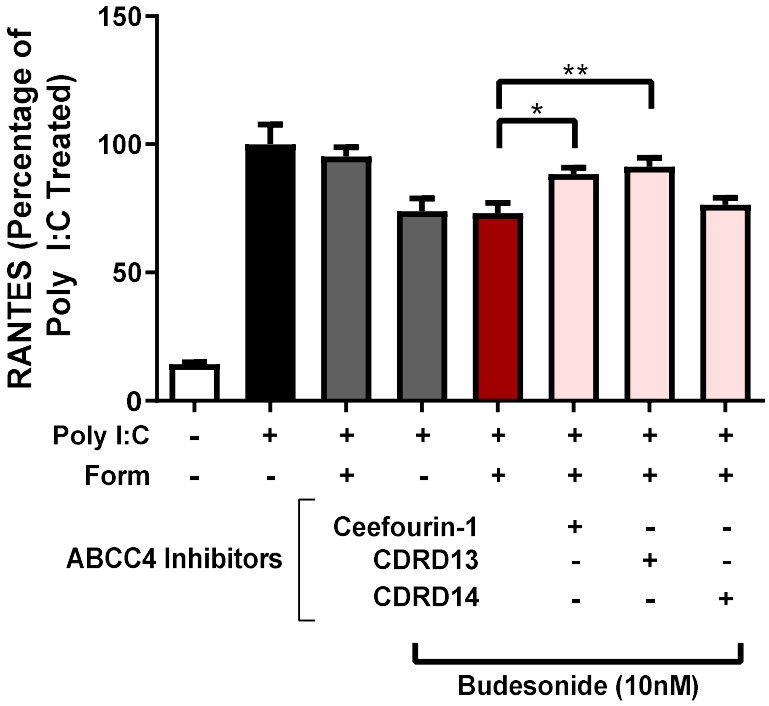

Figure 12 - ABCC4 inhibition intervention study – RANTES.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of RANTES was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$*, $P < 0.01$** compared with LABA/GCS to LABA/GCS with ABCC4 inhibitors). n=5

ABCC4 Inhibition Experiment (GM-CSF)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	50.3			
LABA/GCS	1.5	12.8	87.2 (p<0.0001)	
LABA/GCS + CF-1	1.3	13.8	86.2 (p<0.0001)	-1.0 (ns)
LABA/GCS + CDRD13	1.2	12.8	87.2 (p<0.0001)	0.0 (ns)
LABA/GCS + CDRD14	1.4	13.3	86.7 (p<0.0001)	-0.5 (ns)
ABCC4 Inhibition Experiment (IL-6)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	561.2			
LABA/GCS	412.6	75.0	25.0 (p<0.05)	
LABA/GCS + CF-1	294.1	53.3	46.8 (p<0.0001)	21.8 (p<0.01)
LABA/GCS + CDRD13	223.9	40.6	59.4 (p<0.0001)	34.4 (p<0.0001)
LABA/GCS + CDRD14	371.4	67.1	32.9 (p<0.01)	7.9 (ns)
ABCC4 Inhibition Experiment (IL-8)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	322.8			
LABA/GCS	213.7	67.6	32.4 (p<0.01)	
LABA/GCS + CF-1	203.3	64.7	35.3 (p<0.01)	2.9 (ns)
LABA/GCS + CDRD13	142.26	45.0	55.0 (p<0.0001)	22.6 (p<0.05)
LABA/GCS + CDRD14	154.9	49.7	50.3 (p<0.0001)	17.9 (ns)
ABCC4 Inhibition Experiment (IP-10)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	3326.9			
LABA/GCS	2176.5	67.9	32.1(p<0.05)	
LABA/GCS + CF-1	2516.9	77.6	22.4 (ns)	-9.8 (ns)
LABA/GCS + CDRD13	1176.6	35.8	64.2 (p<0.0001)	32.1 (p<0.01)
LABA/GCS + CDRD14	1554.9	48.3	51.7 (p<0.001)	19.6 (ns)
ABCC4 Inhibition Experiment (RANTES)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	703.1			
LABA/GCS	508.8	73.2	26.8 (p<0.01)	
LABA/GCS + CF-1	619.7	88.4	11.6 (ns)	-15.2 (p<0.05)
LABA/GCS + CDRD13	636.4	91.3	8.7 (ns)	-18.0 (p<0.01)
LABA/GCS + CDRD14	533.1	76.5	23.5 (p<0.01)	-3.3 (ns)

Table 1- Quantitative measures to complement Figures 8-12 – ABCC4 inhibition intervention study.

PDE4 inhibition

GM-CSF: LABA/GCS suppressed Poly I:C induced GM-CSF by about 92.2%, and the further addition of PDE4 inhibitors did not significantly impact GM-CSF release (Figure 13).

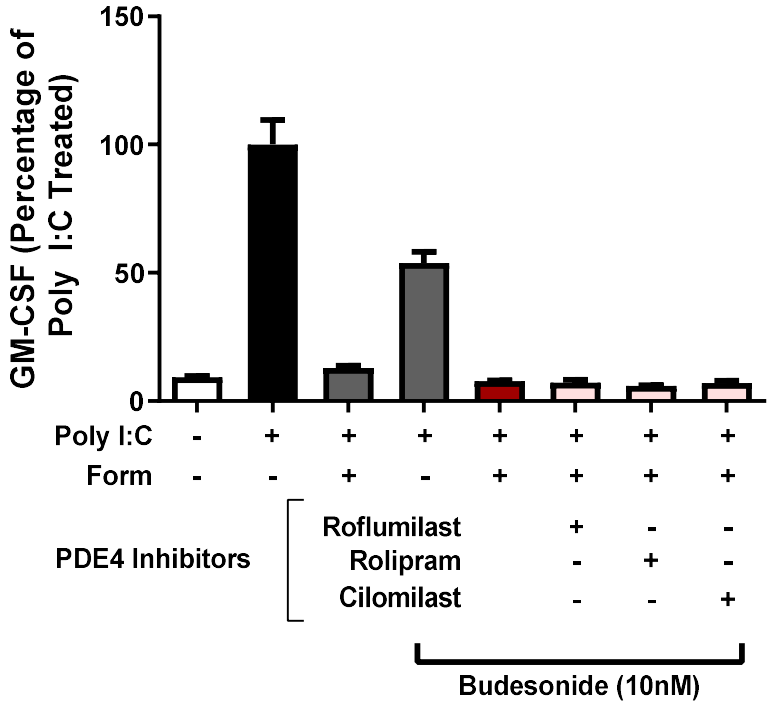

Figure 13 - PDE4 inhibition intervention study – GM-CSF.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitors (Roflumilast [1uM], Rolipram [10uM], and Cilomilast [1uM]) and release of GM-CSF was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (no significance was found comparing LABA/GCS to LABA/GCS with PDE4 inhibitors). n=5

IL-6: LABA/GCS suppressed Poly I:C induced IL-6 by 29.9% compared to the positive control. Addition of Rolipram was able to suppress Poly I:C induced IL-6 by 60.1% compared to positive control (30.2% further suppression, $p<0.05$ compared to LABA/GCS)(Figure 14).

