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## The Effect of Glucocorticosteroids on Th2 cells

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Graduate Program in Pathology  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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## Abstract

The abundance of Th2 cells in both the airways and circulation associates with asthma severity. Activation of CRTh2 expressed by Th2 cells mediates production of Th2 cytokines and inhibits apoptosis. Glucocorticosteroid effects occur through suppression of Th2 cytokines and induction of apoptosis. However, recent evidence shows that the total daily dose of inhaled glucocorticosteroids was positively correlated with the percentage of circulating Th2 cells. As such, we chose to examine regulation of Th2 cells by glucocorticosteroids. We used CCRF-CEM cells as a Th2 cell model and *in vitro* differentiated primary Th2 cells to study the effect of dexamethasone. Low dose dexamethasone treatment reduced IL-13 but surprisingly increased CRTh2, while only high dose induced apoptosis. Interestingly, T cell activation reduced glucocorticosteroid receptor signaling genes FKBP5 and GR transcript levels. Our results suggest that glucocorticosteroids at insufficient doses to trigger apoptosis may sustain CRTh2<sup>+</sup> Th2 cells and thereby maintain inflammation.

## Keywords

CRTh2, Glucocorticosteroids, Th2 cells, Allergy, Asthma

## Dedication

To my parents for their unwavering support and being the inspiration for all my accomplishments. To Arani for being my rock through all the ups and downs, and the reason I have achieved any level of success.

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## List of Abbreviations

AHR	Airway hyperresponsiveness
AMP	Adenosine 5'-monophosphate
APC	Antigen presenting cell
ATS	American Thoracic Society
BAD	BCL activated death promotor protein
BAL	Bronchoalveolar lavage
BPT	Bronchial provocation test
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRTh2	Chemoattractant homologous molecule expressed on Th2 cells
C <sub>t</sub>	Cycle threshold
CysLT	Cysteine leukotrienes
DK-PGD <sub>2</sub>	13, 14-dihydro-15-keto Prostaglandin D <sub>2</sub>
DP	D Prostanoid
ENCODE	Encyclopedia of DNA elements
FACS	Flow Activated Cell Sorting
FCεR1	Fragment crystallizable Epsilon Receptor 1
FEV <sub>1</sub>	Forced expiratory volume in one second

FKBP	FK506 Binding Protein
GC	Glucocorticosteroid
GITR	Glucocorticoid- induced TNFR related protein
GPCR	G-protein coupled receptor
GR	Glucocorticosteroid receptor
GRE	Glucocorticosteroid response element
HDM	House Dust Mite
HSD	Honest Significant Difference
HSP90	Heat Shock Protein 90
IFN $\gamma$	Interferon Gamma
IL	Interleukin
ILC	innate lymphoid like cells
iT <sub>regs</sub>	Induced T regulatory cells
MFI	Mean fold intensity
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
PBS	Phosphate Buffered Saline
PC <sub>20</sub>	Provocative concentration for 20% fall in FEV <sub>1</sub>
PER	Period circadian protein homolog
PGD <sub>2</sub>	Prostaglandin D <sub>2</sub>

PMA	Phorbol 12-myristate 130 acetate
PS	Phosphatidylserines
qRT-PCR	Quantitative real-time polymerase chain reaction
SE	Standard Error
SNPS	Single nucleotide polymorphisms
TCR	T Cell Receptor
Tfh	Follicular helper T cells
Th	Helper T cell
Th2	Type 2 helper T cells
T <sub>M</sub>	Memory T cells
TNF $\alpha$	Tumour Necrosis Factor $\alpha$
TSLP	Thymic Stromal Lymphopoietin

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## Chapter 1 : Introduction

### 1 Overview

Asthma is a chronic inflammatory disease of the airways that affects over 3 million Canadians (8% of the population) over the age of 12 [1]. Asthma is a growing problem as it is expected to affect over 100 million people worldwide by the year 2025 [1]. Although most types of asthma are well controlled by medication, asthma has an annual cost to our healthcare system of 600 million dollars [1]. The majority of the asthma related healthcare costs occur from patients suffering from severe difficult-to-treat asthma [2]. The pathogenesis of severe forms of asthma is considered largely related to type 2 inflammation. The inflammation in asthma patients influences response to glucocorticosteroid (GC), the main anti-inflammatory medication for asthma [2]. Indeed, much research has examined the question of why severe asthmatics exhibit persistent inflammation and overall poor control of their disease despite taking the highest doses of GC [2]. As such, this study focused on understanding how GCs influence Th2 cells, a T lymphocyte subset elevated in severe asthma [3, 4].

#### 1.1 Asthma

Asthma patients present clinically with symptoms such as wheezing, shortness of breath, and chest tightness [5]. Diagnosis of asthma is based primarily on symptoms and reversible airflow obstruction [5]. Asthma diagnosis can be determined by objective measures such as forced expiratory volume in one second (FEV<sub>1</sub>) [6]. A spirometer is commonly used to assess lung function by measuring changes in the volume of air inhaled and exhaled [5, 6].

Asthma is classified as an obstructive lung disease due to the narrowing of the airways [5]. Thus, asthma patients often have difficulty exhaling air at a normal rate. FEV<sub>1</sub> assesses lung function by determining the maximum volume of air exhaled in one second [6]. FEV<sub>1</sub> values are represented as percent predicted, which compares measured FEV<sub>1</sub> values relative to expected FEV<sub>1</sub> based on height, sex, race and age [6]. An FEV<sub>1</sub> of 80% of the percent predicted is considered normal [5, 6]. Asthma diagnosis requires performing FEV<sub>1</sub> before and after administration of a bronchodilator and increases of FEV<sub>1</sub> by 15% and/or 200 mL identify reversible airflow obstruction [6, 7]. In this circumstance, reversibility of airflow

obstruction is used to diagnose asthma over the other major chronic disease of the airways, chronic obstructive pulmonary disease (COPD) which does not show airway reversibility [8].

Another method used to assess variable airflow obstruction is through administration of a bronchial constrictor [2]. Airway hyper responsiveness (AHR) in asthma is characteristic of inflammation and smooth muscle cell abnormalities in the airways [9]. Bronchiole provocation tests (BPTs) are used clinically to assess AHR [9]. Adenosine 5'-monophosphates (AMPs) that bind  $A_{2B}$  receptors to induce degranulation of mast cells are used as an indirect BPT [9, 10]. Mast cells release inflammatory mediators such as histamines, leukotrienes and prostaglandins that act on smooth muscle cells leading to bronchoconstriction [11]. Thus, AMPs test inflammatory regulation of AHR which is an indirect measure. Enhanced sensitivity of muscarinic receptors on smooth muscle cells lining the airways contributes to AHR [12]. The methacholine challenge is a type of direct BPT that utilizes methacholine which binds muscarinic receptors and induces bronchial constriction [13]. Thus, asthma patients with enhanced muscarinic receptor sensitivity have bronchioles that constrict at low doses of methacholine [2]. The concentration that causes FEV<sub>1</sub> to drop by 20% is considered the provocation concentration (PC<sub>20</sub>) [2, 7]. Normal airways would have a PC<sub>20</sub> of > 16 mg/ml and an asthmatic patient would have a PC<sub>20</sub> of < 8mg/ml [2].

Measuring lung function and reversibility reflects the effect of the inflammatory process but does not indicate the type of inflammation. The airway inflammation in asthma patients determines a patients' response to treatment.

### 1.1.1 Treatment

Strategies for asthma treatment focus on temporary relief of symptoms and reduction of chronic inflammation. Inhaled GCs are the mainstay for asthma treatment due to their ability to reduce airway inflammation [14]. Oral GCs are prescribed in the cases of more severe forms of asthma despite the high risk for adverse side effects such as osteoporosis, high blood pressure and obesity with long-term use. Treatment strategies for asthma patients often involve combinatorial therapy which involve controller medication such as GCs to reduce inflammation and a reliever medication for temporary relief of symptoms [15]. Reliever medications include short-acting bronchodilators, such as those that act on  $\beta_2$ -adrenergic receptors on smooth muscle cells that alleviate bronchial constriction [16]. Cysteinyl

leukotriene (CysLT) receptor antagonists are another family of controller drugs used to target inflammation mediated symptoms [15]. CysLTs are strong contractile agonists released by mast cells that are increased in active asthma, thus antagonist treatment blocks potential bronchoconstriction, edema and mucous production [17]. Advances in our understanding of asthma have allowed for development of therapeutics with specific immunological targets [15]. For example, Omalizumab an anti-IgE medication has shown to be successful as an add on therapy in patients with IgE mediated asthma [18].

### 1.1.2 Severity

One of the main ways to categorize asthma is based on severity, which is determined based on symptoms, lung function and response to asthma therapy [2]. The American Thoracic Society (ATS) defines mild asthma as having symptoms at least 2 times a week but normal FEV<sub>1</sub> ( $\geq 80\%$ ) and requiring no or only low dose inhaled GCs [2, 19]. Moderate asthma is when patients have daily symptoms accompanied by reduced lung function (FEV<sub>1</sub> between 60-80%), but are taking  $< 800 \mu\text{g}$  of inhaled beclomethasone to control their symptoms [2, 19]. Severe asthmatics, on the other hand, have an FEV<sub>1</sub> below the normal limit at  $< 80\%$  along with persistent symptoms despite taking  $> 800 \mu\text{g}$  of beclomethasone a day, and the need for other controller medications and/or oral GCs [2, 19].

### 1.1.3 Inflammation

Although clinically asthma is categorized as mild/moderate/severe, these subgroups are not well associated with a particular type of inflammation [20]. For instance, asthma has been divided into phenotypes based on the predominance of inflammatory cells such as eosinophils, helper T cells and neutrophils [21]. The underlying pathology of asthma is further diversified by etiology [22]. The most common classifications are known as non-allergic and allergic asthma [22, 23]. Non-allergic asthma occurs in the absence of an allergic reaction and is considered to be due to viral infections, exercise or other primary diseases that influence bronchial obstruction [23]. Non-allergic asthma is often associated with neutrophilic and Type 1 cytokine related inflammation [23, 24]. Allergic asthma is defined by a hypersensitive reaction that occurs in response to an allergen [23, 25] and is commonly associated with T helper 2 cells (Th2) and eosinophils [23, 26]. Th2 high asthmatic patients



have been associated with severe asthma [21]. As research continues to uncover the diversity of asthma this will advance our understanding of its etiology, pathology and treatment [27].

## 1.2 Development of Allergic Asthma

Allergic asthma is the most common type of asthma and is characterized by elevated blood and airway eosinophilia and Th2 inflammation [28-30]. It often develops during childhood following sensitization to an allergen(s) [20]. Both genetic and environmental factors that influence the developing immune system have been associated with becoming sensitized and developing allergic asthma [31].

### 1.2.1 Atopy

Individuals that are genetically predisposed to developing allergic diseases usually become atopic early in life [32]. Atopy, the state of being sensitized to an antigen, is assessed by a positive skin prick test or measuring total serum IgE levels [33]. Exposure to LPS from living on a farm during the first year of life was shown to protect against sensitization, while environmental pollution has been associated with increased risk of developing asthma [34, 35].

### 1.2.2 Epithelium

Allergen sensitization develops as a result of an innocuous environmental antigen known as an allergen activating both the innate and adaptive immune system. The cells of the innate immune system act as the first line of defense against antigens. In the case of allergic asthma, the epithelium lining the airways is the first line of contact and its activation initiates an immune response [36]. The epithelial cells express pattern recognition receptors that are activated upon allergen exposure to promote allergen sensitization and Th2 mediated inflammation [37]. Mutations in proteins regulating epithelial integrity such as E-cadherin,  $\alpha$ -catenin and ZO-1 have been associated with enhanced allergic sensitization [38]. Loss of epithelial integrity by epithelial remodeling also makes asthmatics more sensitive to allergen re-exposure over-time [39]. Even in the absence of any primary epithelial dysfunction, allergens such as house dust mites (HDM) can cleave tight junctions connecting epithelial cells to enter the lung mucosal layer [40].

### 1.2.3 Antigen Presentation

Antigens in the lung mucosa are recognized by antigen presenting cells (APCs), the cells which provide the crucial link between the innate and adaptive immune system [41]. The epithelium secretes factors such as interleukin-33 (IL-33), thymic stromal lymphopoietin (TSLP) and interleukin-25 (IL-25) that promote maturation of APCs in response to allergen exposure [37]. APCs such as dendritic cells, macrophages and B cells take up antigens and process them for presentation to CD4<sup>+</sup> T cells [42]. Dendritic cells are the main APCs responsible for sensitization in allergic asthma [43, 44]. Through phagocytosis or receptor mediated endocytosis APCs take up the allergen and process a peptide from the allergen (antigen) into a complex with major histocompatibility complex (MHC) class II molecules on the surface [42]. These APCs migrate to the lymph nodes and present antigens to naive CD4<sup>+</sup> T cells [42].

### 1.2.4 IgE Production

Interaction of APCs with naïve CD4<sup>+</sup> helper T cells (Th) mediates T cell differentiation into multiple subtypes including Th1, Th2, Th17, induced regulatory T cells (iTreg) and follicular helper T cells (Tfh) [45]. For many years, Th2 cells were thought to be the primary cells interacting with B cells to induce isotype switching to IgE [46, 47]. More recently, however, it has been shown that dendritic cell activated CD4<sup>+</sup> T cells first differentiate into Tfh cells [48]. Tfh cells express Th2 cytokines (IL-4 and/or IL-13) required to induce B cells to form specialized B cells called plasma cells to produce IgE [49]. Plasma cells migrate to the site of allergen exposure and produce IgE, which binds to fragment crystallizable Epsilon Receptor 1 (FcεR1) present on mast cells [50]. This binding of IgE to mast cell represents allergen sensitization, where an individual is primed to respond to subsequent exposure to allergen.

### 1.2.5 Th2 Cell Differentiation

Helper T cell differentiation depends on factors such as type of antigen, level of antigen stimulation, and cytokine environment [45]. Moderate T cell receptor (TCR) cross-linking favors differentiation to Th2 cells versus high level stimulation, which favors Th1 cell development [45]. Dendritic cells stimulate naïve CD4<sup>+</sup> T cells to differentiate into Th2 cells through CD3/CD28 crosslinking/co-stimulation, which induces IL-2 and low level IL-4 production from differentiating T cells [51-53]. IL-4 binds to the IL-4R, activating the

transcription factor STAT6, which leads to more IL-4 production through autocrine regulation which promotes Th2 cell differentiation [54]. GATA3 is also upregulated following TCR activation and both STAT6 and GATA3 remodel the chromatin to open enhancer regions within genes that enable maintenance of the Th2 phenotype [55]. Despite this low level of IL-4 production, an external source of IL-4 is likely required to promote full Th2 differentiation alongside TCR activation, which has been suggested to be basophils and/or lung tissue residing type 2 innate lymphoid like cells (ILC2s) [56, 57]. A recent study has suggested that the major source of Th2 cells is not through direct differentiation from CD4<sup>+</sup> T cells but from development of precursor Tfh cells [58]. Dendritic cells and B cells are required for the development of IL-4 producing Tfh cells [58]. With exposure to HDMs Tfh cells sustain plasticity to differentiate into type 2 effector helper T cells that can act as precursors for pathogenic Th2 cells [58]. Inhibiting the production of Tfh cells reduced the abundance of Th2 cells residing in the lung following HDM exposure [58]. Thus, it appears that although CD4<sup>+</sup> T cells can differentiate into Th2 cells, parallel production from precursor type 2 Tfh cells is also a source of Th2 cells [48, 58].

### 1.2.6 Other Avenues to Allergic Sensitization

Though allergic sensitization has been largely considered to be the main driver of type 2 inflammation, recently factors other than allergens have been shown to induce expression of these cytokines. The epithelium has been shown to produce IL-25 and IL-33 not only in response to allergen, but also following exposure to virus and pollutants such as cigarette smoke [59-61]. IL-25 can bind to the IL-25 receptor on naïve T cells to promote Th2 differentiation and induce IL-5 and IL-13 expression [62, 63]. Similarly, IL-33 has been shown to stimulate basophils to secrete IL-4 and ILC2s to secrete IL-13 and IL-5 [64]. The IL-4 production by basophils is thought to serve as a source of IL-4 initiating Th2 differentiation [56, 65]. ILC2 secretion of IL-13 has been shown to promote dendritic cell migration to lymph nodes to promote Th2 differentiation [65]. As such, there is now an appreciation for the fact that ‘type 2 inflammation’ can develop from allergic sensitization or following microbial exposures which creates a cytokine milieu that could support subsequent allergic sensitization.