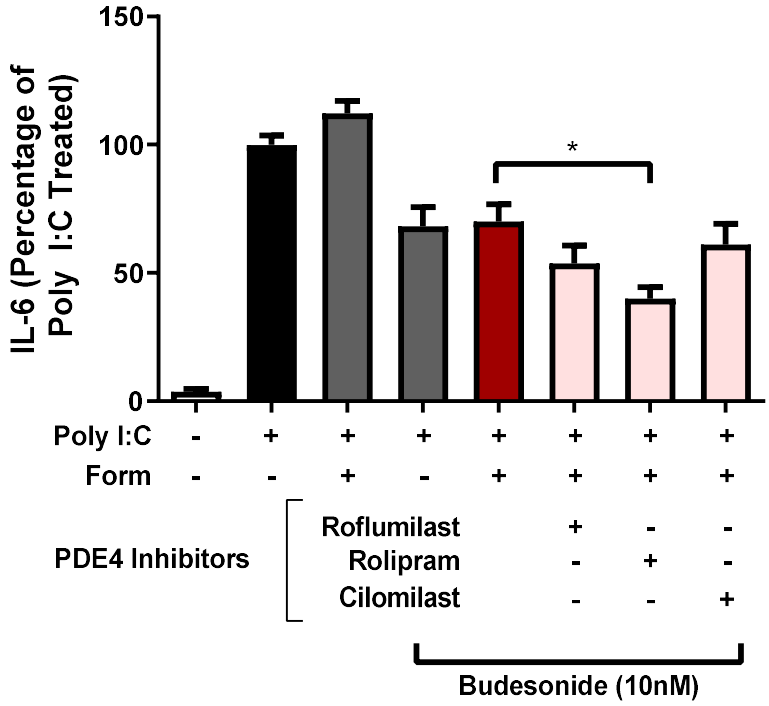

Figure 14 - PDE4 inhibition intervention study – IL-6.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitors (Roflumilast [1uM], Rolipram [10uM], and Cilomilast [1uM]) and release of IL-6 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$* compared with LABA/GCS to LABA/GCS with PDE4 inhibitors). n=5

IL-8: LABA/GCS suppressed Poly I:C induced IL-8 by 57.2% compared to the positive control. Addition of Rolipram was able to suppress Poly I:C induced IL-8 by 73.7% compared to positive control (16.5% further suppression, $p<0.05$ compared to LABA/GCS)(Figure 15).

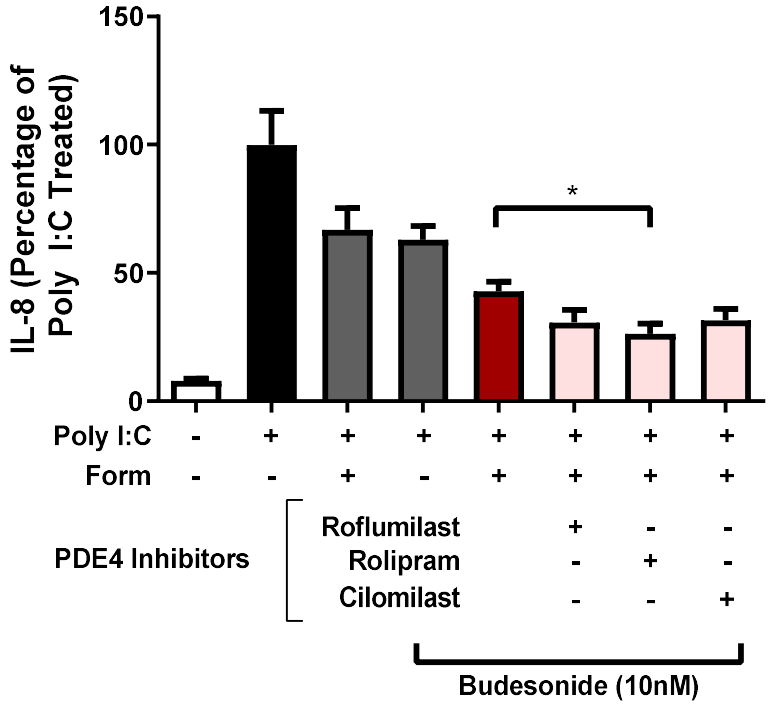

Figure 15 - PDE4 inhibition intervention study – IL-8.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitors (Roflumilast [1uM], Rolipram [10uM], and Cilomilast [1uM]) and release of IL-8 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$* compared with LABA/GCS to LABA/GCS with PDE4 inhibitors). n=5

IP-10: LABA/GCS did not alter Poly I:C induced IP-10 to a statistically significant level compared to the positive control. Addition of any PDE4 inhibitors did not significantly change IP-10 readouts when compared to both the positive control and LABA/GCS (Figure 16).

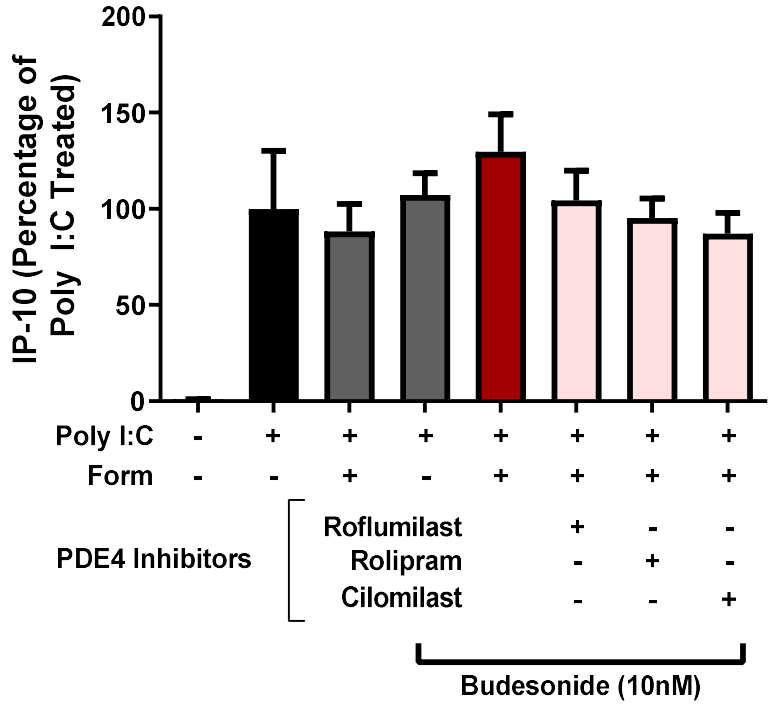

Figure 16 - PDE4 inhibition intervention study – IP-10.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitors (Roflumilast [1uM], Rolipram [10uM], and Cilomilast [1uM]) and release of IP-10 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (no significance was found comparing LABA/GCS to LABA/GCS with PDE4 inhibitors). n=5

RANTES: LABA/GCS did not alter Poly I:C induced RANTES to a statistically significant level compared to the positive control. Addition of any PDE4 inhibitors did not significantly change RANTES readouts when compared to both the positive control and LABA/GCS(Figure 17).

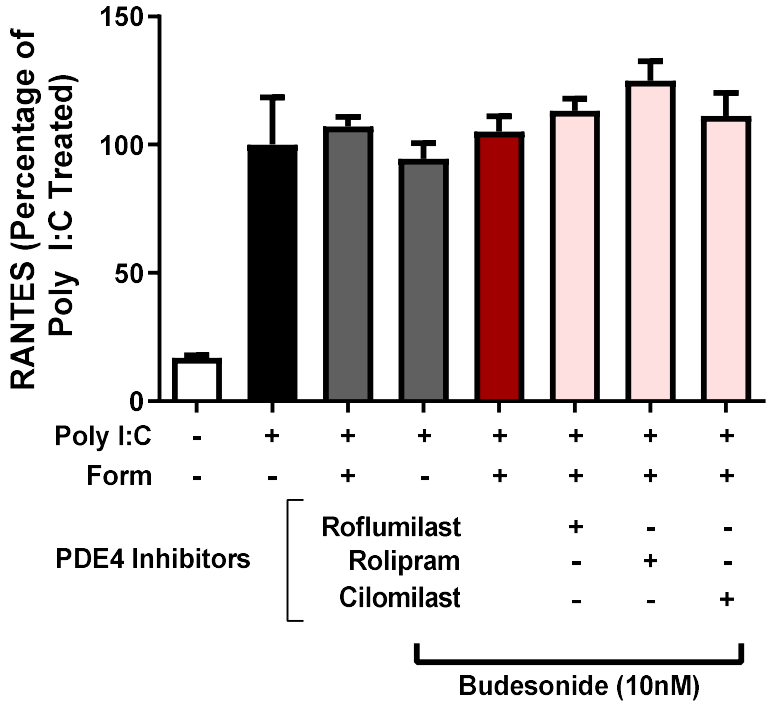

Figure 17 - PDE4 inhibition intervention study – RANTES.
An intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitors (Roflumilast [1uM], Rolipram [10uM], and Cilomilast [1uM]) and release of RANTES was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (no significance was found comparing LABA/GCS to LABA/GCS with PDE4 inhibitors). n=5

PDE4 Inhibition Experiment (GM-CSF)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	121.3			
LABA/GCS	9.5	7.8	92.2 (p<0.0001)	
LABA/GCS + Roflumilast	8.4	7.1	92.9 (p<0.0001)	0.7 (ns)
LABA/GCS + Rolipram	7.1	5.9	94.1 (p<0.0001)	1.9 (ns)
LABA/GCS + Cilomilast	8.1	7.0	93.0 (p<0.0001)	0.8 (ns)
PDE4 Inhibition Experiment (IL-6)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	1030.9			
LABA/GCS	723.7	70.1	29.9 (p<0.05)	
LABA/GCS + Roflumilast	554.7	53.7	46.3 (p=0.001)	16.4 (ns)
LABA/GCS + Rolipram	413.9	39.9	60.1 (p<0.0001)	30.2 (p<0.05)
LABA/GCS + Cilomilast	635.3	61.0	39.0 (p=0.001)	9.1 (ns)
PDE4 Inhibition Experiment (IL-8)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	474.2			
LABA/GCS	194.0	42.8	57.2 (p<0.0001)	
LABA/GCS + Roflumilast	134.8	30.7	69.3 (p<0.0001)	12.1 (ns)
LABA/GCS + Rolipram	114.8	26.3	73.7 (p<0.0001)	16.5 (p<0.05)
LABA/GCS + Cilomilast	139.4	31.6	68.4 (p<0.0001)	11.2 (ns)
PDE4 Inhibition Experiment (IP-10)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	1355.3			
LABA/GCS	1671.2	129.7	-29.7 (ns)	
LABA/GCS + Roflumilast	1460.3	104.4	-4.4 (ns)	25.3 (ns)
LABA/GCS + Rolipram	1307.6	95.2	4.8 (ns)	34.5 (ns)
LABA/GCS + Cilomilast	1228.4	87.2	12.8 (ns)	42.6 (ns)
PDE4 Inhibition Experiment (RANTES)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	1134.2			
LABA/GCS	1152.6	105.0	-5 (ns)	
LABA/GCS + Roflumilast	1250.9	113.2	-13.2 (ns)	-8.1 (ns)
LABA/GCS + Rolipram	1393.0	124.9	-24.9 (ns)	-19.9 (ns)
LABA/GCS + Cilomilast	1229.6	111.1	-11.1 (ns)	-6.1 (ns)

Table 2 - Quantitative measures to complement Figures 13-17 – PDE4 inhibition intervention study.

ABCC4 + PDE4 inhibition

GM-CSF: LABA/GCS suppressed Poly I:C induced GM-CSF by about 88.7%, compared to the positive control. Addition of CF-1+Roflumilast was able to suppress Poly I:C induced GM-CSF by 94.3% compared to positive control (5.6% further suppression, $p<0.0001$ compared to LABA/GCS). Addition of CDRD13+Roflumilast was able to suppress Poly I:C induced GM-CSF by 94.9% compared to positive control (6.2% further suppression, $p<0.0001$ compared to LABA/GCS). Addition of CDRD14+Roflumilast was able to suppress Poly I:C induced GM-CSF by 95.4% compared to positive control (6.7% further suppression, $p<0.0001$ compared to LABA/GCS)(Figure 18).

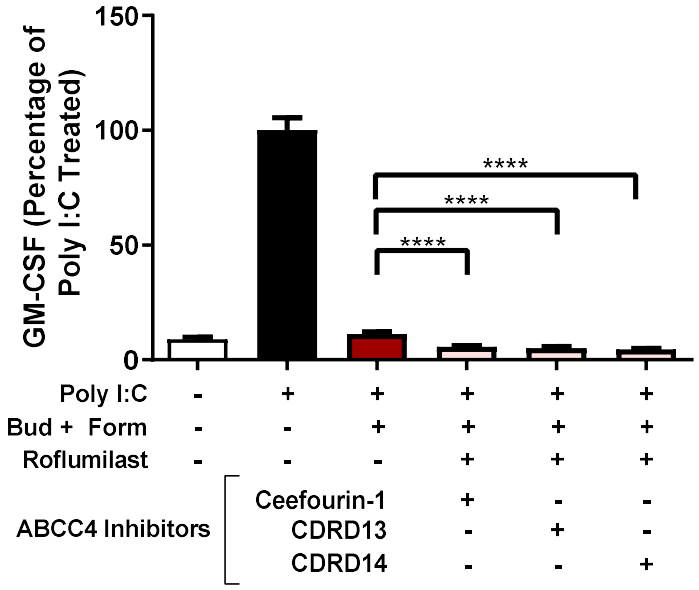

Figure 18 - ABCC4 + PDE4 inhibition intervention study – GM-CSF.
A combination intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitor (Roflumilast [1uM]) and ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of GM-CSF was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.0001$**** compared with LABA/GCS to additional PDE4 and ABCC4 inhibitors). n=5

IL-6: LABA/GCS suppressed Poly I:C induced IL-6 by about 22.5%, compared to the positive control. Addition of CF-1+Roflumilast was able to suppress Poly I:C induced IL-6 by 52.5% compared to positive control (30.0% further suppression, $p<0.05$ compared to LABA/GCS). Addition of CDRD13+Roflumilast was able to suppress Poly I:C induced IL-6 by 64.7% compared to positive control (42.2% further suppression, $p<0.001$ compared to LABA/GCS). Addition of CDRD14+Roflumilast was able to suppress Poly I:C induced IL-6 by 49.7% compared to positive control (27.2% further suppression, $p<0.05$ compared to LABA/GCS)(Figure 19).

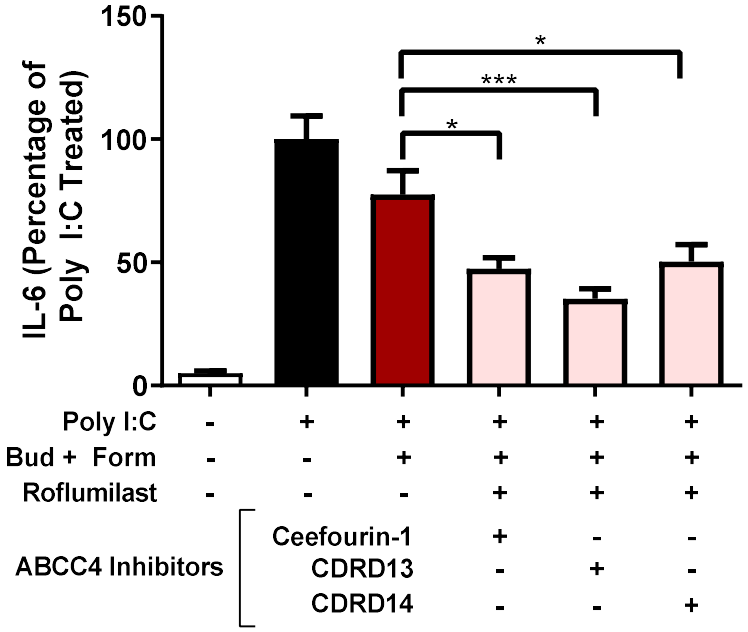

Figure 19 - ABCC4 + PDE4 inhibition intervention study – IL-6.
A combination intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitor (Roflumilast [1uM]) and ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IL-6 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.05$*, $P < 0.001$*** compared with LABA/GCS to additional PDE4 and ABCC4 inhibitors). n=5

IL-8: LABA/GCS suppressed Poly I:C induced IL-8 by about 49.5%, compared to the positive control. Addition of CF-1+Roflumilast was able to suppress Poly I:C induced IL-8 by 67.7% compared to positive control (18.2% further suppression, $p<0.005$ compared to LABA/GCS). Addition of CDRD13+Roflumilast was able to suppress Poly I:C induced IL-8 by 76.3% compared to positive control (26.8% further suppression, $p<0.0001$ compared to LABA/GCS). Addition of CDRD14+Roflumilast was able to suppress Poly I:C induced IL-8 by 74.9% compared to positive control (25.4% further suppression, $p=0.0001$ compared to LABA/GCS)(Figure 20).