### 1.2.7 Developing Chronic Inflammation

Once an individual is sensitized, reintroduction of the allergen initiates a type 1 hypersensitivity reaction [50]. The early phase of this response is elicited by mast cell degranulation induced by allergen binding IgE. Mast cell degranulation releases histamines, chemokines and the lipid mediator prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), which resolves within the 30 minutes [50, 66]. The late phase response, 6 - 24 hours following exposure, is mediated by inflammatory cell infiltration and amplification of type 2 cytokine expression [67]. During this response memory T cells develop from re-stimulation by antigens [44].

Four main types of memory T cells develop, stem (T<sub>SM</sub>), central (T<sub>CM</sub>), effector (T<sub>EM</sub>) and resident memory T cells (T<sub>RM</sub>) [68]. T<sub>SM</sub> cells were recently identified in humans as memory T cells that express FAS, CD122 and naïve markers such as CD45RA [69]. T<sub>SM</sub> cells have high proliferative capacity and can differentiate into T<sub>CM</sub> or T<sub>EM</sub> cells upon re-stimulation by antigens [69]. T<sub>CM</sub> cells have minimal effector function and in response to antigen stimulation proliferate and differentiate to produce T<sub>EM</sub> [68]. T<sub>EM</sub> express CCR4 receptors required to infiltrate into lung tissue from circulation, while T<sub>CM</sub> express CD62L, CCR7 and CD27, which mediate emigration out of tissues and migration back to secondary lymphoid organs [67, 68]. T<sub>CM</sub> cells differentiation into T<sub>EM</sub> cells require the loss of CCR7 expression to prevent migration into lymph nodes [67]. T<sub>EM</sub> cells can differentiate into T<sub>RM</sub> cells that reside in tissue [70]. T<sub>RM</sub> cells derived from CD4<sup>+</sup> T cells have been shown to secrete IL-2, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interferon gamma (IFN $\gamma$ ) in the lungs [71]. Memory T cells are detrimental for chronic inflammation as re-stimulation causes a much more rapid and effective response than non-memory T cells, thus for this reason these cells have been targets for immunotherapy [72].

### 1.2.8 Type 2 Cytokines

If exposure is continual, inflammation can become chronic with persistent infiltration of eosinophils and Th2 cells with the secretion of type 2 cytokines IL-4, IL-5 and IL-13 [26]. Each of these cytokines plays an important role in type 2 inflammation. In addition to the initiation, maintenance and proliferation of Th2 cells, both IL-4 and IL-13 activate the endothelium to mediate inflammatory cell infiltration [51, 54, 73, 74]. IL-13 also stimulates epithelial cells to express the MUC5AC gene and produce mucous and smooth muscle cells

to enhance contractility through elevated calcium signaling [75, 76]. IL-5 regulates eosinophils [77], stimulating their production in the bone marrow and migration from the bone marrow to the site of allergen exposure through binding of the IL-5 receptor [78, 79]. Overall IL-4, IL-5 and IL-13 play significant roles in the inflammatory process leading to narrowing of the airways of asthmatic patients.

### 1.3 Chemokine Receptor Homologous Molecule Expressed on Th2 cells (CRTh2) is a marker for Th2 cells

In a subtractive RNA hybridization screen of Th2 vs Th1 human cells, Nagata et al showed the differential expression of a transcript called G protein coupled receptor 44 [80]. Since this gene has homology with chemokine receptors, they called it chemokine receptor homologous molecule expressed on Th2 cells (CRTh2) [81]. Their characterization of the receptor showed surface expression of CRTh2 on Th2, but not Th1 cells, and that CRTh2<sup>+</sup> CD4<sup>+</sup> T cells were those that produced IL-4, IL-5 and IL-13 in response to allergen challenge [80, 82]. Despite its name, CRTh2 is also expressed by eosinophils, basophils, ILC2s and was reported to be present within mast cells [83-85]. However, a subsequent study revealed that within the CD4<sup>+</sup> T helper cell subset, CRTh2 was the most reliable marker of cells expressing the Th2 cytokine profile [86].

#### 1.3.1 CRTh2<sup>+</sup> Th2 cells are elevated in allergic disease

CRTh2<sup>+</sup> Th2 cells are increased in patients with atopic dermatitis, nasal mucosa of patients with allergic rhinitis and the lungs of patients with asthma [4, 87, 88]. Not only are Th2 cells elevated within the sites of exposure, but also in the circulation of patients with allergic airway disease [3, 44]. Single nucleotide polymorphisms (SNPs) in CRTh2 have been associated with higher susceptibility to develop phenotypes of allergic disease, including allergic asthma, further supporting the importance of CRTh2 in the development of allergic inflammatory diseases [89-91].

#### 1.3.2 CRTh2<sup>+</sup> Th2 cells are central memory T cells

Wang et al identified that Th2 cells are central memory T cells that are sustained in circulation and form memory against allergens [44]. Memory Th2 cells have been identified as dominant inflammatory cells involved in the pathogenesis of allergic asthma [44]. The

majority of memory Th2 cells are at resting state and upon activation form effector memory cells [68]. Research has identified various pathogenic subsets of memory Th2 cells that enhance inflammation through secretion of type 2 and non-type 2 cytokines. For example, IL-5<sup>+</sup> memory CRTh2<sup>+</sup> Th2 cells have been correlated with blood eosinophils and shown to regulate AHR [92]. IL-17 producing Th17 cells that are marked by CCR6 expression have been implicated in steroid resistance, thus may be involved in difficult to treat severe asthma [93]. CCR6<sup>+</sup> CRTh2<sup>+</sup> Th2 cells produce both IL-17 and IL-4 and were associated with recruitment of neutrophils, macrophages, eosinophils at levels superior to Th2 or Th17 cells independently [94]. The maintenance of memory cells *in-vivo* provides a challenge in eliminating inflammation through the reduction of just cytokines and other inflammatory proteins. While immune memory is beneficial for pathogen elimination, in the case of allergic disease long lived central memory Th2 cells are one of the main factors mediating persistence of disease.

## 1.4 Functions of the CRTh2 Signaling Pathway

CRTh2 is a G-protein coupled receptor (GPCR) that uses G<sub>oi</sub> proteins and therefore inhibits adenylyl cyclase activity [95]. The ligand for CRTh2 is PGD<sub>2</sub>, released by mast cells following allergen-IgE crosslinking and is also considered the second D prostanoid receptor (DP2) [96, 97]. To date, activation of CRTh2 has been shown to regulate Th2 cells by three main ways, secretion of cytokines, chemotaxis and inhibition of apoptosis (Figure 1) [95, 98, 99].

### 1.4.1 CRTh2 Mediates Th2 Cytokine Production

*In vitro* culture of Th2 cells with PGD<sub>2</sub>, showed that CRTh2 activation results in secretion of type 2 inflammatory cytokines IL-4, IL-5 and IL-13, but not IL-10 [99]. The mechanism has been shown to be through Nuclear factor of activated T cells (NFAT) signaling [100]. *In vivo*, mice deficient in CRTh2, either through gene knockout (CRTh2<sup>-/-</sup>) or using CRTh2 antagonists had reduced production of type 2 cytokines in response to allergen sensitization and challenge [101, 102]. Also, overexpression of lipocalin PGD synthase in mice, the enzyme responsible for PGD<sub>2</sub> production, mediated increase in type 2 cytokines in the bronchoalveolar lavage (BAL) [103]. CRTh2 mediated release of cytokines is considered to be through PI3K activation and calcium mobilization [100]. Calcium mobilization in

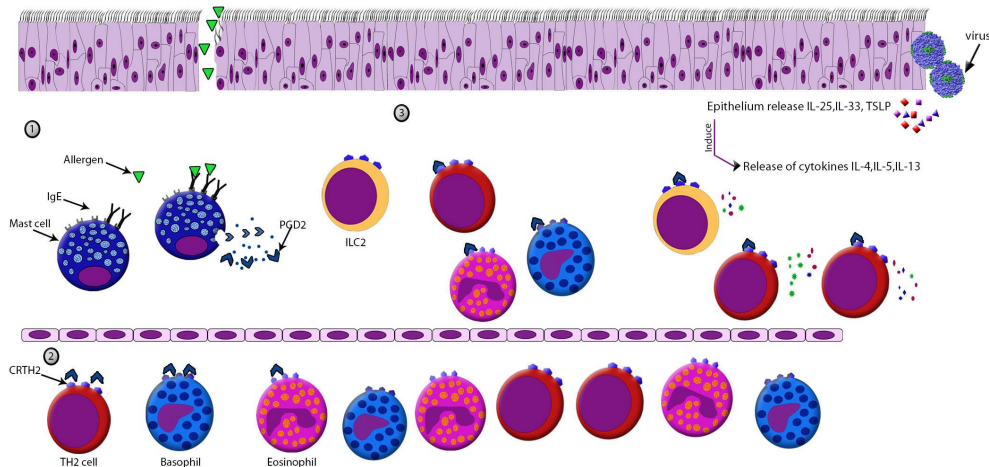
response to CRTh2 activation induces calcineurin activity, which dephosphorylates NFAT allowing for nuclear import leading to transcription of IL-4, IL-5 and IL-13 [100]. The PGD<sub>2</sub>-CRTh2 activation of PI3K also activates AKT, which in turn phosphorylates and inhibits GSK3 $\beta$  [100]. GSK3 $\beta$  can inhibit NFAT through phosphorylation to inhibit NFAT's ability to bind DNA and induce transcription [104].

#### 1.4.2 CRTh2 Mediates Th2 Cell Chemotaxis

CRTh2 also induces Th2 cell chemotaxis by PGD<sub>2</sub>-mediated PI3K signaling pathway [95]. Overexpression of lipocalin PGD synthase in mice showed increased inflammatory cells in BAL after allergen challenge [103]. AKT phosphorylation leads to F-actin polymerization which induces chemotaxis in Th2 cells [100]. This mechanism of PGD<sub>2</sub> mediated chemotaxis has also been shown in eosinophils and basophils [95]. Interestingly, though, inhibition of AKT in Th2 cells showed only partial inhibition of chemotaxis. ERK activation may be a partial regulator of chemotaxis as ERK facilitates CRTh2 mediated chemotaxis of eosinophils [100, 105].

#### 1.4.3 CRTh2 Inhibits Apoptosis in Th2 cells

PGD<sub>2</sub>-CRTh2 activation has been shown to inhibit apoptosis in response to IL-2 starvation and therefore CRTh2 appears to regulate survival [98]. CRTh2 is a GPCR utilizing G $\alpha_i$  protein, so activation results in influx of Ca<sup>2+</sup> from cellular calcium stores [95]. This calcium influx has been shown to activate PI3K, phosphorylation of AKT and BCL-2 associated death promotor protein (BAD), which interferes with anti-apoptotic proteins BCL-2 and BCL-xL [98]. Interference of BCL-2 and BCL-xL would initiate caspase-3 mediated apoptosis, but AKT signaling downstream of CRTh2 activation inhibits this pathway [98]. Xue et al showed that increasing amounts of PGD<sub>2</sub> reduced percentage of Annexin V<sup>+</sup> Th2 cells induced by IL-2 starvation [98]. Annexin V staining is an assay for detecting viable cells undergoing early apoptosis through binding of phosphatidylserines (PS) [106]. During initiation of apoptosis XRP8 lipid scramblase disrupts the membrane and causes PS to flip from inner membrane leaflet to outer membrane leaflet [107]. PS can be bound by calcium dependent protein Annexin V which in combination with nuclear dye 7AAD or propidium iodide is used to assess early apoptosis and cell death, respectively [106].



**Figure 1: CRTh2 mediated chronic inflammation**

Chronic airway inflammation is initiated by allergen surpassing the epithelial barrier and binding to IgE present on mast cells to cause mast cell degranulation release of PGD<sub>2</sub> (1). PGD<sub>2</sub> binds to inflammatory cells in circulation leading to chemotaxis of these cells into the lung mucosal layer (2). Th2 cells recruited into the lung mucosa and ILC2s can bind CRTh2 and release type 2 cytokines to enhance inflammation (3). This process occurs chronically leading to narrowing of the airways.

## 1.5 Glucocorticosteroid Mechanisms of Action

Since its introduction in 1950, treatment with synthetic GCs has been the main method of reducing chronic inflammation in asthma [108]. Oral GCs have a broad range of effects on various tissues leading to obesity, osteoporosis and hypertension which led to the development of inhaled GCs [108]. Inhaled GCs reduce inflammation in the airways with minimal systemic absorption. Continued development of more effective inhaled GCs aims to enhance receptor potency while minimizing off-site systemic effects [108, 109]. Ultimately, the best approach would be a treatment with no/low side effects and long-lasting effects.

GCs function through binding the GC receptor (GR), which resides in the cytoplasm in a heterocomplex that includes the chaperone proteins Heat Shock Protein 90 (HSP90) and FK506 Binding Protein 5 (FKBP5) [110]. Binding of GCs to GR results in a conformational change and FKBP5 losing affinity for the GR [110]. At this time, FKBP4 then replaces FKBP5 in the heterocomplex and interacts with dynein motor proteins to transport the GR



into the nucleus [110, 111]. The ability of GCs to reduce inflammation is mediated through many mechanisms including binding directly to DNA, affecting mRNA stability and protein-protein interactions (Figure 2) [14].

### 1.5.1 Glucocorticosteroid Mediated Transcriptional Regulation

The GR enters the nucleus and dimerizes with itself or other transcription factors to regulate gene transcription [112]. GR homodimers bind to positive and negative glucocorticoid response elements (GREs), to activate and repress transcription, respectively [113]. Interestingly, the GR can upregulate expression of proteins involved in the GR signaling pathway such as GR and FKBP5 [114, 115]. GR represses transcription of pro-inflammatory genes such as JUN, IL-11 and MAP3K14 [113]. The GR can also activate transcription of anti-inflammatory genes such as MKP-1, I $\kappa$ B, and GILZ [113, 116, 117]. One of the benefits of combination therapy with GCs and  $\beta$  agonists is due to the ability of GR to increase transcription of  $\beta$ 2 adrenergic receptor which is reduced in SMCs lining the airways of asthmatics [118]. Typically, the GR is thought to increase anti-inflammatory gene transcription and reduce pro-inflammatory gene transcription. However, GCs are also known to play a key role in T cell maturation and differentiation of T<sub>regs</sub> through transcriptional up-regulation of FOXP3 and glucocorticosteroid induced tumor necrosis factor receptor (GITR) [119].

Generally, GCs are thought to negatively regulate cytokine expression. Naïve T cells treated with GCs and CD3 showed a reduction in IL-4 expression [120]. GC treatment in patients with allergic diseases reduced mRNA expression of type 2 cytokines IL-4, IL-5 and IL-13 [121-124]. The reduction of IL-5 by GCs also reduced eosinophil recruitment [122]. TSLP contains negative GREs that the GR binds to block transcription [125]. Post-transcriptional function of the GR also has been shown to reduce mRNA levels by reducing mRNA stability of IL-4 and TNF- $\alpha$  [126, 127].

However, other studies have reported the contrary. Ramirez *et. al.* showed that pre-treatment with GCs enhanced type 2 cytokine expression following T cell activation [128], while another study showed a positive regulation of cytokines IL-4 and IL-10 when T<sub>EM</sub> cells were treated with GC in combination with CD3 stimulation [120]. Therefore, the effect of GC on cytokines appears to be more complex than simple suppression.