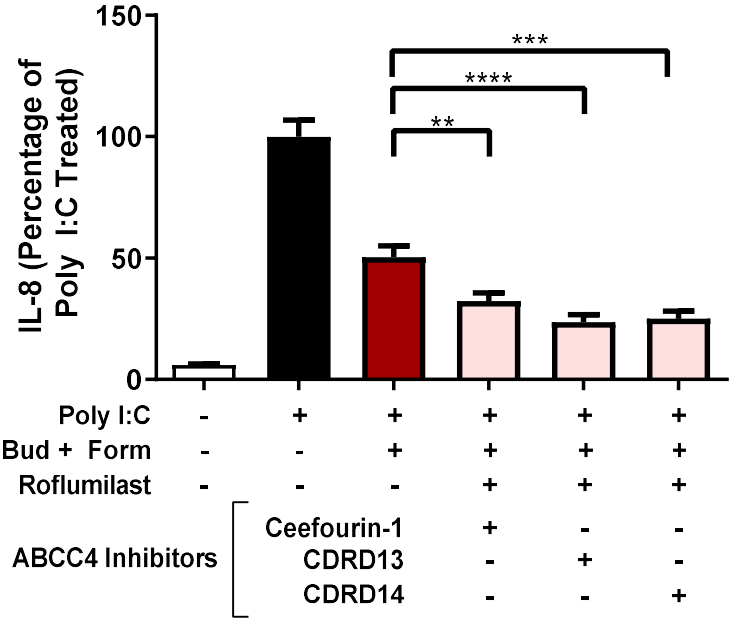

Figure 20 - ABCC4 + PDE4 inhibition intervention study – IL-8.
A combination intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitor (Roflumilast [1uM]) and ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IL-8 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.01$**, $P < 0.001$***, $P < 0.0001$**** compared with LABA/GCS to additional PDE4 and ABCC4 inhibitors). n=5

IP-10: LABA/GCS did not alter Poly I:C induced IP-10 to a statistically significant level compared to the positive control. Addition of CDRD13+Roflumilast was able to suppress Poly I:C induced IP-10 by 52.8% compared to positive control (52.1% further suppression, $p<0.01$ compared to LABA/GCS). Addition of CDRD14+Roflumilast was able to suppress Poly I:C induced IP-10 by 42.9% compared to positive control (42.1% further suppression, $p<0.01$ compared to LABA/GCS)(Figure 21).

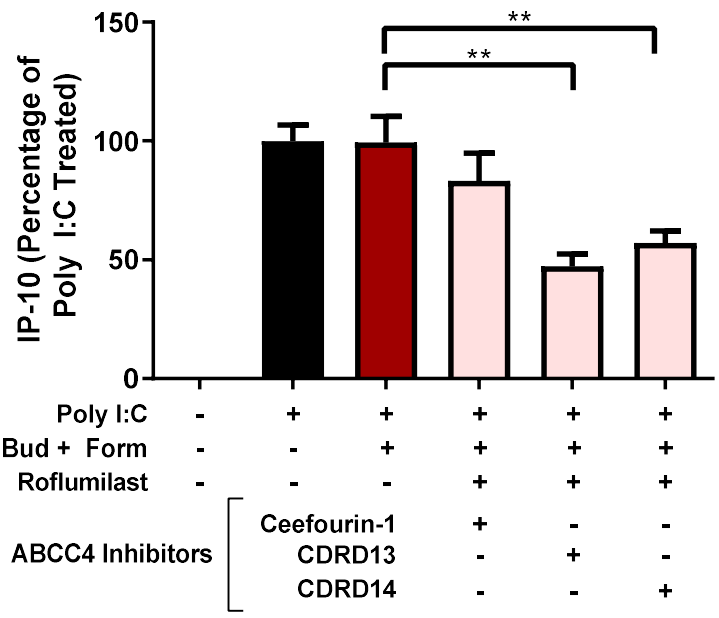

Figure 21 - ABCC4 + PDE4 inhibition intervention study – IP-10.
A combination intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitor (Roflumilast [1uM]) and ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of IP-10 was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. ($P < 0.01$** compared with LABA/GCS to additional PDE4 and ABCC4 inhibitors). n=5

RANTES: LABA/GCS did not alter Poly I:C induced IP-10 to a statistically significant level compared to the positive control. Addition of any ABCC4 inhibitors and Roflumilast did not significantly change RANTES readouts when compared to LABA/GCS (Figure 22).

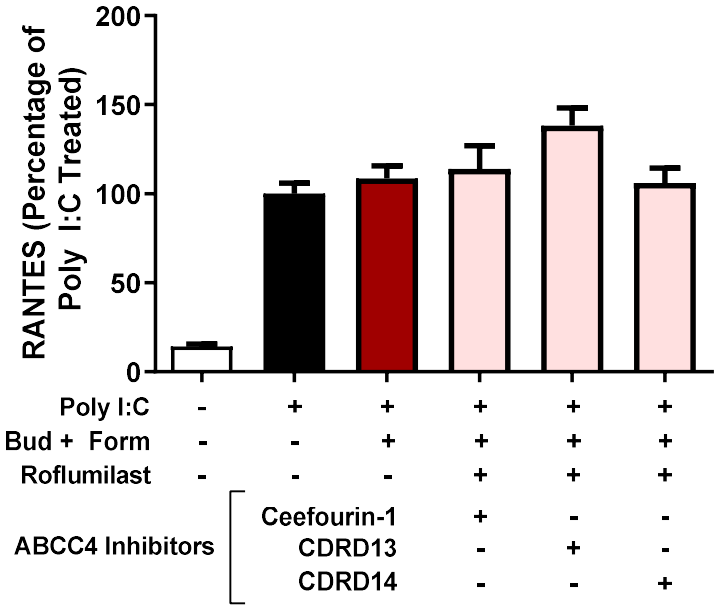

Figure 22 - ABCC4 + PDE4 inhibition intervention study – RANTES.
A combination intervention study was performed with Poly I:C. Formoterol (0.01nM) and budesonide (10nM) were used in combination with PDE4 inhibitor (Roflumilast [1uM]) and ABCC4 inhibitors (CF-1 [10uM], CDRD13 [10uM] and CDRD14 [10uM]) and release of RANTES was measured using ELISA assays. All measures were normalized to the positive control (Poly I:C) ± SEM. One-way ANOVAs were performed with a post hoc Bonferroni test for multiple comparisons. (no significance was found comparing LABA/GCS to additional PDE4 and ABCC4 inhibitors). n=5

ABCC4+PDE4 Inhibition Experiment (GM-CSF)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	119.9			
LABA/GCS	13.5	11.3	88.7 (p<0.0001)	
LABA/GCS + Rolipram + CF-1	6.7	5.7	94.3 (p<0.0001)	5.6 (p<0.0001)
LABA/GCS + Rolipram + CDRD13	6.1	5.1	94.9 (p<0.0001)	6.2 (p<0.0001)
LABA/GCS + Rolipram + CDRD14	5.4	4.6	95.4 (p<0.0001)	6.7 (p<0.0001)
ABCC4+PDE4 Inhibition Experiment (IL-6)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	1045.4			
LABA/GCS	775.1	77.5	22.5 (ns)	
LABA/GCS + Rolipram + CF-1	483.5	47.5	52.5 (p<0.001)	30.0 (p<0.05)
LABA/GCS + Rolipram + CDRD13	359.3	35.3	64.7 (p<0.0001)	42.2 (p<0.001)
LABA/GCS + Rolipram + CDRD14	510.2	50.3	49.7 (p<0.001)	27.2 (p<0.05)
ABCC4+PDE4 Inhibition Experiment (IL-8)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	525.9			
LABA/GCS	263.8	50.5	49.5 (p<0.0001)	
LABA/GCS + Rolipram + CF-1	165.1	32.3	67.7 (p<0.0001)	18.2 (p<0.01)
LABA/GCS + Rolipram + CDRD13	120.7	23.7	76.3 (p<0.0001)	26.8 (p<0.001)
LABA/GCS + Rolipram + CDRD14	128.2	25.1	74.9 (p<0.0001)	25.4 (p<0.001)
ABCC4+PDE4 Inhibition Experiment (IP-10)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	2582.9			
LABA/GCS	2525.9	99.3	0.7 (ns)	
LABA/GCS + Rolipram + CF-1	2113.1	83.2	16.8 (ns)	16.1 (ns)
LABA/GCS + Rolipram + CDRD13	1192.63	47.2	52.8 (p<0.001)	52.1 (p<0.01)
LABA/GCS + Rolipram + CDRD14	1447.6	57.1	42.9 (p<0.01)	42.1 (p<0.01)
ABCC4+PDE4 Inhibition Experiment (RANTES)				
Conditions	Raw Value (pg/mL)	% to Positive control	Δ % Positive Control (p-value)	Δ LABA/GCS (p-value)
Poly I:C	1038.8			
LABA/GCS	1112.7	108.6	-8.6 (ns)	
LABA/GCS + Rolipram + CF-1	1162.5	113.8	-13.8 (ns)	-5.2 (ns)
LABA/GCS + Rolipram + CDRD13	1409.0	138.2	-38.2 (p<0.05)	-29.6 (ns)
LABA/GCS + Rolipram + CDRD14	1076.4	105.9	-5.9 (ns)	2.7 (ns)

Table 3 - Quantitative measures to complement Figures 18-22 – ABCC4 + PDE4 inhibition intervention study.

Discussion

Objective 1: Characterizing ABCC4 expression and localization patterns at the gene and protein level and developing novel ABCC4 inhibitors

Characterization of ABCC4

In this thesis, we used several techniques and resources, including publicly available gene expression datasets, Nanostring gene expression data, and archived human lung tissue to characterize the gene and protein expression and localization of ABCC4. All of this was to determine whether ABCC4 is present and could be a viable target for therapeutic purposes in human airway epithelial cells. Also, we verified that the cell line model (HBEC-6KT) that we were working with has a similar expression level of ABCC4 when compared with primary airway samples. These results are consistent with published literature (84,87).

Ten airway tissues from healthy individuals were analyzed for ABCC4 mRNA transcript detected using RNAscope probes and chromogen in situ hybridization. The representative image displayed in Figure 1A depicts ABCC4 mRNA transcripts by red punctate dots. To complement this qualitative measure, we used a publicly available dataset from GEO(94) and performed Nanostring analysis on HBEC-6KT cells. The GSE11906 dataset contains gene expression from primary bronchial brushings from healthy subjects (n=20). The gene expression in these data are measured using probe intensity and are normalized by using MAS 5.0, an Affymetrix algorithm used for producing gene expression signal. We measured ABCC4 as a target and E-Cadherin as an epithelial cell marker to serve as a frame of reference for the magnitude of transcript

expression. The ratio between ABCC4 and E-Cadherin in these primary samples was approximately 1:18.8. Whereas, the ratio of the ABCC4 to E-Cadherin gene expression Nanostring data on HBEC-6KT came up to be approximately 1:19.7. Based on the similarity of the ratio between primary samples and our HBEC-6KT cells, we were able to validate that our cell line model may have similar ABCC4 expression compared to what would be found in primary cells. We had to compare the ratio instead of the raw count because the data from GSE11906 represents signal intensity, while the Nanostring data is actual transcript counts. Also, the GSE11906 dataset also held the expression of ABCC7 (cystic fibrosis transmembrane conductance regulator), a well known functionally active transporter, to have a similar level of expression to ABCC4, further shedding light onto functional capability of ABCC4 (data not shown).

We then moved onto characterizing ABCC4 protein expression using *in situ* via IHC and *in vitro* with western blot. Using the same ten healthy airway samples, we performed IHC staining for ABCC4 protein expression. The representative image displayed in Figure 1B, which is also from the same patient sample but a different sliced section from our RNAscope in Figure 1A, depicts ABCC4 protein expression to be localized to airway epithelial cells in the basal regions of the pseudostratified epithelium. To ensure that our HBEC-6KT cells also expressed ABCC4 at a protein level, we performed a western blot. Figure 1C shows the expression of ABCC4 in respect to the total protein reading. Once we have established that ABCC4 is expressed in both gene and protein expression in primary and immortalized human airway epithelial cells, we sought to characterize small molecules that could inhibit ABCC4.

Developing ABCC4 Inhibitors

Unlike PDE4 inhibitors which are currently in use as therapeutic interventions, ABCC4 inhibitors have not yet been interrogated to the extent of PDE4 inhibitors. As such, we had to ensure that the small molecules that we are using are performing as ABCC4 inhibitors. Currently, two small molecules, CF-1, and Ceefourin-2 (CF-2), have been identified as highly selective inhibitors of ABCC4 through high throughput screening(95). Additional active compounds that may interact with ABCC4 were searched using a ligand-based approach, which uses similarity searching, pharmacophore mapping, and machine learning methods. The generation of a pharmacophore was used to inform our selective curating and purchasing of commercially available compounds for our downstream in vitro screening. Over 3 million compounds in the eMolecules database were screened, resulting in the identification of 39 compounds of interest. Candidates were purchased from commercial vendors, screened at 10μM in an *in vitro* cAMP efflux assay with HBEC-6KT cells, with top candidates undergoing a dose-response analysis (0.01, 0.1, 1, 10, 100μM). From this assay, seven compounds reduced forskolin-stimulated cAMP-efflux signal by greater than 50%, 26 reduced cAMP efflux signal to between 50% and 100% and 6 compounds increased cAMP efflux signal. The top 9 candidates selected for subsequent analysis were chosen based on their ability to reduce cAMP-efflux. The final two candidates that we chose were CDRD13, and CDRD14, both of which are based on CF-1. We were able to successfully show that our three small molecules, CF-1, CDRD13, and CDRD14 have the capability of blocking ABCC4 by measuring the extracellular cAMP levels with forskolin to stimulate cAMP production

and differing concentrations of our small molecules. However, caution must be taken as our CDRD13, and CDRD14 compound has not been tested for ABCC4 selectivity and may have other non-ABCC4 inhibiting properties that may cause unintended effects.

Objective 2: Determine whether the addition of cAMP-elevating agents can improve LABA/GCS control of Poly I:C induced inflammation

Optimizing stimulus, LABA, and GCS concentrations in HBEC-6KT

Before we could test our hypothesis, we had to first establish an appropriate model that would allow us to pursue our research question. We had already demonstrated that our HBEC-6KT cells expressed ABCC4 genes and protein. As our main concern is with viral inflammation, we used Poly I:C, a dsRNA viral mimic as our stimulus. Poly I:C, a synthetic analog of double-stranded RNA mimic, was selected due to its ability to mimic the inflammatory responses associated with a viral infection, leading to increased cytokine production, excessive mucus secretion, loss of epithelial integrity, and impaired ciliary function(96). Furthermore, Poly I:C-induced inflammation was also studied and validated in a variety of airway epithelial cell lines, including BEAS-2B and A549(97,98). In addition, as most viruses in the course of their infection activate toll-like receptor 3 (TLR3) through a secondary mediator, we felt that Poly I:C would be an appropriate stimulus for our initial investigation being a potent TLR3 agonist capable of upregulating inflammatory cytokines such as IL-1β, TNF-α, GM-CSF, IL-6, and IL-8, as well as inducing the production of anti-viral sensors IP-10 and RANTES(99,100). We performed a Poly I:C dose response with three different concentrations and chose 1μg/mL to be sufficient enough to induce a strong release of GM-CSF, IL-6, and IL-8. We then performed a LABA and GCS dose response to find the concentration that results in a sub-optimal suppression of inflammation. Since we were interested in investigating the ability of ABCC4 or PDE4 inhibitors' ability to suppress inflammation to a greater effect, we needed to ensure that despite LABA/GCS being on board, the concentration is not strong

enough to completely attenuate all cytokine production. In a previous study that we have published, we demonstrated that 10nM for budesonide and 0.01nM for formoterol was a suboptimal concentration in another human airway epithelial cell line, BEAS-2B(87). Using those two concentrations as our reference point, we performed the dose response by going two log concentrations lower and higher. Our results showed that 10nM for budesonide and 0.01nM for formoterol did not fully suppress IL-6 and IL-8, but GM-CSF was found to be greatly LABA/GCS sensitive.