### 1.5.2 Glucocorticosteroid Receptor Regulated Protein-Protein Interactions

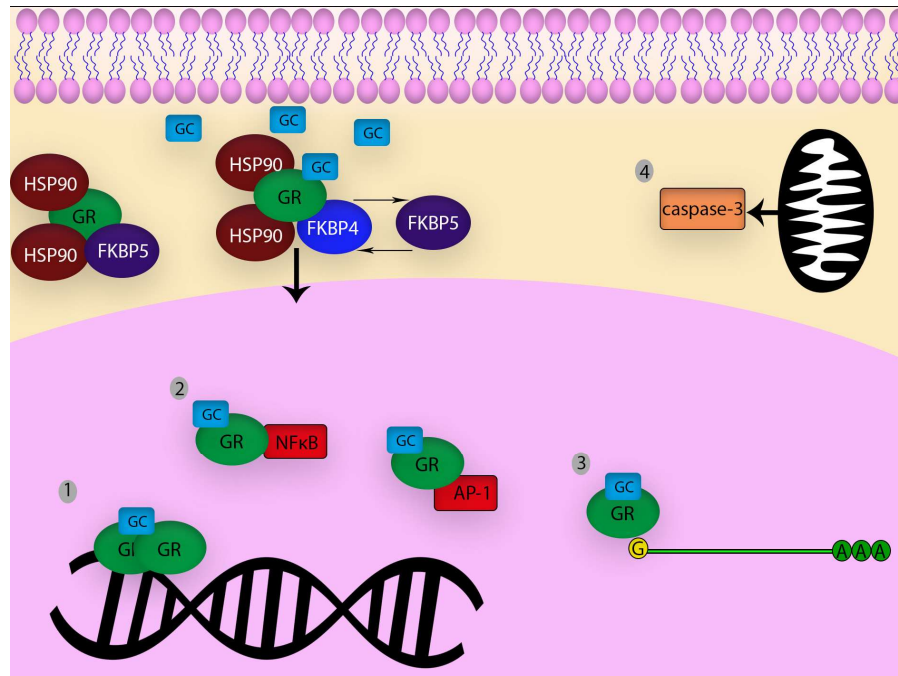
The anti-inflammatory action also occurs through protein-protein interactions by binding AP-1 and NF $\kappa$ B. These transcription factors are up-regulated during T cell activation to promote inflammatory function of lymphocytes [129]. NF $\kappa$ B is a pro-inflammatory transcription factor that regulates transcription of pro-inflammatory genes such as cyclooxygenase-2 (COX-2) and TNF- $\alpha$  [130, 131]. NF $\kappa$ B also up-regulates expression of cell adhesion molecules on lymphocytes to allow lymphocytes into and through the tissues [132]. The GR can inhibit inflammation by binding directly to NF $\kappa$ B [133]. Even with the GR protein binding domain mutated, NF- $\kappa$ B was still inhibited by GR activation most likely through transcriptional regulation of I $\kappa$ B proteins [134]. AP-1 interacts with NFAT to regulate transcription of cytokines IL-5 and IL-13, while AP-1 can regulate transcription of IL-13 independent of NFAT [135]. AP-1 and GR can mutually inhibit each other, as demonstrated by studies overexpressing AP-1 and GR.

### 1.5.3 Glucocorticosteroid Mediated Apoptosis

Apoptosis is dependent on the regulation of BCL family proteins and caspases to induce death [136]. Caspases- 2,3,6,7,8,9 and 10 all have the potential to be active during apoptosis depending on whether mode of cell death is triggered intrinsically or extrinsically [137]. For example, caspase-8 is activated during Fas mediated apoptosis and caspase-9 is activated during GC-induced apoptosis [138, 139]. Both caspase-8 and caspase-9 cleave procaspase-3, activating caspase-3 and leading to apoptosis [140].

GC-induced apoptosis is regulated by BCL-2 and BCL-xL expression in cells [141, 142]. Studies show that their reduction leads to apoptosis and BCL-2 and BCL-xL overexpression has been shown to produce GC resistant T cells [141, 142]. Overexpression of GILZ, a GC inducible gene, has been shown to be correlated with downregulation of BCL-xL mRNA and protein [141]. The ability of GCs to induce apoptosis is dependent on transcriptional upregulation of genes that inhibit anti-apoptotic proteins and downregulation of homeostatic proteins [141, 143]. Eosinophils showed dose and potency dependent apoptosis in response to GC treatment [144]. GCs such as hydrocortisone that have lower affinity for the GR were not able to induce apoptosis [144]. Interestingly, GCs inhibit apoptosis in neutrophils, with more potent GCs having a greater inhibitory effect [145]. Thus, the ability of GCs to regulate

apoptosis is dependent on GC potency and dose. GCs have also been shown to inhibit T cell activation induced apoptosis through up-regulation of MKP-1 and GILZ [146]. T cell activation induces both AP-1 and NF $\kappa$ B to increase FAS-ligand expression leading to Fas-mediated apoptosis [147]. Whether GCs induce or inhibit apoptosis is dependent on the specific pathways activated, which may explain the ability of GCs to induce apoptosis in eosinophils and inhibit apoptosis in neutrophils.



**Figure 2: Glucocorticosteroid mechanisms of action**

GCs binding to the GR leads to the nuclear translocation of the receptor by FKBP4. Once the receptor is in the nucleus it can homodimerize and bind to DNA to regulate transcription (1), bind transcription factors to inhibit transcription (2) or reduce mRNA stability (3). GR can also up-regulate transcription of genes that lead to apoptosis (4).

#### 1.5.4 Glucocorticosteroid Resistance in Asthma

Severe asthmatics are deemed GC resistant because despite taking the highest recommended dose of GCs these patients show little to no control over their symptoms [2]. One of the difficulties of identifying mechanisms of GC resistance is the complexity GC- GR signaling in the various inflammatory cells.

Previous studies have reported that asthmatic patients have an elevated level of the dominant negative isoform of the GR beta (GR $\beta$ ) in peripheral blood mononuclear cells (PBMCs) and T cells [148, 149]. GR $\beta$  binds to GREs and prevents GR $\alpha$  (commonly referred to as GR) from binding GREs, therefore inhibiting GC-mediated transcription [150]. Work completed in our laboratory showed that the levels of GR $\beta$  in CCRF-CEM cells are very low, ~10,000 times less than GR $\alpha$ , and therefore unlikely to play a major role in T cells. This is a limitation of the previous studies identifying elevated GR $\beta$  in asthmatic patients, as they did not look at total GR levels [148, 149].

In terms of T cells, GCs have been shown to induce apoptosis in resting T cells, but T cell activation inhibits GC induced apoptosis through NFAT and AP-1 up-regulation [129, 151]. IL-2, a T cell survival factor has also been shown to inhibit GC-induced apoptosis in T cells [152, 153]. IL-2 activates the AKT pathway similar to CRTh2 to inhibit GC-induced apoptosis [154]. IL-2 can also activate STAT5 which can bind and inhibit the GR [155]. T<sub>EM</sub> cells unable to produce IL-2 were shown to be more sensitive to GC-induced cell death [156]. Interestingly, severe asthmatics have also been shown to have higher levels of IL-2 relative to mild/moderate asthmatics [157] and the type 2 cytokine IL-4 was shown to render Th2 cells GC resistant [74].

## 1.6 Rationale

Asthma is a disease characterized by chronic inflammation and is mostly controlled by GC treatment. Allergic asthma, the most common type of asthma is dominated by Th2 cell inflammation and Th2 dominant phenotypes are associated with the most severe form of asthma [26]. Severe asthmatic patients despite being prescribed the highest recommended dose of inhaled GCs and oral GCs show reduced lung function and lack of disease control. The anti-inflammatory function of GCs is mediated by transcriptional regulation, protein binding and apoptosis [14].

To better understand why severe asthmatics are GC resistant, research has focused on identifying unique features of this asthma phenotype. Severe asthmatics have been shown to have higher abundance of CRTh2 mRNA and higher percentage of CRTh2<sup>+</sup> cells in BAL relative to mild/moderate asthmatics [4]. The percentage of Th2 cells in the circulation was also higher in severe asthmatics relative to mild/moderate asthmatics [3]. While the

prevailing thought for GC mechanism of action in asthma is that they reduce Th2 cytokine production, our laboratory has observed a positive correlation between the percentage of Th2 cells in the blood of asthmatic patients and the total daily dose of inhaled GCs [3, 121, 123]. Collectively, these studies suggest there may be a link between the level of Th2 cells present in the lungs/circulation of asthmatic patients and their response to GC treatment. GCs could potentially promote Th2 cell phenotype similar to GC promotion of T<sub>regs</sub>, or promote survival similar to the phenomenon seen with neutrophils [119, 145]. Understanding the function of GCs in Th2 cells can provide insight into the method of GC action and potentially GC resistance.

## 1.7 Hypothesis & Objectives

### 1.7.1 Hypothesis

GC function in Th2 cells is dose dependent and varies based on state of T cell activation.

### 1.7.2 Objectives

#### **Objective 1: To investigate dose dependent effects of GC on pro-inflammatory gene expression**

Daily dose of GCs showed a positive correlation with the percentage of Th2 cells in the blood of asthmatic patients [3]. Regulation of CRTh2 expression is an important determinant of Th2 cell function [96]. GCs primarily downregulate transcription of pro-inflammatory genes, but some studies have indicated otherwise [113, 119]. Understanding how GCs regulate CRTh2 and IL-13 expression can provide insight into how GCs reduce inflammation and/or how Th2 cells exhibit GC resistance.

**Hypothesis:** Low dose GCs regulate CRTh2 and IL-13 expression.

#### **Objective 2: To determine if CRTh2 activation inhibits GC-mediated apoptosis**

GCs induce apoptosis in T cells through caspase-3 activation, the same pathway inhibited by CRTh2 activation [98, 142]. PGD<sub>2</sub> and Th2 cells have been shown to be correlated with asthma severity, indicating that severe asthmatics have the highest level of PGD<sub>2</sub> and Th2 cells [3]. If PGD<sub>2</sub> can inhibit GC-induced apoptosis this may be a way that Th2 cells become GC resistant and results in Th2 cells being sustained *in vivo*.

**Hypothesis:** High dose GCs induce apoptosis that is regulated by PGD<sub>2</sub>.

**Objective 3: To assess how T cell activation influences GR-mediated gene regulation**

T cell activation via dendritic cells is a major step in the development of chronic inflammation in allergic asthma [44]. Interestingly, pathways activated by CD3/CD28 activation are in direct competition with pathways activated by GCs [129, 158]. GCs have been shown to downregulate secretion of type 2 cytokines by activated T cells [120]. T cell activation on the other hand has been shown to inhibit GC mediated transcription, but it is unclear if this also affects genes involved in GR signaling [159]. This can provide further clarification on the role T cell activation plays in GC resistance.

**Hypothesis:** T cell activation regulates GR signaling genes.

## Chapter 2

### 2 Materials and Methods

#### 2.1 Cell Culture

##### 2.1.1 CCRF-CEM cells

An immortalized leukemia CD4<sup>+</sup> T cell line CCRF-CEM cells (CCL- 119) was purchased from American Type Culture Collection (VA, USA). These cells were seeded every 2 days at  $0.2 \times 10^6$  cells/mL in RPMI 1640 media (Sigma Aldrich, ON, Canada) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone Scientific, ON, Canada) and 0.5% Penicillin, Streptomycin and glutamine (Gibco, ON, Canada).

##### 2.1.2 *In Vitro* Differentiated Primary Human Th2 cells

For this thesis, experiments were performed on previously frozen *in vitro* differentiated Th2 cells. Blood was obtained from healthy donors and PBMCs were isolated using Ficoll histopaque Plus (GE Healthcare, Sweden). CD4<sup>+</sup> cells were isolated by negative selection with a CD4<sup>+</sup> cell Isolation Kit II (Miltenyi Biotech, CA, USA). CD4<sup>+</sup> T cells were then differentiated first through activation for 3 days on plate bound anti-CD3 (clone UCHT1, 1 $\mu$ g/mL) and anti-CD28 (clone 37407, 1 $\mu$ g/mL) in the presence of recombinant human 5ng/mL rhIL-2 (R&D Systems, MN, USA), 10-20ng/mL rhIL-4 (R&D Systems, MN, USA), blocking antibody for IFN $\gamma$  (polyclonal, 1 $\mu$ g/mL) and IL-12 (Clone C8.6, 1 $\mu$ g/mL). After activation, cells were proliferated with cytokines (IL-2 and IL-4) and blocking antibodies (IFN $\gamma$  and IL-12) for 4 days. Following the 7 days of differentiation, a CRTh2<sup>+</sup> selection kit (Milteny Biotech, CA, USA) was used to enrich CRTh2<sup>+</sup> cells using CRTH2-PE conjugated antibody (Miltenyi Biotech, CA, USA). CRTh2<sup>+</sup> cells were sorted using a positive selection kit (CRTh2<sup>+</sup> cell selection kit, Miltenyi Biotech, CA, USA) to obtain a purity of 98%. *In vitro* differentiated primary Th2 cells were cultured at  $2 \times 10^6$  cells/mL in X-Vivo 15 media (Lonza, MD, USA) supplemented with 10% Fetal Bovine Serum (Wisent, QC, Canada), 0.5% Penicillin, Streptomycin and glutamine (Gibco, ON, Canada) and 5ng/mL of IL-2 (MN, USA) with cells cycled on plate bound 1 $\mu$ g/mL of  $\alpha$ CD3/ $\alpha$ CD28 for 3 days or just 5ng/mL IL-2 for 4 days.

## 2.2 Experimental Set-up

### 2.2.1 Resting Cell Experiments

Experiments were set-up at  $0.2 \times 10^6$  cells/mL for CCRF-CEM cells and  $1.3 \times 10^6$  cells/mL with 0-5ng/mL of IL-2 for *in vitro* differentiated Th2 cells. Cells were treated with  $0.01 \mu\text{M}$ - $1 \mu\text{M}$  dexamethasone (Sigma Aldrich, ON, Canada), 13, 14-dihydro-15-keto Prostaglandin D<sub>2</sub> (DK-PGD<sub>2</sub>) (Caymen Chemicals, MI, USA) and  $0.01 \mu\text{M}$ - $1 \mu\text{M}$  of GC antagonist RU486 (Sigma Aldrich, ON, Canada). Cells were harvested by washing with phosphate buffered saline (PBS) for RNA isolation or PBS flow cytometry activated cell sorting (FACS) (0.5% bovine serum albumin, 0.1% sodium azide, 3% FBS) for flow cytometry.

### 2.2.2 Activated Cell Experiments

Experiments were set-up at  $0.2 \times 10^6$  cells/mL during peak growth phase, day after cells were seeded. Cells were treated with  $0.01 \mu\text{M}$ - $1 \mu\text{M}$  dexamethasone (Sigma Aldrich, ON, Canada), 20ng/mL of phorbol 12-myristate 130 acetate (PMA) (Sigma Aldrich, ON, Canada), and  $1 \mu\text{M}$  ionomycin (Sigma Aldrich, ON, Canada). Cells were harvested by washing with Phosphate Buffered Saline (PBS) before pelleting cells for RNA isolation.

## 2.3 Gene Expression

### 2.3.1 Quantitative Real Time PCR (qRT-PCR)

Taqman quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine relative gene expression using complementary DNA (cDNA). This method uses a fluorescent reporter dye (5') and a quencher (3') attached to the reporter probe. As the Taq Polymerase elongates the template strand it cleaves the reporter from the probe, once separated from the quencher the probe fluoresces. This fluorescence is measured and reflects the mRNA abundance amplified at the end of the assay which is represented by cycle threshold ( $c_t$ ).

To quantify mRNA levels, mRNA was extracted using RNeasy mini plus extraction kit (Qiagen, ON, Canada). RNA concentrations and quality were assessed using a nano-drop (Thermo Scientific). RNA samples with a 260/280 and 260/230 ratio greater than 1.8 were used for complimentary DNA (cDNA) synthesis. cDNA was synthesized using 400ng of mRNA template, iScript reverse transcriptase (BioRad, CA, USA), and RNase/DNase free



water (Lonza, MD, USA). cDNA synthesis was generated by priming for 5 min (25°C), reverse transcription 30 minutes (42°C) followed by RT inactivation 5 minutes (85°C). Resulting cDNA was stored short term at 4°C and long-term in -20°C prior to qRT-PCR. Taqman gene expression assays (Life Technologies, CA, USA) for GAPDH (Hs02786624\_g1), CRTh2 (Hs00173717\_m1), IL-13 (Hs00174379\_m1), FKBP5 (Hs01561006\_m1) and GR (Hs00353740\_m1) were used. qRT-PCR reactions contained 2µL of cDNA, 10µL of Taqman Mastermix (Applied Biosystems, CA, USA) and 7µL of RNase/DNase free water. Amplification was performed with the full reaction on a CFX 96: UNG Activation 2 minutes (50°C), polymerase activation 20 seconds (95°C), denature within 3 seconds, anneal/extend 30 seconds (60°C) (for 40 cycles). Data was analyzed using cycle threshold ( $C_t$ ) relative to housekeeping gene GAPDH. Fold increase relative to control condition was assessed for experimental treatments using  $2^{-\Delta\Delta C_t}$ .