Drug intervention: Evaluate whether ABCC4 and/or PDE4 inhibition enhances LABA/GCS anti-inflammatory responses in HBEC-6KT

Our intervention studies were performed to elucidate whether the combination of ABCC4 and/or PDE4 inhibition can further potentiate LABA/GCS ability suppress Poly I:C induced inflammation. We tested LABA/GCS in combination with ABCC4 inhibitors, LABA/GCS in combination with PDE4 inhibitors, and finally with LABA/GCS in combination with both ABCC4 and PDE4 inhibitors on board. We measured GM-CSF, IL-6, IL-8, IP-10, and RANTES.

ABCC4 inhibition + LABA/GCS

We chose 10μM for all of our ABCC4 inhibitors as there is no clear data that have used these small molecules, let alone their anti-inflammatory effect on human airway epithelial cells. Using the same concentration for all of the ABCC4 inhibitors will allow us to make comparisons between their respective ability to further suppress inflammation when added to LABA/GCS.

It is difficult to conclude whether the addition of ABCC4 inhibitors has the potential to decrease GM-CSF levels further when compared to LABA/GCS since LABA/GCS alone effectively brought down GM-CSF to a significant degree. This significant suppression of GM-CSF by LABA/GCS was not what we expected since our previous published data showed that the concentration of LABA/GCS at 0.01nM/10nM did not attenuate GM-CSF in BEAS-2Bs. This difference is most likely attributable to the difference in cell line that we were using(87). Our results seem to indicate that HBEC-6KT cells are more responsive to LABA/GCS for suppression of GM-CSF compared to BEAS-2Bs. Further experiments using primary cells and perhaps a lower dose of LABA/GCS or human airway epithelial cells with less sensitive GM-CSF should be completed to better shed light on LABA/GCS + ABCC4 inhibition effects on GM-CSF. In contrast, IL-6 was further suppressed with both CF-1 and CDRD13 compared to LABA/GCS alone, whereas CDRD14 did not impact IL-6 greatly. Similarly, IL-8 was further suppressed with CDRD13 compared to LABA/GCS alone. However, both CF-1 and CDRD14 failed to augment IL-8 levels in a statistically significant manner. Interestingly, CDRD13 greatly suppressed IP-10 levels, unlike the other ABCC4 inhibitor, which could be due to CDRD13 having potential non-specific binding to other receptors. As described before, IP-10 is an established viral biomarker associated with the severity of the viral infection. However, it is also a chemokine that attracts immune cells such as macrophages, T cells, NK cells, and dendritic cells. Also, a study shows IP-10 mRNA expression is expressed at a higher level in severe asthmatic subjects as well as severe asthma model mouse suggesting that IP-10 might be correlated to persistent type 1

inflammation(30). Based on this observation, suppressing the high levels of IP-10 may lessen the inflammatory burden that severe asthmatics face. However, as there is insufficient data presently on the interactions of IP-10 to LABA/GCS and improved airway functions, it is difficult to conclude yet whether the decrease in IP-10 is promising or not. Surprisingly, in contrast to IP-10, RANTES, yet another anti-viral chemokine, was shown to increase with the addition of both CF-1 and CDRD13. RANTES has been shown to be expressed at an elevated level in asthmatic children compared to healthy children, and in studies conducted in adult asthmatics, RANTES has been implicated in asthma exacerbations (101–104). However, when RANTES was investigated as a potential therapeutic target, one study showed that using a RANTES antagonist (Met-RANTES) in mice was able to decrease inflammation but resulted in delayed viral clearance and increased cellular infiltration during reinfection(55). As such, although RANTES may be a key contributor to virally induced inflammation, it is a key player in viral clearance and targeting it may lead to worse outcomes.

Stimulating airway epithelial cells with influenza A virus has been shown to activate TLR3 and the retinoic acid-inducible gene I (RIG-I) pathway, which activates NF-κB and interferon regulatory transcription factor 3 (IRF-3), respectively(105). Our results show some specificity of the pathway that our novel combination therapy may be acting on. The relationship between PKA and NF-κB have been shown to be inhibitory, where increased PKA activity results in decreased expression of NF-κB(106). IL-6 and IL-8 were successfully further suppressed, as such, since IL-6 and IL-8 are cytokines released in response to NF-κB, it strengthens the idea that our combination therapy is

inhibiting NF-κB through amplified PKA activity induced by increased cAMP levels(105). As of now, there is limited research on the interaction between IRF-3 and PKA activity. Our results potentially shed light on this interaction or lack thereof, as IP-10 and RANTES are cytokines that are induced by IRF-3(107). Although the IP-10 signal seemed to decrease with CDRD14, this was not the case for the other ABCC4 inhibitors, and RANTES seemed to not be responsive at all with the addition of ABCC4 inhibitors.

PDE4 inhibition + LABA/GCS

The PDE4 inhibitors that we used were Roflumilast, Rolipram, and Cilomilast. Roflumilast (trade names: Daxas, Daliresp) is currently used in the clinical setting for the management of COPD exacerbations but has been indicated to have a high incidence of side effects such as nausea and vomiting (108). Rolipram and Cilomilast are both selective PDE4 inhibitors that have failed clinical trials. Based on previous literature that evaluated their anti-inflammatory effects in human airway epithelial cells, we chose 1μM for Roflumilast, 10μM for Rolipram, and 1μM for Cilomilast(80,109,110).

Similar to ABCC4 inhibition, it is hard to conclude whether GM-CSF can be further suppressed by the addition of PDE4 inhibitors in combination with LABA/GCS since our model effectively suppressed GM-CSF level with LABA/GCS alone. Interestingly, Roflumilast, the clinically available drug, was not able to significantly alter LABA/GCS effectiveness in any of the cytokine measurements. This trend was also seen in Cilomilast. However, Rolipram displayed similar trends as those seen in CDRD13, where it was able to suppress both IL-6 and IL-8 levels further. IP-10 and RANTES

readout was not further augmented with the addition of any of the PDE4 inhibitors. As it stands, it would be erroneous to conclude that ABCC4 inhibitors "work better" than PDE4 inhibitors as the concentration of the seemingly ineffective Roflumilast and Cilomilast were both 10-fold lower than the ABCC4 inhibitors and their more effective PDE4 counterpart, Rolipram. The difference in the PDE4 inhibitors may be due to its preferential selectivity to specific isoforms. Rolipram favourably inhibits PDE4A (IC_{50} = 3nM) over other isoforms such as B and D (IC_{50} = 130nM and IC_{50} = 240nM respectively)(111). Whereas, Roflumilast has a preferential inhibition of PDE4B and PDE4D ($IC_{50} \leq$ 1nM for both)(112). To support this claim, Cilomilast also shows 10-fold selectivity toward PDE4D compared with PDE4A and PDE4B(113). Perhaps, the most relevant PDE4 isoform for our hypothesis may be PDE4A, but more investigation must be done to find more conclusive results.