## 2.4 Fluorescent Staining

### 2.4.1 Surface Expression

Flow cytometry uses laser technology to assess physical and chemical properties of single cells suspended in fluid. Fluorescent labelled antibodies are used to identify specific protein or chemical markers. The fluorochromes are excited by lasers of specific wavelength and the light emitted is then detected and assessed. Presence and intensity of light emission is used to determine existence and relative abundance of a protein or chemical marker.

Surface expression of CRTh2 was assessed using flow cytometry. Cells ( $0.5 \times 10^6$  cells) were washed (PBS FACS) at 900rpm. Cells were pelleted and re-suspended (PBS FACS) at  $5 \times 10^6$  cells/ml. Cells were blocked for 45 minutes (room temperature) with 3mg/mL of Rat IgG (Cederlane ON, Canada). Blocking was followed by cell staining with 75ng/mL of CRTh2 Alexa647 antibody (BM16-rat IgG2a, Cederlane ON, Canada) or 75ng/mL of isotype rat IgG2a Alexa647 (Cederlane ON, Canada) for 30 minutes on ice. Cells were then washed with 1mL of PBS FACS for 5 minutes at 900rpm and then re-suspended in 200uL of PBS FACS and fixed with 200uL of 4% paraformaldehyde (final 2%). Data were presented as % parent and mean fold intensity (MFI). MFI was calculated controlling for isotype (MFI = [Mean Intensity Antibody – Mean intensity Isotype] / Mean intensity Isotype).

### 2.4.2 Apoptosis

Experiments assessing apoptosis were set-up the day cells were re-seeded. Cells were treated with 0.1 $\mu$ M-1 $\mu$ M dexamethasone treatment in the presence or absence of antagonist RU486 for 24 or 48 hours. Experiments testing PGD<sub>2</sub> were pre-treated with DK-PGD<sub>2</sub> 30 minutes prior to dexamethasone treatment.

Caspase 3/7(Cederlane ON, Canada) assay uses a fluorescently tagged peptide which enters the cell and is cleaved by active caspase 3/7. The fluorescent dye is cleaved from the peptide and can bind directly to DNA to emit fluorescence. The Sytox assay (Cederlane ON, Canada) is a dye that can bind directly to DNA under conditions of cell death when cellular and nuclear membrane integrity is compromised.

Cells (1mL) were collected and placed into 1.5mL Eppendorf tubes. 2 drops of caspase 3/7 and 1 $\mu$ L of Sytox was added to cells and mixed by inversion. Stains were incubated for 40 minutes (room temperature). Data were presented as percentage of cells positive for each fluorescent stain.

Apoptosis and cell death following experimental treatments was assessed using Annexin V and 7AAD, respectively. Cells were collected (0.5x10<sup>6</sup>cells) and washed with PBS FACS at 900rpm for 10 minutes. Cells were re-suspended in 100 $\mu$ L of Annexin V binding buffer (10mM HEPES, 140mM NaOH, 2.5mM CaCl<sub>2</sub>) at 5x10<sup>6</sup>cells/mL. Double stain of 1 $\mu$ L of Annexin V and 10 $\mu$ L of 7AAD was added to each condition. Cells were incubated for 15 minutes (room temperature). After incubation cells were diluted with 400 $\mu$ L of Annexin V binding buffer. Data were presented as percentage of positive cells of parent population.

## 2.5 Statistical Analysis

All data were represented as mean  $\pm$  standard error. Unpaired and paired t tests or one-way Anova were used to identify significance between conditions. Tukey's Honest Significant Difference (HSD) test was used to identify significant differences between conditions. *P* values lower than 0.05 were considered significant differences.

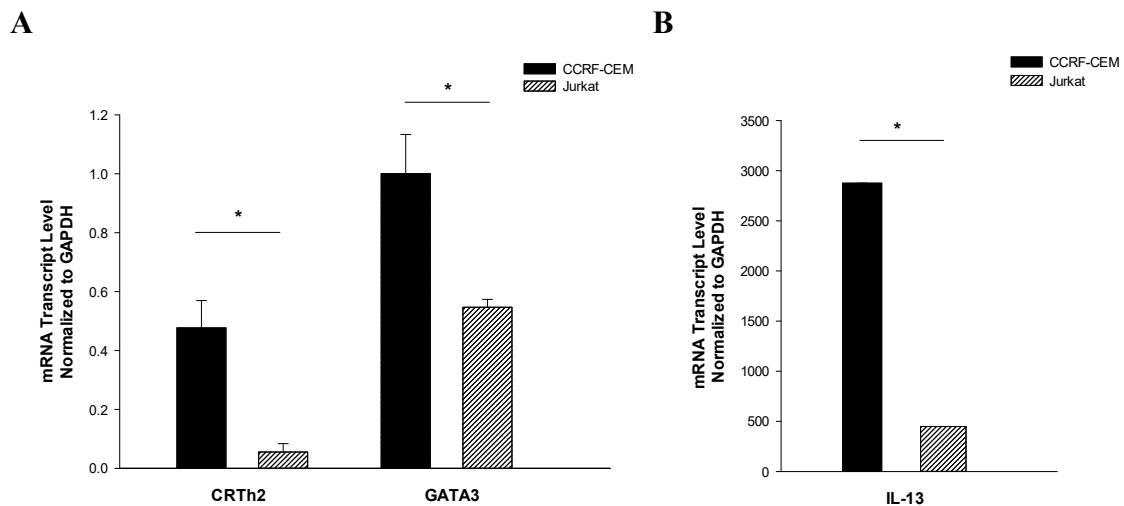
## Chapter 3

### 3 Results

Allergic asthma is mostly dominated by Th2 cell mediated inflammation [26]. GCs are the most common treatment for allergic asthma but the regulation of Th2 cells by GCs has not been well characterized. In this study, we examined the effect of GCs on Th2 cells with a T cell line (CCRF-CEM) that models a Th2 gene expression profile and validated these findings with an *in vitro* differentiated primary Th2 cell line. Two major functions of the GR are regulating mRNA transcripts either through transcription or stability and inducing apoptosis [14]. The GR can regulate transcription of genes by binding positive GREs or negative GREs to increase or decrease transcription, respectively [113]. GR mediated apoptosis is initiated by activation of the caspase-3 enzyme [160]. Interestingly, CRTh2 activation by PGD<sub>2</sub> has been shown to inhibit caspase-3 dependent apoptosis in response to IL-2 starvation [98]. This indicates that GR mediated apoptosis may be inhibited in Th2 cells by CRTh2 activation. To have a complete understanding of the regulation of Th2 cells by GCs, we must determine GC regulation of mRNA in Th2 cells activated via CD3/CD28. T cell activation has been shown to inhibit both GR mediated transcription and apoptosis through the up-regulation of AP-1 and NFκB [133, 134, 161]. GR self-regulation through transcription of FKBP5 and GR is essential to sustaining GR function, whether this is affected by T cell activation is unclear [115, 162]. The study aims to understand the regulation of Th2 cells by GCs to enhance our knowledge of how GCs affect Th2 dominant inflammation in the airways.

### 3.1 CCRF-CEM Cells – A Th2-like cell line

We chose to initiate our study of GC using a cell line because of the relative ease of cell culture and their accelerated growth (typically doubling daily). To choose a T cell line we reviewed T cells commonly used in the literature. The CCRF-CEM cell line was chosen based on its CRTh2 expression relative to other CD4<sup>+</sup> T cells (Figure 3). CCRF-CEM cells are an acute lymphoblastic leukemia CD4<sup>+</sup> T cell line derived from a 4-year-old female patient. Expression of CRTh2 was confirmed and other important Th2 genes (GATA3 and IL-13) were assessed relative to Jurkat T cells, another cell line commonly used to study T cell function [163]. We found that CCRF-CEM cells had higher mRNA transcript levels of CRTh2 and GATA3 compared to Jurkat cells (Figure 3A). Activation using PMA and ionomycin of CCRF-CEM cells demonstrated higher IL-13 mRNA levels relative to Jurkat cells (Figure 3B). These results identified that CCRF-CEM cells are a better model to study Th2 cell regulation by GCs compared to the Jurkat T cell line.



**Figure 3: Th2 gene expression of Th cell lines**

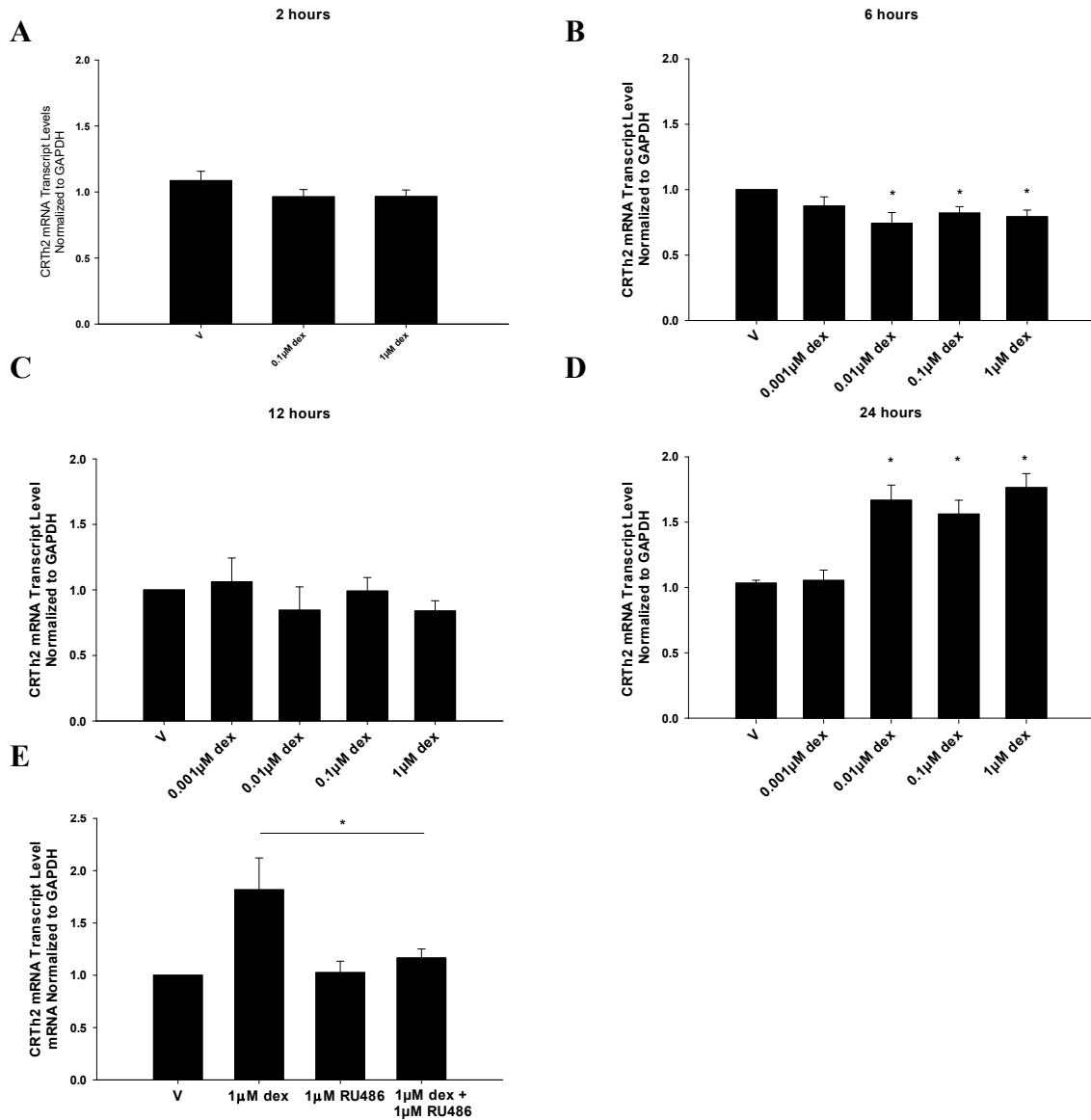
Expression of Th2 genes in CCRF-CEM and Jurkat T cells were assessed using qRT-PCR. The basal expression of CRTh2 and GATA3 (A) and expression of IL-13 in response to T cell activation (B) was determined. Transcript levels are presented as  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH and represent as mean fold difference  $\pm$  standard error (SE). Statistical difference ( $p < 0.05$ ) detected by Student's t-test.  $n=5$ .

## 3.2 GC Regulation of CRTh2 Expression

CRTh2 is a marker of Th2 cells and its expression exemplifies a fully differentiated helper T cell subset [86, 164]. CRTh2 expression by Th2 cells is important because CRTh2 mediates chemotaxis, cytokine production and cell survival [95, 98, 99]. So far, GATA3 has been the sole transcription factor shown to regulate CRTh2 transcription [165]. Sequence variants of CRTh2 transcripts in the African American population have been associated with increased stability and greater risk of asthma development [91]. These findings build on the data identifying higher CRTh2 mRNA and CRTh2<sup>+</sup> cells in the lungs of severe asthmatics that take the highest dose of GCs [3, 4]. A positive correlation was also seen between the daily dose of GCs and percentage of Th2 cells in circulation [3]. One of the main functions of the GR is regulation of transcription by binding GREs [113]. Interestingly, preliminary data in our lab generated using the Encyclopedia of DNA Elements (ENCODE) show that DNASE 1 hypersensitive sites on the CRTh2 promotor contain putative GRE (Naghdi, unpublished). This provided further incentive to determine whether GCs regulate transcription of CRTh2.

### 3.2.1 Dexamethasone Regulates CRTh2 mRNA in CCRF-CEM cells

Dexamethasone is among the most potent oral GCs available clinically and has a half-life of 36-54 hours [166, 167]. Compared to prednisone which is a common oral GC prescribed for asthma treatment, dexamethasone is 5 times more potent and has double the half-life [167]. Thus, we thought dexamethasone would be the best GC to use to help us understand the potential of the GR signaling pathway to regulate transcription. The CRTh2 locus has been shown to contain putative GR binding sites. Determining whether the GR regulates CRTh2 transcription will help us to understand how GCs regulate Th2 cell function. The level of CRTh2 mRNA transcripts in response to kinetic and titration experiments of dexamethasone were performed using CCRF-CEM cells (Figure 4). No changes in CRTh2 mRNA transcript levels were seen at 2 hours (Figure 4A), there were reduced levels at 6h (Figure 4B) and no change at 12 hours (4C) of dexamethasone treatment. CRTh2 mRNA levels were higher in response to GC treatment of 0.01 $\mu$ M-1 $\mu$ M after 24 hours (Figure 4D). Interestingly the increase in CRTh2 transcript levels plateaued at 0.01 $\mu$ M dexamethasone. This increase in CRTh2 mRNA transcripts was inhibited by RU486 (Figure 4E), an antagonist that blocks GRE binding, indicating that the GR directly regulates transcription of CRTh2.

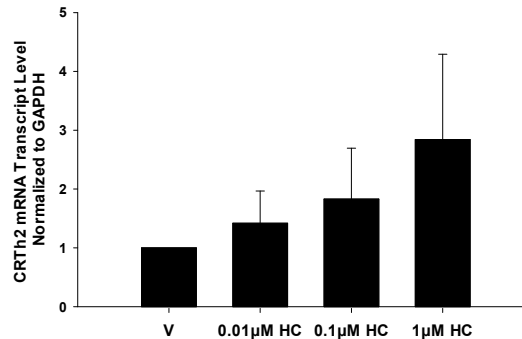


**Figure 4: CRTh2 mRNA transcript levels in response to dexamethasone treatment**

CRTh2 mRNA transcript levels were assessed by qRT-PCR after 2 hours (A), 6 hours (B), 12 hours (C), and 24 hours (D) of dexamethasone treatment in CCRF-CEM cells. The antagonist RU486 was added with dexamethasone to inhibit GR activity (E). Transcript levels are presented as  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH and represent mean fold difference relative to vehicle  $\pm$  SE. Statistical difference ( $p < 0.05$ ) detected by one way ANOVA. V=vehicle (ETOH 0.004%); n=3-19.

### 3.2.2 Hydrocortisone Regulation of CRTh2 mRNA in CCRF-CEM cells

Hydrocortisone is found naturally in the body and is produced in response to stress. Compared to dexamethasone, hydrocortisone is a weaker GR agonist [166]. We wanted to determine whether GR regulation of CRTh2 mRNA was dependent on GC potency. The level of CRTh2 mRNA transcripts appeared to increase, though this change was not statistically significant, due to the lack of statistical power.

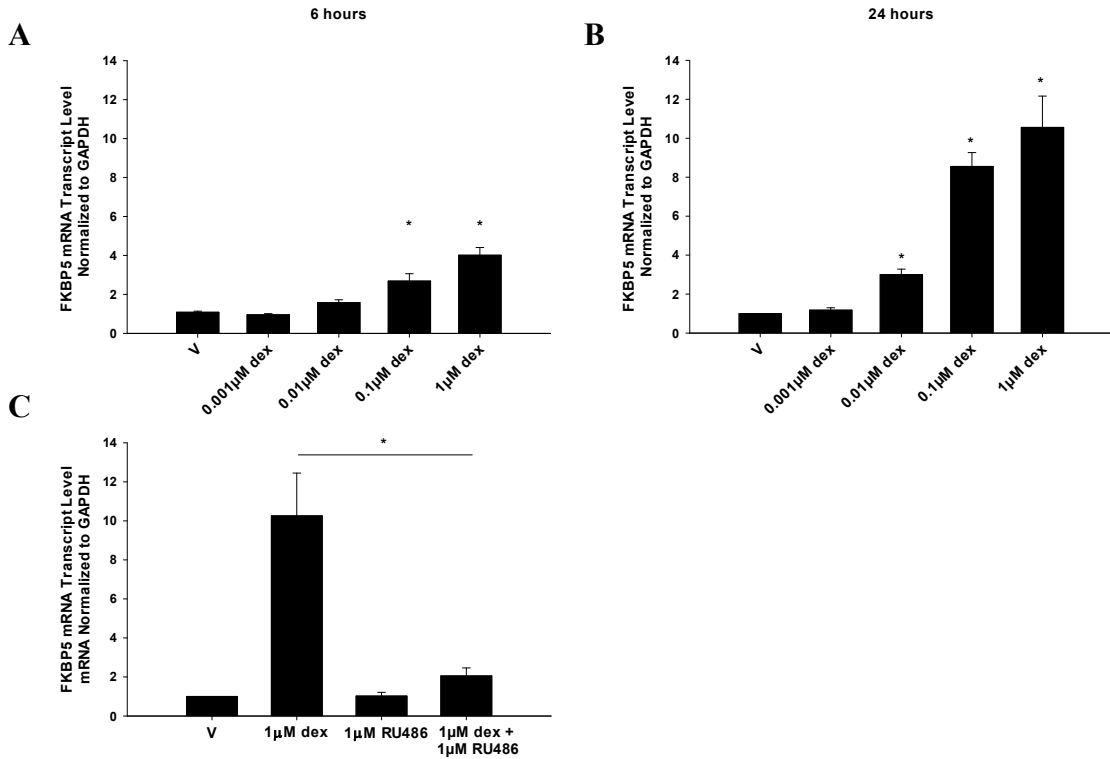


**Figure 5: CRTh2 mRNA transcript levels in response to hydrocortisone treatment**

CRTh2 mRNA transcript levels were assessed by qRT-PCR after 24 hours of hydrocortisone treatment in CCRF-CEM cells. Transcript levels are presented as  $2^{-\Delta\Delta CT}$ , normalized to housekeeping gene GAPDH and represented as mean fold difference relative to vehicle  $\pm$  SE. HC= hydrocortisone; V=vehicle (ETOH 0.004%); n =3.

### 3.2.3 Dexamethasone Regulates FKBP5 mRNA levels

FKBP5 which is a chaperone protein that keeps the GR in the cytoplasm and in an active state within the GR heterocomplex [110]. To sustain GR function, there is feed-forward regulation by the GR that increases FKBP5 transcription [115]. FKBP5 was used as a positive control for GR-induced effect on transcripts to assess the level of GR function at different concentrations. We assessed FKBP5 mRNA transcript levels to determine GC regulation in CCRF-CEM cells (Figure 6). A dose dependent increase in FKBP5 mRNA transcript levels was seen, with the first increase at 6 hours (Figure 6A) and continual increase after 24 hours (Figure 6B). Incorporation of RU486 with dexamethasone for 24 hours inhibited the increase in FKBP5 mRNA transcript levels (Figure 6C). FKBP5 mRNA transcript levels showed a dose dependent induction in response to dexamethasone treatment in CCRF-CEM cells.



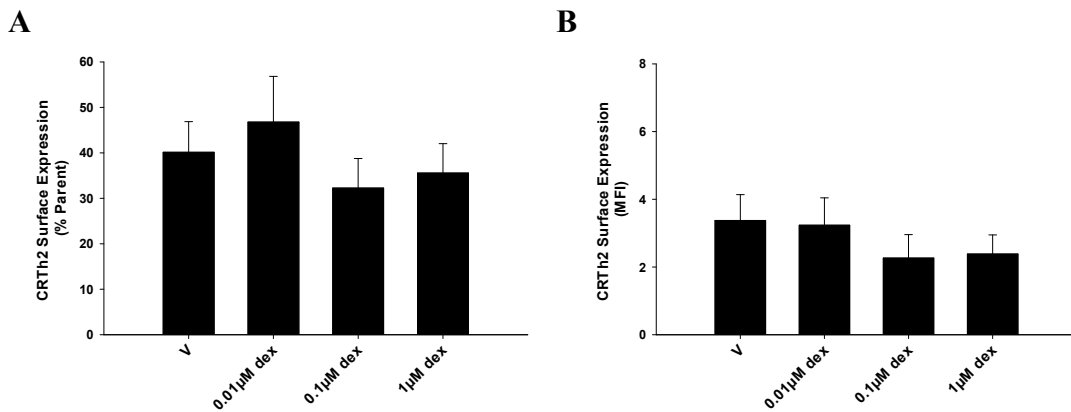
**Figure 6: FKBP5 mRNA transcript levels in response to dexamethasone treatment**

FKBP5 mRNA transcript levels were assessed over time with qRT-PCR after 6 hours (A) and 24 hours (B) of dexamethasone treatment in CCRF-CEM cells. The antagonist RU486 was added with dexamethasone to inhibit GR activity (C). FKBP5 mRNA transcript levels were assessed using  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH. Data represented as mean fold difference relative to vehicle  $\pm$  SE. Statistical difference ( $p < 0.05$ ) detected by one-way ANOVA. V=vehicle (ETOH 0.004%);  $n = 3-6$ .



### 3.2.4 Dexamethasone Regulation of CRTh2 Surface Expression in CCRF-CEM cells

CRTh2 is a seven-transmembrane spanning G-protein coupled receptor, thus its expression on the membrane requires the work of multiple folding and transport proteins [168]. Previous research using transfected T cells demonstrated that CRTh2 mRNA levels are reflected by total protein levels in the cell [80]. We treated CCRF-CEM cells with dexamethasone and assessed whether the change in CRTh2 mRNA levels we detected (Figure 4D) are reflected by the CRTh2 surface expression (Figure 7). After 24 hours of dexamethasone treatment there were no significant changes in the percentage of cells positive for CRTh2 (Figure 7A) or the abundance of receptors per cell, represented by mean fold intensity (Figure 7B). These results indicate that the changes in CRTh2 mRNA did not translate to surface expression.

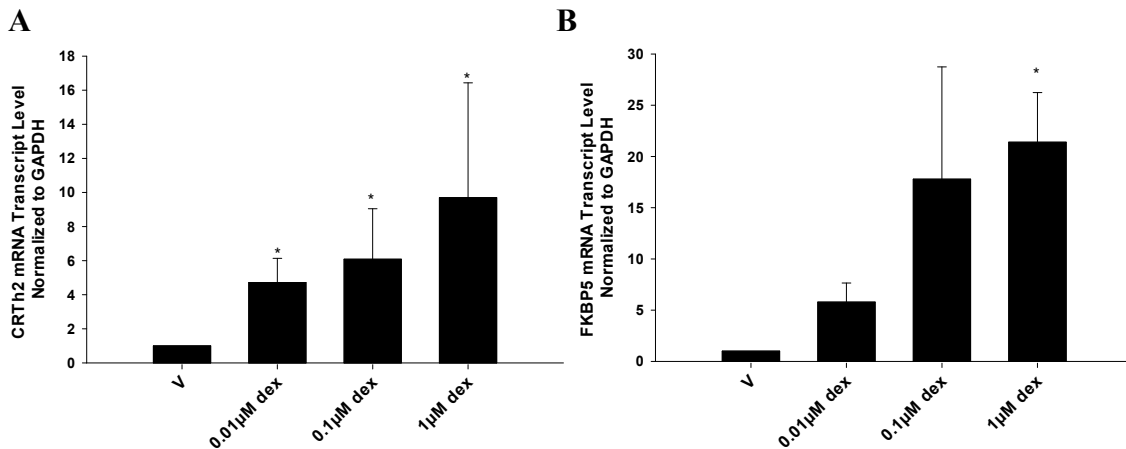


**Figure 7: CRTh2 surface expression in response to dexamethasone treatment**

Expression of CRTh2 on the cell surface was assessed using flow cytometry. CRTh2 levels were determined in response to dexamethasone treatment in CCRF-CEM cells for 24 hours. The data were represented as percentage of cells expressing CRTh2 (A) and mean fold intensity representing the abundance of CRTh2 receptors per cell (B). Data represented as means  $\pm$  SE. V=vehicle (ETOH 0.004%); n = 7.

### 3.2.5 Dexamethasone Regulates CRTh2 mRNA in Primary Th2 cells

Due to the lack of agreement between CRTh2 mRNA and protein in CCRF-CEM cells, we next turned to *in vitro* differentiated Th2 cells as a more physiologically relevant cell type to assess the GC effect on CRTh2 expression. Transcript levels of CRTh2 and FKBP5 in response to dexamethasone treatment for 24 hours were assessed (Figure 8). CRTh2 mRNA levels (Figure 8A) were higher in response to dexamethasone treatment at concentrations of 0.01 $\mu$ M and above but did not differ between doses. The fold induction in primary Th2 cells was higher (~4 fold) than what was previously seen with dexamethasone treatment of CCRF-CEM cells (~2 fold; Figure 4D). FKBP5 mRNA transcript levels also increased in response to dexamethasone in a dose dependent manner. The results replicate the mRNA changes seen in CCRF-CEM cells, but show that in primary Th2 cells GC upregulates CRTh2 mRNA to an even greater extent.



**Figure 8: mRNA transcript levels in primary Th2 cells**

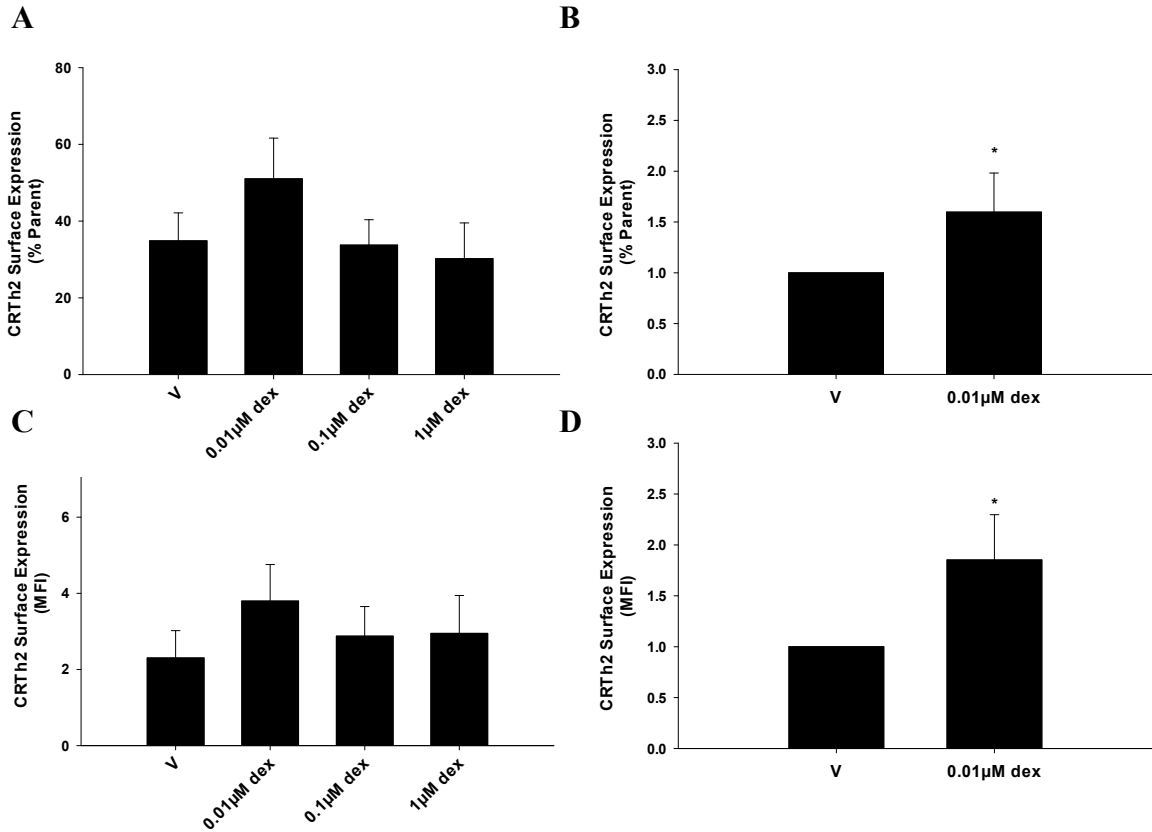
CRTh2 (A; n=5) and FKBP5 (B; n=3) mRNA transcript levels were assessed using qRT-PCR in response to dexamethasone. Transcript levels are presented as  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH and represent mean fold difference relative to vehicle  $\pm$  SE. Statistical difference ( $p < 0.05$ ) detected by one-way ANOVA. V=vehicle (ETOH 0.004%).

### 3.2.6 Dexamethasone Regulates CRTh2 Surface Expression in Primary Th2 cells

CRTh2 mRNA transcripts were increased in both CCRF-CEM and primary Th2 cells in response to 24 hours of dexamethasone treatment. Although previous studies have indicated that CRTh2 mRNA changes are reflected at the protein level, this was not seen with CRTh2 surface expression in CCRF-CEM cells (Figure 7) [80]. To determine whether changes in CRTh2 mRNA transcript levels are reflected with CRTh2 surface expression we treated primary Th2 cells with dexamethasone (Figure 9). When assessing all doses of dexamethasone together we saw that with a one-way ANOVA there were no significant changes in percentage of cells positive for CRTh2 (Figure 9A) or the abundance of receptors per cell, represented by mean fold intensity (Figure 9C). On the other hand, when we looked at the vehicle in comparison to the lowest concentration of dexamethasone needed to see mRNA changes (Figure 8A) we saw a significant difference using a Paired t-test in both the percentage of cells positive for CRTh2 (Figure 9B) and abundance of CRTh2 receptors per cell (Figure 9D). This indicates that at low concentrations of dexamethasone CRTh2 surface expression is also increased.

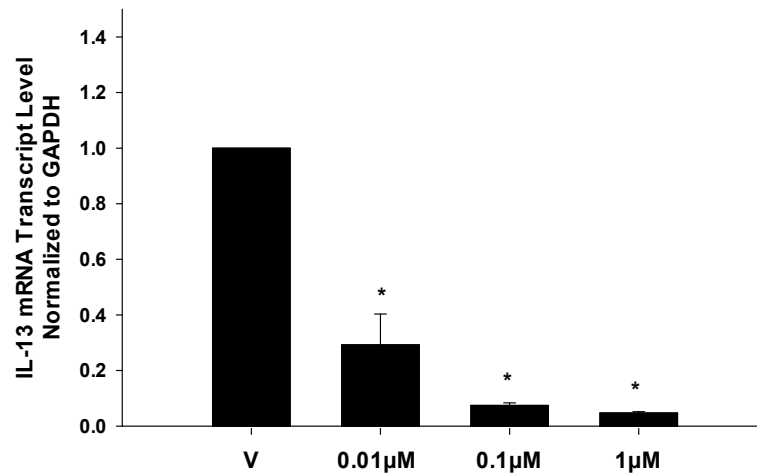
### 3.2.7 Dexamethasone Regulates IL-13 Expression in Primary Th2 cells

The ability of GCs to regulate CRTh2 expression may help promote Th2 inflammation, which is contrary to previous *in vivo* studies showing that GCs reduce type 2 cytokines [127, 169]. Even in the case of Th2 high asthma the reduction of IL-13 in response to GCs is an indicator of steroid sensitivity [26]. As such, we wanted to determine whether GCs reduce IL-13 mRNA levels despite increasing CRTh2 mRNA. To test whether GCs can reduce IL-13 mRNA levels we treated primary Th2 cells with dexamethasone for 24 hours (Figure 10) and found that there was a dose dependent reduction in IL-13 mRNA levels at the lowest concentration of dexamethasone (0.01 $\mu$ M). These data show GCs can reduce IL-13 expression but at the same time increase CRTh2 expression.