In addition, although we used the concentration that has been previously used in literature, it may have proven to be more useful if we administered all of the cAMP-elevating agents at the same concentration to be able to compare relative efficacy. However, since the goal of this thesis is not to find the best agent, but to interrogate our hypothesis, this may be a future avenue to be studied later.

If we work under the assumption that the concentrations that we used our ABCC4 and PDE4 inhibitors are relatively close in terms of potency, a reason for the difference in phenotype could be due to the difference in the location of ABCC4 and PDE4. PDE4 are present throughout the cell with different isoforms localized to different compartments of the cells such as the cytosol, Golgi body, nucleus as well as being coupled to plasma

membrane proteins(114,115) Figure 23. In contrast, ABCC4 is confined in location to the plasma membrane, which could indicate the presence of a more compartmentalized increase in cAMP. A study looking at the spatiotemporal coupling of ABCC4 to CFTR in gut epithelial cells have shown that blocking ABCC4 enhances CFTR function at lower concentrations of adenosine, an adenylyl cyclase agonist, but not at higher concentrations(82). This suggests that blocking ABCC4 leads to compartmentalized cAMP accumulation. This also means that blocking ABCC4 does not lead to a global increase in cAMP throughout the cell, and this is complemented by the fact that overexpression of ABCC4 does not lead to a substantial decrease in intracellular cAMP levels(116). PKA activities can be localized through A-kinase anchor proteins (AKAPs), which binds to the regulatory subunit of PKA and confining the holoenzyme to discrete locations in the cell(117). AKAPs have isoforms that dictate the foci for PKA signalling. For example, AKAP isoforms RIα typically is cytoplasmatic, RIβ is enriched in the mitochondria, and RIIs are localized to membranes(118). It may be that the phenotype that we are seeing could be due to the localized activation of specific isoforms of AKAP since ABCC4 inhibition is more localized near the membrane, AKAP isoform RII may have some influence in the further suppression of NF-κB induced IL-6 and IL-8.

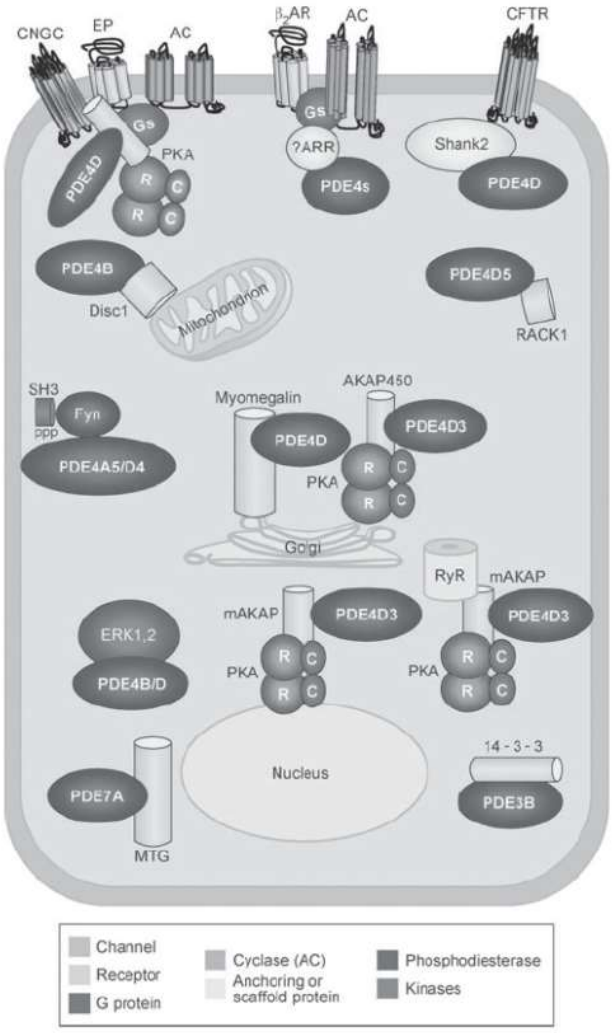

Figure 23 - Localization of PDE4.
A schematic of the proposed localization of different PDE4 isoforms eluding to their ability to perform compartmentalized cAMP regulation in the cell (90).

ABCC4 + PDE4 inhibition + LABA/GCS

Inhibiting ABCC4 and PDE4 singularly may lead to one overcompensating for the other. To account for this, we blocked the export of cAMP by inhibiting ABCC4 and prevented the degradation of cAMP by inhibiting PDE4. We used all three of our ABCC4 inhibitors in combination with Roflumilast. The reason we did not carry forward with the other two PDE4 inhibitors is that we felt that using Roflumilast would be more clinically relevant as Rolipram and Cilomilast would not be used beyond the lab bench. By inhibiting both, we wanted to see if we could improve the efficacy of our drugs for anti-inflammation.

Surprisingly, we were able to see a *further* decrease in GM-CSF levels with the addition of ABCC4/PDE4 inhibitors when compared with LABA/GCS alone. In fact, in all three of our combinations, we were able to decrease GM-CSF even lower than the basal concentration of GM-CSF. However, it is hard to see the extent of the combination ABCC4/PDE4 inhibitors effect on LABA/GCS attenuation of GM-CSF since once again, LABA/GCS suppressed GM-CSF to the basal level. Both IL-6 and IL-8 were further suppressed in all three of our combinations to a significantly greater degree. Most notably, CDRD14, which we didn't see a potentiation of anti-inflammation when administered alone, showed a greater degree of suppression of GM-CSF, IL-6, and IL-8 compared to when either roflumilast or CDRD14 were administered alone. IP-10 signal was brought down in CDRD13+Roflumilast and CDRD14+Roflumilast group compared to LABA/GCS alone. RANTES readout showed similar trends as seen in ABCC4 inhibitor alone experiments, except that the effect was not as pronounced and failed to meet

statistical significance. We believe that the ABCC4 and PDE4 inhibitors work synergistically to increase cAMP, as shown in the figure below (Figure 24).