**Figure 9: CRTh2 surface expression in response to dexamethasone treatment**

Expression of CRTh2 on the surface of the cell was assessed using flow cytometry. CRTh2 levels were determined in response to dexamethasone treatment for 24 hours. The data were represented as percentage of cells expressing CRTh2 (A, C) and mean fold intensity representing the abundance of CRTh2 receptors per cell (B, D). Data represented as means  $\pm$  SE V=vehicle (ETOH 0.004%); Statistical difference ( $p < 0.05$ ) detected by using paired T-test.  $n = 7$ .



**Figure 10: IL-13 mRNA levels in primary Th2 cells in response to dexamethasone**

IL-13 mRNA levels were assessed using qRT-PCR in primary Th2 cells treated with dexamethasone for 24 hours. Transcript levels are presented as  $2^{-\Delta\Delta CT}$ , normalized to housekeeping gene GAPDH and represented as mean fold difference relative to vehicle  $\pm$  SE. V=vehicle (ETOH 0.004%); n =5.

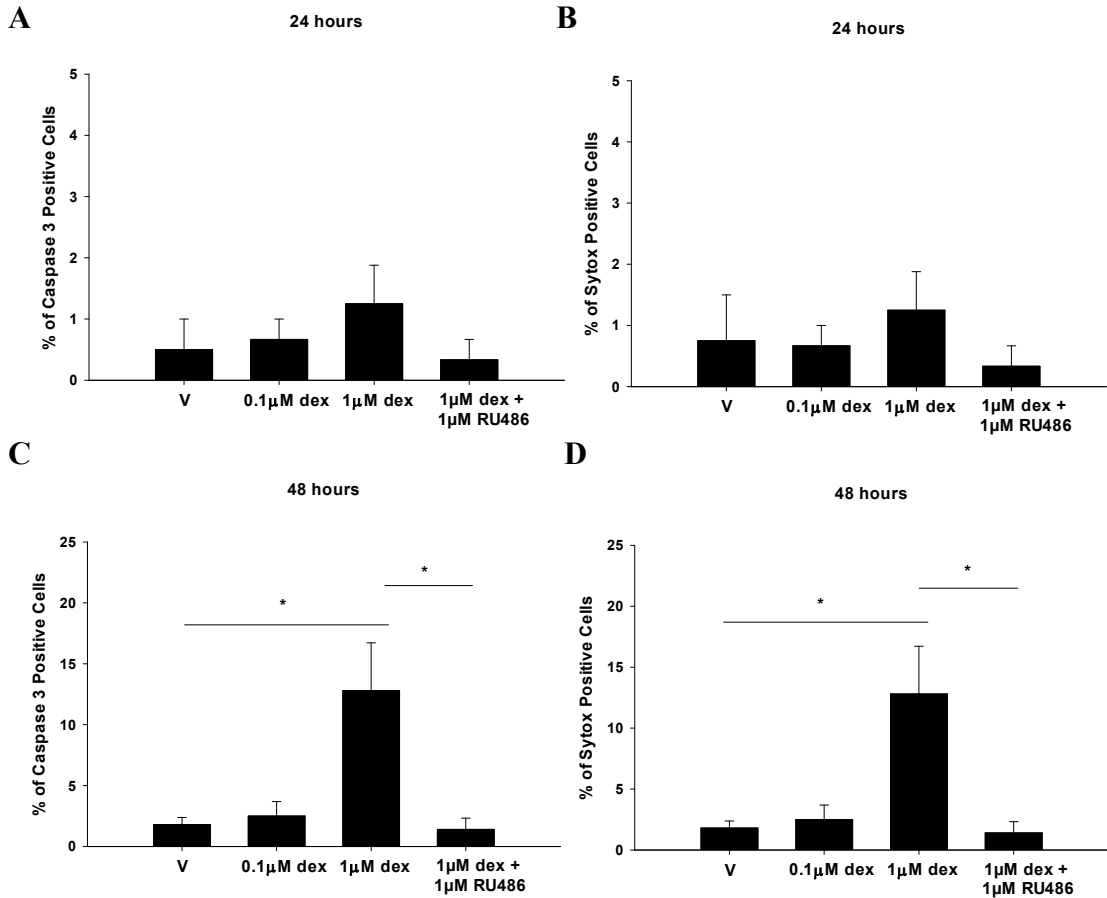
### 3.3 Glucocorticosteroid Induced Apoptosis in Th2 cells

In addition to suppressing Th2 cytokines, another function of GCs is the ability to induce apoptosis in inflammatory cells in a dose and time dependent manner [144]. GC-induced apoptosis in naïve and memory T cells has been well documented but no studies have been completed specifically in Th2 cells [141-143]. PGD<sub>2</sub> binding CRTh2 activates the PI3K pathway which has been shown to inhibit apoptosis induced by IL-2 starvation in Th2 cells by inhibiting BAD interference of anti-apoptotic BCL proteins [98]. Downregulation of BCL proteins induces GC-induced apoptosis, thus there is competition between pathways activated by GCs and PGD<sub>2</sub> [98, 141, 151]. Severe asthmatics who take the highest dose of GCs have been shown to have a higher level of PGD<sub>2</sub> relative to other asthmatics [4]. If PGD<sub>2</sub> can inhibit GC-induced apoptosis, Th2 cells may be sustained in severe asthmatics even though they take high dose GCs. As such, we wanted to test whether CRTh2 signaling could inhibit GC-induced apoptosis and render Th2 cells GC resistant.

### 3.3.1 Dexamethasone Regulates Apoptosis in CCRF-CEM cells

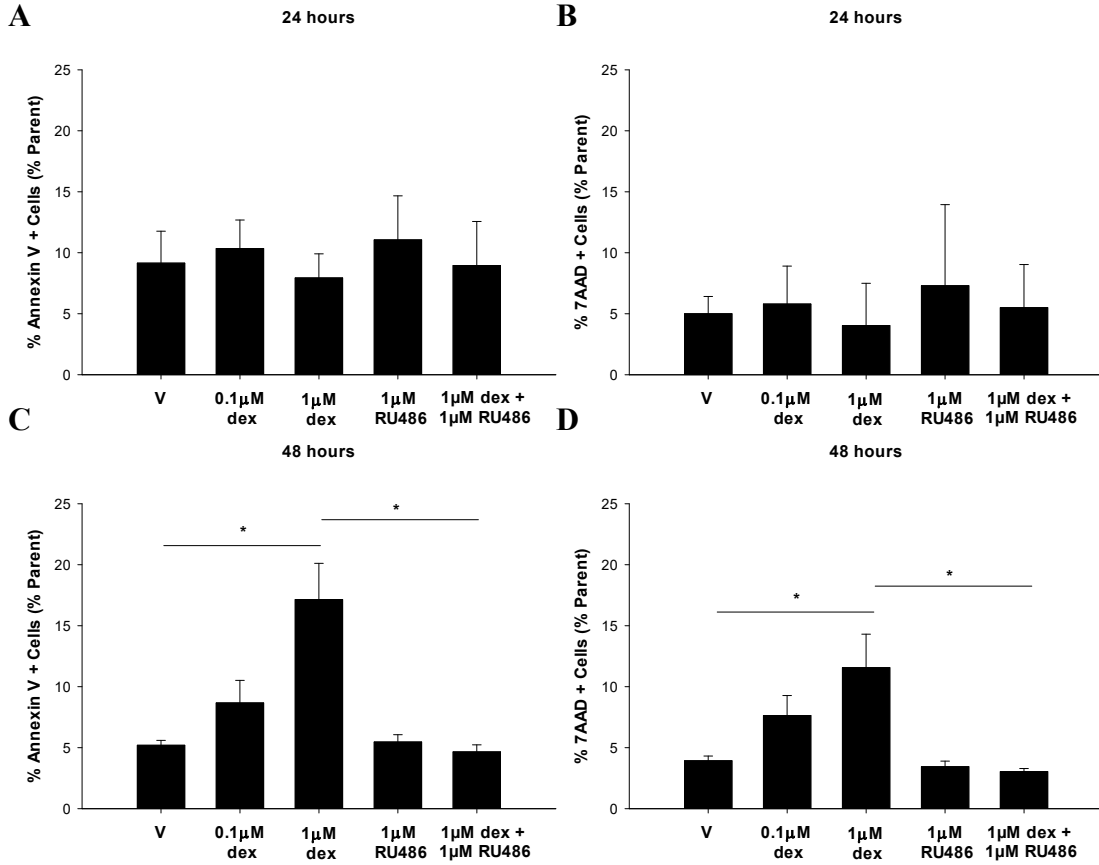
The effect of GCs is dependent on dose and exposure time and most importantly dependent on cell type as demonstrated by opposing apoptotic effects seen in eosinophils versus neutrophils [144, 145]. To determine whether PGD<sub>2</sub> can inhibit GC-induced apoptosis it was important to first establish the dose and time of exposure needed to induce apoptosis. CCRF-CEM cells were treated with dexamethasone for 24 and 48 hours to assess the ability of GCs to induce apoptosis. To test whether cells were undergoing apoptosis and cell death we used Caspase 3/7 and Sytox fluorescent stains, respectively. After 24 hours of exposure to various concentrations of dexamethasone treatment there were no changes in apoptosis (Figure 11A) or cell death (Figure 11B). After 48 hours of 1 $\mu$ M dexamethasone there was a significant increase in apoptosis (Figure 11C) and cell death (Figure 11D). Induction of apoptosis was related directly to the ability of GCs to influence transcription as the effect was inhibited by RU486.

GC-induced apoptosis results from the release of cytochrome C and downstream activation of caspase-3 to induce cell death [170]. We wanted to use a more sensitive assay to ensure we did not miss any apoptotic effects after 24 hours of dexamethasone treatment. We used the stain Annexin V which binds phosphatidylserines to detect early apoptotic changes at the cell surface of viable cells [106]. To do this CCRF-CEM cells were treated with various concentrations of dexamethasone (24 and 48 hours) and stained for Annexin V and 7AAD (Figure 13). Similar to Figure 11 there was no significant apoptosis or cell death after 24 hours of dexamethasone treatment (Figure 12A,12B), but again we see that after 48 hours of 1 $\mu$ M dexamethasone treatment there is an increase in both apoptosis and cell death (Figure 12C, D). Induction of apoptosis by GCs was inhibited by antagonist RU486.



**Figure 11: Dexamethasone induced apoptosis in CCRF-CEM cells**

CCRF-CEM cells were treated with dexamethasone for 24 and 48 hours with and without antagonist RU486 to determine apoptosis and cell death using fluorescent stains. After 24 hours the percentage of caspase 3/7 positive cells (A) and Sytox positive cells (B) indicated apoptosis and cell death, respectively. After 48 hours the percentage of caspase 3/7 positive cells (C) and Sytox positive cells (D) indicated apoptosis and cell death, respectively. Data represented as means  $\pm$  SE. Dex = dexamethasone; V=vehicle (ETOH 0.004%); Statistical difference ( $p < 0.05$ ) detected by using one way ANOVA.  $n = 4$ .



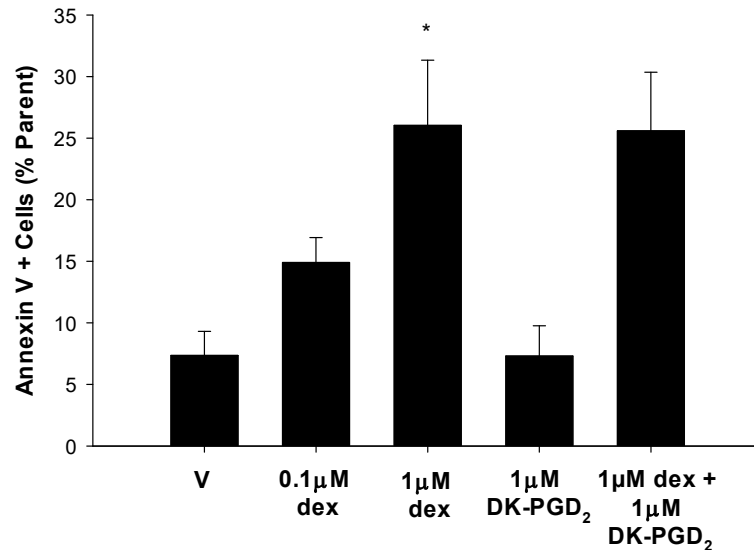
**Figure 12: Dexamethasone induced apoptosis**

CCRF-CEM cells were treated with dexamethasone for 24 and 48 hours with and without antagonist RU486 to determine apoptosis and cell death using flow cytometry. Percentage of Annexin V positive cells identified dexamethasone induced apoptosis for 24 hours (A) and 48 hours (C). Cell death was assessed by cells positive for 7AAD after 24 hours (B) and 48 hours (D). Data represented as mean fold difference relative to vehicle  $\pm$  SD; dex = dexamethasone; V=vehicle (ETOH 0.004%); Statistical difference ( $p < 0.05$ ) detected by using one way ANOVA.  $n = 4$ .



### 3.3.2 PGD<sub>2</sub> Does Not Regulate Dexamethasone Induced Apoptosis in CCRF-CEM cells

GR induction of apoptosis and CRTh2 inhibition of apoptosis are both regulated by similar pathways but in the opposite manner [98, 141]. PGD<sub>2</sub> binding to CRTh2 activates AKT signaling which inhibits caspase-3 mediated apoptosis, while GCs induce caspase-3 mediated apoptosis [98, 141]. CRTh2 has been shown to inhibit apoptosis but only in the context of IL-2 starvation [98]. To determine whether CRTh2 and GC activated pathways compete to maintain Th2 cell survival, we pre-treated CCRF-CEM cells with CRTh2 specific agonist, the PGD<sub>2</sub> metabolite DK-PGD<sub>2</sub> before treating with dexamethasone for 48 hours (Figure 13). DK-PGD<sub>2</sub> did not reduce the percentage of Annexin V<sup>+</sup> cells in response to GC treatment. These results may be due to a limitation of the CCRF-CEM cells ability to regulate CRTh2.

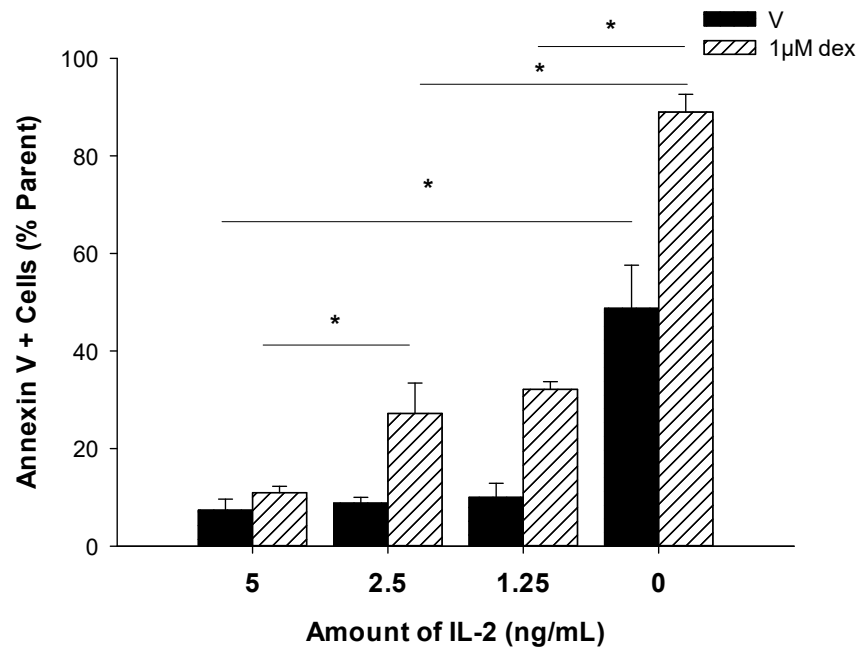


**Figure 13: DK-PGD<sub>2</sub> does not inhibit GC-induced apoptosis in CCRF-CEM cells**

CCRF-CEM cells were pre-treated with DK-PGD<sub>2</sub> and then dexamethasone for 48 hours. Data represented as means  $\pm$  SE. Early apoptosis was determined by Annexin V positive cells. V=vehicle (ETOH 0.004%); Statistical difference ( $p < 0.05$ ) detected by using one-way ANOVA. n=5.

### 3.3.3 IL-2 Inhibits Glucocorticosteroid Induced Apoptosis in Primary Th2 cells

IL-2 is a survival factor that is required to maintain T cells in culture [154]. IL-2 signals through AKT to inhibit apoptosis induced by GCs [171]. We have determined that 1 $\mu$ M dexamethasone was required to induce apoptosis in CCRF-CEM cells, but these cells were not cultured with IL-2. We needed to determine the concentration of IL-2 needed to maintain Th2 cells but still leave them susceptible to apoptosis. We treated cells with decreasing concentrations of IL-2 with and without dexamethasone and assessed percentage of Annexin V<sup>+</sup> cells (Figure 14). Cells treated with decreasing concentrations of IL-2 (5-0ng/mL) show a dose response to 1 $\mu$ M dexamethasone induced apoptosis. 0ng/mL of IL-2 had significant apoptosis in the absence of dexamethasone. Thus, these results indicate that 1.25ng/mL of IL-2 sustained Th2 cells while still permitting GC sensitivity.

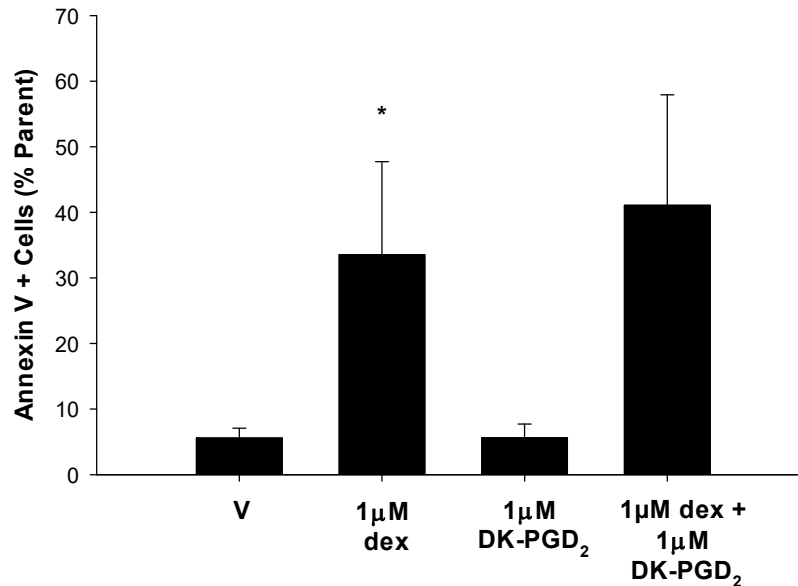


**Figure 14: IL-2 inhibits dexamethasone induced apoptosis**

The ability of dexamethasone to induce apoptosis with varying amounts of IL-2 was identified by Annexin V positive cells using flow cytometry. Primary Th2 cells were treated with dexamethasone and decreasing amounts of IL-2 for 48 hours to assess cell apoptosis. Data represented as means  $\pm$  SE. V= vehicle (0.04% ETOH). Statistical difference ( $p < 0.05$ ) detected by using one way ANOVA and Tukey's HSD test.  $n = 3$ .

### 3.3.4 PGD<sub>2</sub> Does Not Regulate Dexamethasone Induced Apoptosis in Primary Th2 cells

CCRF-CEM cells that were pre-treated with DK-PGD<sub>2</sub> did not show any changes in dexamethasone induced apoptosis. Primary Th2 cells were used to replicate the previous experiments assessing PGD<sub>2</sub> inhibition of apoptosis. In these experiments, Th2 cells were cultured in 1.25ng/mL of IL-2 which we determined was sufficient to maintain Th2 cells and render cells susceptible to GCs (Figure 14). Primary Th2 cells were pre-treated with DK-PGD<sub>2</sub> followed by dexamethasone treatment and assessed for apoptosis using Annexin V staining. However, there were no differences in the percentage of Annexin V<sup>+</sup> cells in response to dexamethasone treatment after DK-PGD<sub>2</sub> pretreatment (Figure 15).



**Figure 15: DK-PGD<sub>2</sub> does not inhibit dexamethasone induced apoptosis**

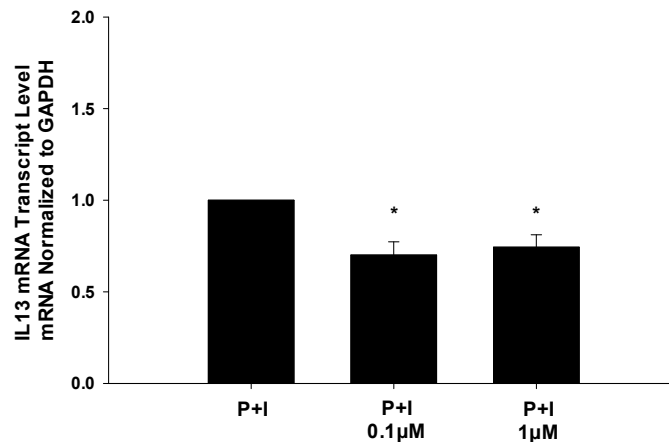
To assess the ability of CRTh2 signaling to inhibit apoptosis, primary Th2 cells cultured with 1.25ng/mL of IL-2 were pre-treated with DK-PGD<sub>2</sub> and then treated with dexamethasone. Early apoptosis was determined by Annexin V positive cells using flow cytometry. Data represented as means  $\pm$  SE; V=vehicle (ETOH 0.004%); n = 3.

### 3.4 Glucocorticosteroid Regulation of Transcription in Activated T cells

A large component of chronic inflammation in asthma is the constant activation of Th2 cells via dendritic cells [44]. CD3 and CD28 co-stimulation of Th2 cells up-regulates transcription factors NFAT, AP-1 and NF $\kappa$ B to induce cytokine production and regulate inflammation [53, 158, 172]. Production of type 2 cytokines can be inhibited by GCs as indicated in literature and demonstrated with our own work (Figure 10) [127]. Interestingly, AP-1 and NF $\kappa$ B have both been shown to inhibit GR induced transcription [134, 161]. The GR has also been shown to reciprocally inhibit AP-1 and NF $\kappa$ B, as GC treatment of T cells increased the threshold for T cell activation [173]. Thus, GR and the T cell activation pathways compete and interfere with each other. Severe asthmatics have the highest percentage of Th2 cells in circulation, thus if T cell activation inhibits GC regulation of Th2 cells, chronic exposure to allergens may render Th2 cells GC insensitive [3]. To fully understand how GCs regulate Th2 cells we wanted to look at GR function in the context of activated Th2 cells.

#### 3.4.1 Dexamethasone Regulates IL-13 mRNA in CCRF-CEM cells

T cell activation induces expression of type 2 cytokines IL-4, IL-5, and IL-13 [55, 135]. Interestingly, activated T cells have been deemed GC resistant but cytokines can be inhibited by GCs [159, 174]. Our previous results indicated that GCs could reduce IL-13 mRNA levels in primary Th2 cells, even at low dose (0.01 $\mu$ M; Figure 11). If T cell activation inhibits GC function, then the extent of IL-13 regulation by GCs may be repressed. To determine whether GCs can inhibit cytokine expression with T cell activation, CCRF-CEM cells were activated with PMA and ionomycin (Figure 16). IL-13 mRNA transcript levels were reduced in response to dexamethasone treatment though the reduction was only about 30% compared to (80%) reduction seen in primary Th2 cells. These results suggest that the GR still retains its ability to inhibit IL-13 production after T cell activation.



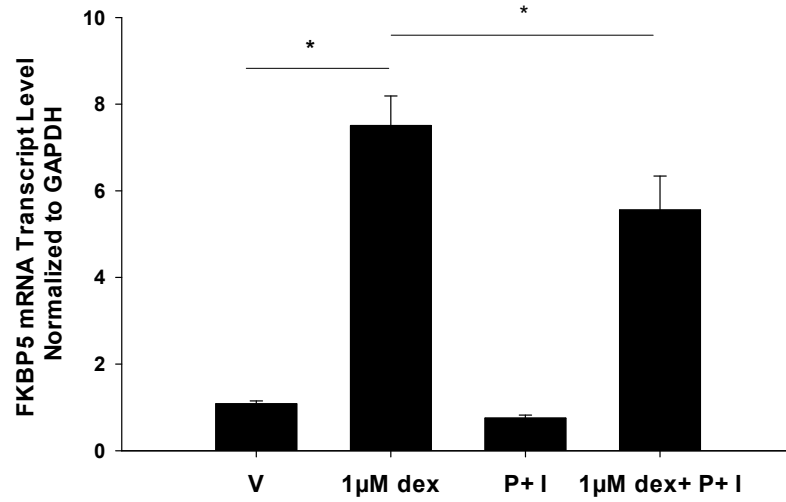
**Figure 16: Dexamethasone reduces IL-13 mRNA transcript levels in activated T cells**

The dexamethasone response of activated CCRF-CEM cells was assessed using qRT-PCR after 24 hours. IL-13 mRNA transcript levels were assessed using  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH. Data represented as mean fold difference relative to vehicle  $\pm$  SE. P= PMA; I= ionomycin; V=0.004% ETOH); Statistical difference ( $p < 0.05$ ) detected by using one-way ANOVA.  $n=5$ .

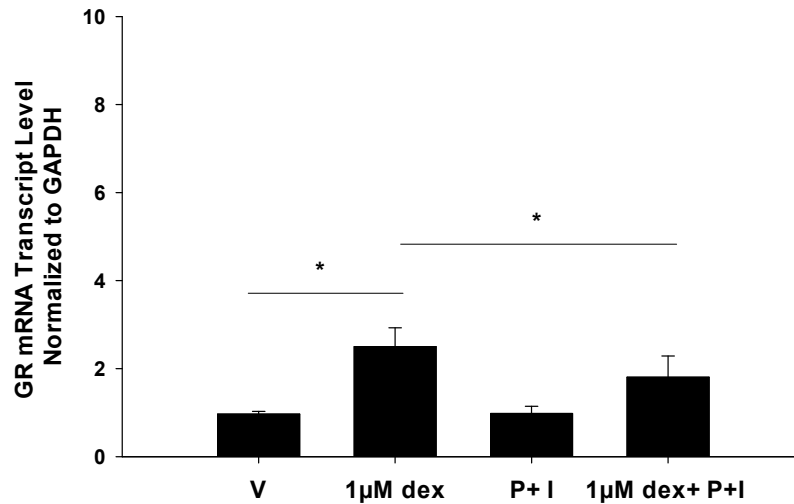
### 3.4.2 T cell Activation Interferes with Dexamethasone Regulation of GR Signaling Genes in CCRF-CEM cells

Upon GC binding, the GR increases transcription of FKBP5 and GR, to sustain GR function. Although T cell activation has been shown to inhibit GR mediated transcription, it has not been shown whether there is a similar effect on the feed forward loop of GCs increasing expression of genes within its own signaling pathway. To test this CCRF-CEM cells were activated with PMA and ionomycin to mimic CD3 and CD28 mediated T cell activation and with or without dexamethasone. The level of FKBP5 and GR mRNA transcripts in response to dexamethasone treatment in resting and activated cells was assessed (Figure 17). As expected both FKBP5 and GR mRNA transcript levels were increased in response to dexamethasone treatment. But, with the addition of PMA and ionomycin the dexamethasone effect on the level of FKBP5 and GR mRNA transcripts were reduced. This builds on previous studies, as these data demonstrate that activation of T cells reduces GR mRNA transcripts and thereby GR activity, to render Th2 cells less sensitive to GCs.

A



B

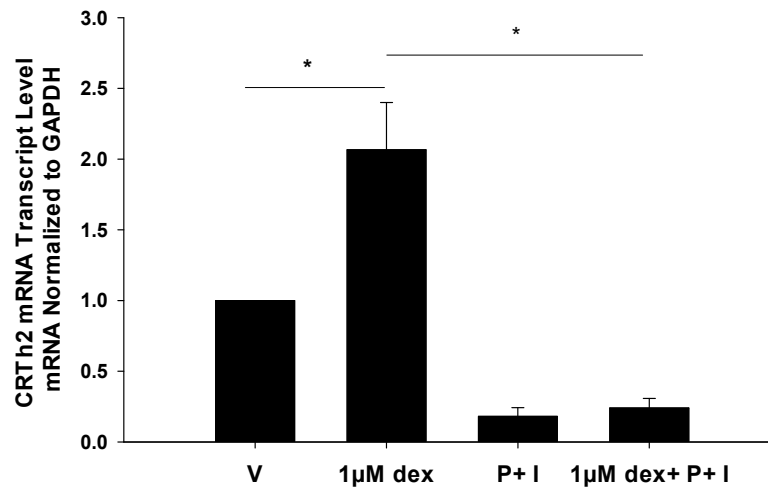


**Figure 17: Dexamethasone regulation is inhibited by T cell activation**

The dexamethasone response in activated CCRF-CEM cells was assessed using qRT-PCR after 24 hours. FKBP5 (A) and GR (B) mRNA transcript levels were assessed using  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH. Data represented as mean fold difference relative to vehicle  $\pm$  SE. V= 0.004% ETOH; P= PMA; I = Ionomycin; Statistical difference ( $p < 0.05$ ) detected by using one way ANOVA.  $n=5$ .

### 3.4.3 Dexamethasone Does Not Regulate CRTh2 Expression in Activated T cells

One of the novel findings of this study was the identification that GCs regulate CRTh2 mRNA levels (Figure 4). This GC regulation of CRTh2 may have vast implications in relevance to Th2 cell function [95, 98, 99]. Interestingly, CRTh2 expression has been shown to be downregulated by T cell activation [80]. We have seen that T cell activation reduces GR activity as demonstrated by lower levels of FKBP5 and GR mRNA transcripts (Figure 18). Despite reduced GR signaling genes these conditions did not completely inhibit the ability of GCs to reduce IL-13 mRNA levels (Figure 16). To determine whether GCs could increase CRTh2 expression in the presence of T cell activation we treated CCRF-CEM cells with dexamethasone and PMA and Ionomycin (Figure 18). As previously shown, CRTh2 mRNA levels were increased with dexamethasone treatment but with the addition of PMA and ionomycin there was significant drop that was reversed by dexamethasone.



**Figure 18: Dexamethasone induction of CRTh2 is inhibited by T cell activation**

The dexamethasone response in activated CCRF-CEM cells was assessed using qRT-PCR after 24 hours. CRTh2 mRNA transcript levels were assessed using  $2^{-\Delta\Delta C_t}$  and normalized to housekeeping gene GAPDH. Data represented as mean fold difference relative to vehicle  $\pm$  SE. V= 0.004% ETOH; P= PMA; I = Ionomycin; Statistical difference ( $p < 0.05$ ) detected by using one way ANOVA. n=5.

## Chapter 4 : Discussion

### 4 Summary

Severe asthmatic patients have a higher percentage of CRTh2<sup>+</sup> cells in the airways and Th2 cells in circulation, relative to mild/moderate asthmatics [3, 4]. Interestingly, Palikhe *et al* identified a positive correlation between the percentage of Th2 cells in the blood of asthmatics and the daily dose of inhaled GCs taken [3]. Naturally, this finding provided incentive to study the effects of GC on Th2 cells.