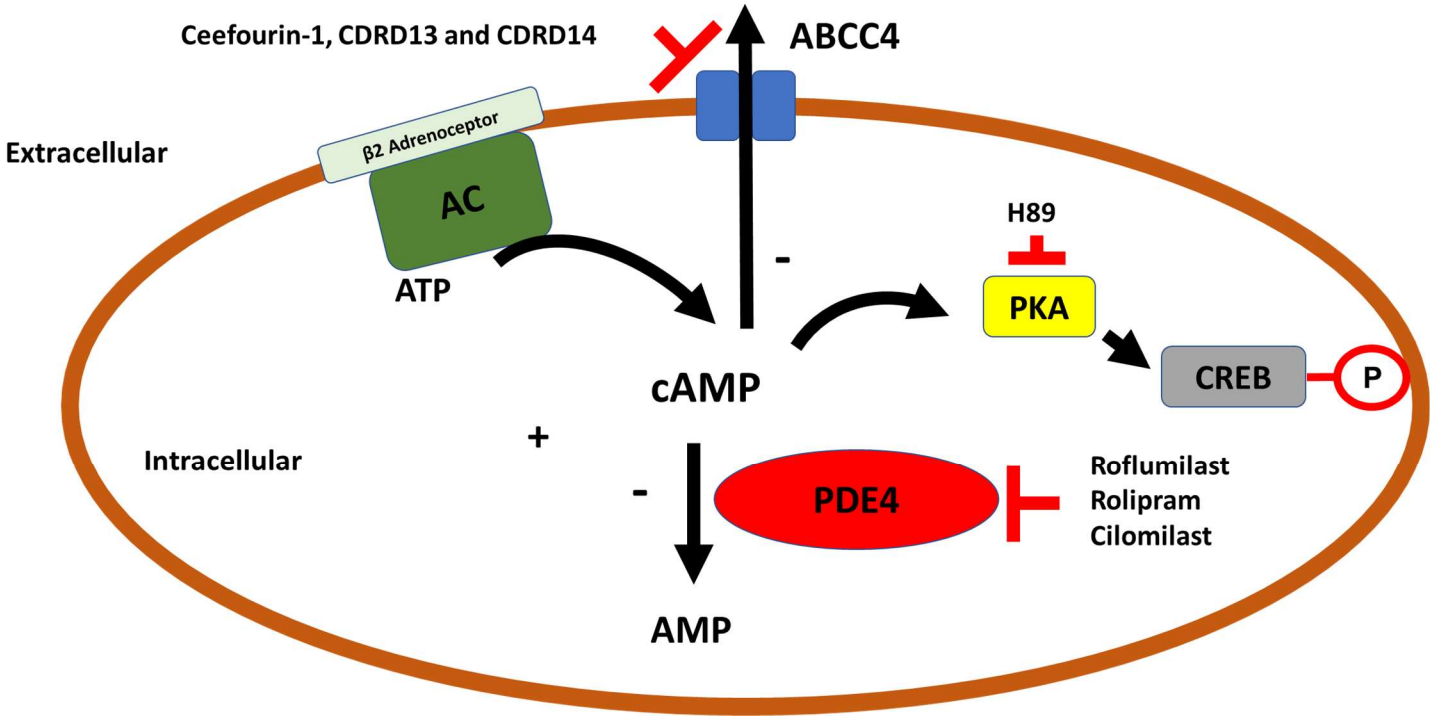

Figure 24 - Schematic of intracellular cAMP regulation.
The adenylyl cyclase (AC) catalyses the generation (+) of cAMP from ATP. cAMP is hydrolyzed (−) to inactive AMP by the activity of PDE4. ABCC4 actively transports cAMP to the extracellular environment (−). The inhibition of PDE4, as well as ABCC4, has the potential to increase intracellular cAMP levels.

Implications

My results show that increasing intracellular cAMP can aid in the suppression of inflammation in a model of virus stimulated HAECs. As the reason for exacerbation, hospitalization, and increased morbidity and mortality are due to the effects of inflammation and not so much the viral load; it is crucial that inflammation is kept in check(119). Interestingly, even amongst asthmatic patients who are well controlled with GCS-treatment, virus-induced inflammation can still lead to exacerbations, which could imply that viral exacerbations may not only be due to corticosteroid insensitivity(120). This is important as our findings can be applied to a broader range of severity of asthmatic patients. In addition, our results showed that the addition of ABCC4 and PDE4 inhibitors could improve the suppression of inflammation with less GCS on board showing therapeutic potential to decrease the intake of steroids for the management of asthma. Although the main disease focus of this thesis was on asthma, our results have implications for the management of COPD as well because they also use LABA/GCS for management of symptoms and have increased risk of exacerbation when infected with viruses(121).

Limitations and Future Directions

There are limitations to take into consideration for this study. Firstly, we were using HBEC-6KT, which is a healthy cell line for all our experiments. Although for the purpose of our research question on the biological response of addition of ABCC4 and PDE4 inhibitors' effect on LABA/GCS suppression of inflammation can be sufficiently answered with these cells, the wider clinical relevance falls short. Additionally, all of our cells were cultured in a submerged monolayer due to HBEC-6KT cells' inability to differentiate when in an air-liquid interface culture (ALI). Furthermore, our findings are limited to epithelial cells despite the diversity of multiple cell types that contribute to the asthmatic and COPD phenotype in a viral exacerbation. An important limitation of our study is our choice in Poly I:C as a viral mimic stimulation. Although Poly I:C has been characterized to be an effective TLR3 agonist; it does not perfectly recapitulate real-live viral infections. For example, real-live viruses such as rhinovirus, influenza virus, respiratory syncytial virus, and adenovirus, activate a variety of TLRs, such as TLR2, 3, 6,7, and 8(122–127). Finally, the cytokines that we chose to measure were all validated to be released via Poly I:C stimulation.

Future directions include more thoroughly investigating what LABA/GCS in combination with cAMP-elevating agents, can have on primary HAEC. Firstly, we will move towards working with HAEC from asthmatic and COPD patient populations to better represent the target population and lead to more accurate and applicable findings. On top of performing the intervention studies as we have done in our HBEC-6KT cells, we are interested in investigating other parameters of immune functions of HAEC in the

context of respiratory health and asthma. For example, our future experiments with primary human airway epithelial cells will be performed in air-liquid interface (ALI) which presents us with opportunities to measure barrier function, through transepithelial electrical resistance (TEER) measurements, and gene and protein expression relevant to epithelial barrier function (Adherens junctions: E-cadherin, β-catenin and α-catenin. Tight junctions: occludins, and claudins. Scaffolding proteins: ZO-1,2,3)(128). There is evidence that PDE4 inhibitors can inhibit the downregulation of cigarette-smoked induced E-cadherin and ZO-1 downregulation, giving us some insight into what further increase in cAMP with the addition of ABCC4 inhibitors could potentially do(129). Additionally, we are interested in measuring the expression of mucus through MUC5AC. PDE4 inhibition has been shown to decrease MUC5AC expression in airway epithelial cells stimulated with epidermal growth factor a, mucin stimulant(130). Again, we want to test to see if this can be applied similarly to ABCC4 inhibition or if it is PDE4 inhibition specific.

Another direction that we wish to move towards is using different stimuli. As mentioned before, Poly I:C, although it gives robust signals, is not the best stimuli. In future experiments, we are interested in including live viruses and perhaps different TLR agonists to define the specificity of the trends that we see with the addition of ABCC4 and PDE4 inhibitors in our TLR3 stimulated model. In addition, we will also move towards using cigarette smoke extract to test the ability of our novel combination therapy could have on cytokine readouts as well as barrier functions. Also, to have a more bias-free readout of inflammatory profiling, we could potentially send our crucial experimental

supernatants for human cytokine array (Eve Technology, Canada) to gain a more inclusive understanding of the impact of our system.

As mentioned before, a great limitation in our system is that we are limited to only our HAECs. To move away from this, we will potentially collaborate with a lab that is looking at intracellular cAMP levels and the expression of bronchodilator genes in airway smooth muscle cells (ASM). Reconciling their expertise with ASM biology and our expertise with increasing intracellular cAMP, it would shed more light onto the topic of how our LABA/GCS/ABCC4+PDE4 inhibitors can have on the bronchodilation aspect as we have only investigated inflammation, which is only one of the two main phenotypes of asthma. In addition, the primary cAMP mechanism of interest for the context of this thesis has been primarily on intracellular, and its effect on the PKA signalling pathway. However, a recent study that looked at extracellular cAMP and its effect on airway smooth muscle in rats have shown that extracellular cAMP, once it has been converted into adenosine by ectoenzymes, induced phasic contractions(131). It would be interesting to see the impact of inhibiting ABCC4 not only to increase intracellular cAMP but decreasing extracellular cAMP on airway smooth muscles. Complimenting this, we have a potential avenue for collaboration with Dr. Newton, who can provide us with C75BL/6 mice with and without RGS2 knockout. This will serve as an excellent segue for us to move towards *in vivo* models, which will enable us to more thoroughly study the effects of a further increase in cAMP in combination with LABA/GCS. We plan on exposing the mice with different stimuli (viruses, and cigarette smoke) and compare the differences between our control group and our intervention group, and between wild type mice and

RGS2 knockout mice. Readouts would include bronchoalveolar lavage fluids for analysis of cells and mediators, lung functions and histological staining of gene and protein of interest.

Conclusion

We demonstrated that the addition of cAMP-elevating agents, ABCC4, and PDE4 inhibitors could potentiate the anti-inflammatory effects of LABA/GCS in our HBEC-6KT cells for Poly I:C induced inflammation. Our findings complement current literature, which suggests that ABCC4 inhibition potentiates LABA/GCS by the up regulation of anti-inflammatory and bronchoprotective genes. Our results form the basis to further explore the potential application of ABCC4 and PDE4 inhibitors for attenuation of inflammation in the context of chronic respiratory diseases.

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