An immortalized CD4<sup>+</sup> T cell line (CCRF-CEM) and *in vitro* differentiated primary Th2 cells were examined with varying concentrations and times of exposure to dexamethasone and its antagonist RU486. We found that the dexamethasone effect on gene expression and apoptosis was concentration dependent. Low concentration of GCs (0.01 $\mu$ M) increased the level of CRTh2 mRNA and surface expression after 24 hours, while reducing the expression of the type 2 cytokine IL-13. Th2 cells exposed to high concentration GC (1 $\mu$ M) for 48 hours triggered apoptosis, but this was not inhibited by PGD<sub>2</sub>. We also examined GC regulation of mRNA transcripts in response to T cell activation, which has been shown to reduce GC mediated transcription and apoptosis [134, 159]. We found that IL-13 expression was reduced (30%) with dexamethasone in activated CCRF-CEM cells and to a greater extent in primary cells (80%). These data suggest that activation may dampen Th2 cell response to GCs. Overall the effect of GCs on Th2 cells proves to be complex and to depend on dose, time of exposure and state of activation. This study provides further insight into the potential mechanisms of GC regulation of Th2 cells. Insufficient dose of inhaled GC *in vivo* may result in higher expression of CRTh2, leading not only to ineffective anti-inflammatory action but enhancing the pro-inflammatory potential of Th2 cells.

#### 4.1 Glucocorticosteroids Regulate Th2 cell expression of CRTh2

GCs regulate transcription by binding GREs [150]. Whether GCs mediate up-regulation or down-regulation of gene transcription is dependent on dose, time of exposure and cell type [113, 166]. Despite its use as an anti-inflammatory drug GCs have been shown to increase transcription of pro-inflammatory genes [119, 175].



We examined the effect of the GC dexamethasone on CCRF-CEM cells at various time points (24-48 hours) and concentration (0.01-1 $\mu$ M). We found that a concentration of dexamethasone of 0.01 $\mu$ M and higher was associated with more CRTh2 mRNA transcripts after 24 hours (2-fold). This upregulation of CRTh2 transcripts was not observed with hydrocortisone, which is a less potent GR agonist than dexamethasone [166]. Further replicates and/or higher concentrations of hydrocortisone may have been necessary to see significant changes in CRTh2 mRNA. The level of CRTh2 mRNA transcript plateaued with the lowest dose of dexamethasone (0.01 $\mu$ M), which was the minimum concentration needed to induce FKBP5, a gene known to be regulated at the transcriptional level by GCs [114]. Interestingly, after 24 hours FKBP5 showed a dose response with a maximum increase (8-fold). This suggests that the GR regulation of CRTh2 and FKBP5 differ. The plateau of CRTh2 mRNA levels at low concentration is similar to that seen with transcriptional regulation of Period circadian protein homolog (PER) [113]. The GR antagonist RU486 inhibited this change in CRTh2 mRNA, which indicates GC mediated transcriptional regulation. Our lab has identified potential GREs on the CRTh2 locus using ENCODE data, in DNase hypersensitive sites that were uniquely present in the Th2 cells (Figure 4) [115]. Thus, direct GR regulation of CRTh2 is possible. This low concentration GC response of CRTh2 mRNA may indicate that another transcription factor interacts with the GR and is the transcription limiting protein. For example, the GR has been shown to interact with ER $\alpha$  and AP-1 [176]. The potential for GR and ER $\alpha$  interaction to regulate CRTh2 could be interesting and provide insight into the disproportionate representation of severe asthma among females [2].

Using flow cytometry, surface expression of CRTh2 was assessed and typically showed ~20-40% positivity. Interestingly, in CCRF-CEM cells surface expression in response to dexamethasone treatment did not reliably reflect the mRNA changes. This discrepancy may be associated with features particular to CCRF-CEM cells, an immortalized cancer T cell line. This was surprising, since the abundance of CRTh2 mRNA in these cells was quite high (Ct ~26). This could be due to the fact that this line grows particularly fast, doubling every 24 hours, and may therefore be constantly shuttling CRTh2 to and from the surface. Alternatively, it is possible the CCRF-CEM cells may not have a robust representation of the machinery needed for membrane transport of CRTh2. For example, mast cells have been

shown to have intracellular expression of CRTh2 but little on the cell surface, presumably due to the lack of machinery [83]. CCRF-CEM cells may be like mast cells and lack sufficient amount of the proteins needed to transport CRTh2. Further work such as performing western blot analysis for total abundance of CRTh2 protein and/or intracellular CRTh2 staining should be done and could help us understand this discrepancy,

To address the limitation in CCRF-CEM cells we chose to examine dexamethasone effects using more physiologically relevant *in vitro* differentiated Th2 cells. To determine whether the CRTh2 mRNA levels are reflected at the surface, we repeated these dexamethasone treatments using primary Th2 cells. I found that after 24 hours, 0.01-1 $\mu$ M dexamethasone treatment increased CRTh2 mRNA transcript levels in primary Th2 cells, to greater extent (4-fold) than CCRF-CEM cells (2-fold). The percentage of cells positive for CRTh2 and abundance of CRTh2 per cell was increased in response to 0.01 $\mu$ M treatment but not at higher concentrations. Interestingly, this was the lowest concentration of dexamethasone and yet was the only dose that had an effect on CRTh2 surface expression. Determining total CRTh2 protein with techniques such as a Western blot may indicate that total CRTh2 changes reflect mRNA transcript changes. Since proteins involved in CRTh2 membrane transport have not been identified the effect GCs have on CRTh2 transport is unclear, but it is plausible that at high doses GCs may affect expression or activity of these proteins.

The ability of GCs to increase CRTh2 transcription is novel and provides new insight into how GCs may regulate Th2 cells. However, these results are contradictory to the anti-inflammatory effects of GCs, particularly the ability of GCs to inhibit type 2 cytokine production [121, 123]. Our finding of increased CRTh2, but inhibition of IL-13, could mean that at low concentration GC can mediate suppression of Th2 inflammation, but maintenance of Th2 cells. Specifically, with patients that have high level of Th2 cells in their airways or blood the low dose GC treatment could worsen the disease over time. If so, this could have significant clinical implications since the GC available *in vivo* is dependent on multiple factors such as amount prescribed, compliance as well as the individual's GC metabolism. Therefore, getting the dose within the therapeutic range for suppression of inflammation is important, as lower amounts could contribute to GC-mediated persistence of Th2 cells.

## 4.2 GCs Induce Apoptosis in Th2 cells

One of the major functions of GCs is the ability to induce apoptosis of T cells [177]. GCs mediate apoptosis by reducing the anti-apoptotic proteins BCL-2 and BCL-xL and activating caspases [141]. The ability of GCs to induce apoptosis is dependent on dose and exposure time, with different cell types varying in sensitivity [144]. To assess the ability of Th2 cells to regulate GC-induced apoptosis we needed to determine the dose and exposure time of GC needed to induce apoptosis.

To determine this, we treated CCRF-CEM cells with dexamethasone and identified that 48 hours (but not 24 hours) of high dose dexamethasone (1 $\mu$ M) treatment induced apoptosis. It is interesting to note that 1 $\mu$ M concentration also resulted in elevated CRTh2 mRNA transcript levels, suggesting that induction of apoptosis likely outweighs any potential pro-inflammatory effect. However, dexamethasone concentrations up to 1 $\mu$ M exposed for 48 hours were not able to induce apoptosis in primary Th2 cells. The GC resistance exhibited by primary Th2 cells were a result of the 5ng/mL of IL-2 used to culture the cells. Similar to PGD<sub>2</sub>-CRTh2 activation, IL-2 activates the AKT pathway which inhibits caspase-3 mediated apoptosis [171]. Previous studies by Xue *et al.* indicated that in the absence of IL-2, Th2 cells undergo apoptosis [98]. Therefore, in order to assess apoptosis in primary Th2 cells we had to first determine the amount of IL-2 that will sustain the cells but will permit a GC response. We found 1.25ng of IL-2 permitted Th2 cells to become responsive to GC-induced apoptosis. At higher doses of GCs the apoptotic ability of GR may outweigh the level of IL-2 present, again going back to the importance of sufficient amounts of GCs. This is relevant clinically because cells from severe asthmatics have been shown to produce high levels of IL-2 and have more Th2 cells relative to mild/moderate asthmatics [3, 30]. Thus, if severe asthmatic patients have more IL-2 this may reduce the Th2 cells response to GC.

## 4.3 PGD<sub>2</sub> Does Not Rescue Th2 cells From Glucocorticosteroid Induced Apoptosis

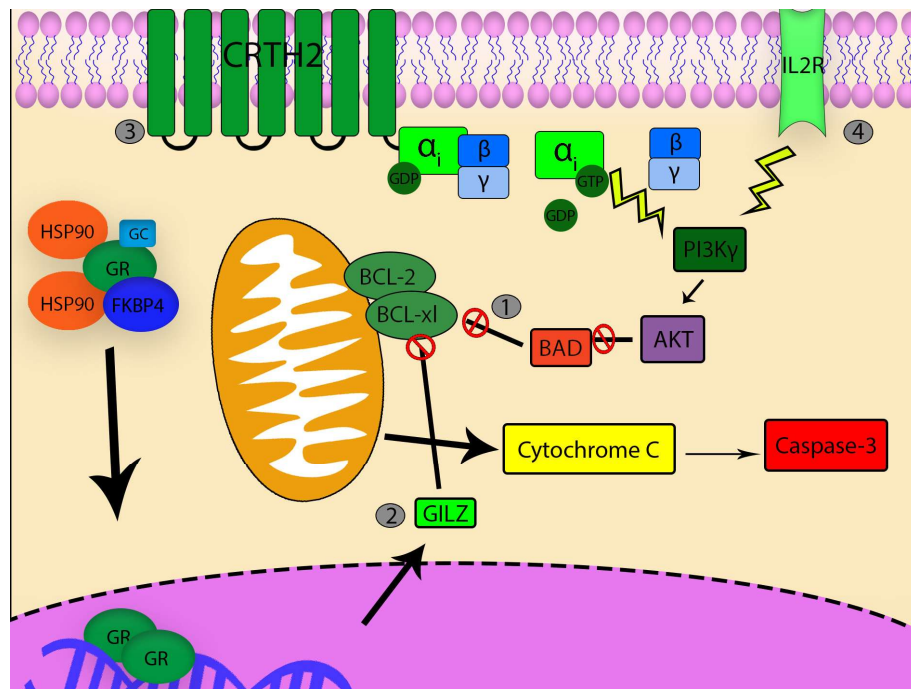
PGD<sub>2</sub> binding to CRTh2 has been shown to inhibit apoptosis induced by IL-2 starvation [98]. CRTh2 activates PI3K signaling pathway which leads to the inhibition of BAD and prevents BAD from interfering with anti-apoptotic BCL proteins (Figure 21) [98], while GCs can

downregulate BCL proteins to induce apoptosis. Therefore, these two pathways overlap and compete [142]. This is significant because PGD<sub>2</sub> levels correlate with asthma severity, indicating patients with the highest level of Th2 cells have the highest levels of PGD<sub>2</sub> [4]. If PGD<sub>2</sub> can inhibit GC-induced apoptosis, then this may be a reason for Th2 cells to be sustained in circulation.

To test this, we pre-treated CCRF-CEM cells with DK-PGD<sub>2</sub> for 30 minutes in the presence and absence of 1 $\mu$ M dexamethasone. However, we found that DK-PGD<sub>2</sub> did not inhibit GC-induced apoptosis. We considered whether this was related to the CCRF-CEM cell line and whether these cells were unable to respond to PGD<sub>2</sub> stimulation. To test this we assessed the ability of DK-PGD<sub>2</sub> to drive type 2 cytokine expression [99]. Preliminary data from the lab shows that CCRF-CEM cells did not have detectable levels of IL-13 mRNA transcripts following PGD<sub>2</sub> treatment (3, 6, 8 12, and 24 hours). CRTh2 is a GPCR, thus when activated results in calcium mobilization [95]. However, preliminary data of calcium flux, showed the absence of calcium mobilization following stimulation of CCRF-CEM cells with DK-PGD<sub>2</sub>. We also used the more potent agonist 15-R-Methyl PGD<sub>2</sub> and found similar results. The reason for our inability to activate CRTh2 signaling is difficult to pinpoint and again may be related to using a leukemia cell line.

To determine whether the lack of response from DK-PGD<sub>2</sub> was a limitation of the cell line, we examined the effect of DK-PGD<sub>2</sub> with Th2 cells cultured with 0 or 1.25 ng/mL of IL-2. We found that the percentage of Annexin V<sup>+</sup> cells was not reduced with DK-PGD<sub>2</sub> pre-treatment. However, there are many factors to consider with these experiments and we are still not confident in this negative result. Reducing the concentration of IL-2 renders Th2 cells more responsive to GC-induced apoptosis, which may alter the optimal time to detect apoptotic effects. Indeed, after only 24 hours we observed a high proportion of apoptotic cells (40%) with 0ng of IL-2 (data not shown). Therefore, future experiments could reassess the ability of PGD<sub>2</sub> to inhibit apoptosis at earlier time points. Secondly, the fact that IL-2 and PGD<sub>2</sub> are both survival factors activating the PI3K/AKT pathway suggests that perhaps the lack of PGD<sub>2</sub> effect may be related to growth factors in the media, though experiments completed with 0, 0.5 and 5% serum, showed no difference in the ability of DK-PGD<sub>2</sub> to reduce apoptosis (preliminary data). Finally, it is important to recognize that Th2 cells express HPGDS, the enzyme that produces PGD<sub>2</sub> [4]. For this reason, we considered the

possibility that endogenous PGD<sub>2</sub> production was reducing the ability of Th2 cells to respond to exogenous PGD<sub>2</sub>. To test this, we performed a set of experiments where we cultured the cells in cyclooxygenase inhibitor, to inhibit PGD<sub>2</sub> production (preliminary data). However, this too failed to alter the ability of PGD<sub>2</sub> to inhibit GC-induced apoptosis or induce IL-13 expression (data not shown). Another possibility is that these cells have upregulated proteins that inhibit CRTh2 signaling. For instance,  $\beta$ -arrestin has been shown to bind to the c-terminal end of CRTh2 and inhibit its activity [178]. Ultimately, western blot co-immunoprecipitation experiments are needed to assess CRTh2- $\beta$ -arrestin interaction to confirm this possibility. If they do interact then we could utilize siRNA to knockdown  $\beta$ -arrestin expression to confirm this recovers PGD<sub>2</sub>-CRTh2 signaling and then re-assess the effect PGD<sub>2</sub> on GC-induced apoptosis.



**Figure 19: Regulation of Apoptosis in Th2 cells**

Th2 cell regulation of apoptosis involves different factors but all converge to anti-apoptotic BCL family proteins. Apoptosis induced by IL-2 starvation allows BAD to inhibit BCL proteins allowing for cytochrome C release from the mitochondria (1). GCs upregulates expression of GILZ which downregulates BCL proteins to induce apoptosis (2). Apoptosis can be inhibited by activation of CRTh2 (3) and IL-2 (4) through PI3K signaling which inhibits the BAD protein.

## 4.4 T Cell Activation Inhibits Glucocorticosteroid Signaling

The effect of GCs on Th2 cells is important in both the resting and activation state. Th2 cells *in vivo* are activated by dendritic cells to elicit an inflammatory response to an allergen [44]. CD3 and CD28 engagement activates pro-inflammatory transcription factors such as NFAT, AP-1 and NF $\kappa$ B [158, 172]. Signaling pathways activated in T cells and pathways activated by GCs overlap and thus may interfere with each other. For instance, GR and AP-1 have been shown to demonstrate reciprocal inhibition [133].

Therefore, to determine the effect of GCs on activated T cells, CCRF-CEM cells were treated with dexamethasone in the presence or absence of PMA and Ionomycin, chemicals that activate PKC and flux calcium, and therefore mimic CD3 and CD28 cross linking. We had seen that in primary Th2 cells GCs were able to reduce IL-13 mRNA transcript levels. CCRF-CEM cells do not have basal expression of IL-13 thus they had to be activated in order to produce cytokines. We wanted to assess whether T cell activation would inhibit the GR from reducing IL-13 mRNA levels. We found that after dexamethasone treatment IL-13 mRNA levels were significantly reduced in activated T cells. However, there was only 30% reduction of IL-13 mRNA in activated cells compared to the 80% reduction seen in resting primary Th2 cells.

To assess whether the blunted regulation of IL-13 by GCs was a result of reduced GR activity we treated CCRF-CEM cells with dexamethasone along with PMA and ionomycin. As expected, we found that FKBP5 mRNA transcript levels were increased in response to dexamethasone, but the effect was significantly less in activated cells. This phenomenon was similar with GR transcript levels, which were higher in resting cells in response to dexamethasone treatment compared to activated cells. These data suggest that activated Th2 cells are less responsive to GC, due to downregulation of GR signaling genes. This is significant because regulation of FKBP5 and GR are essential for maintaining GR function. Reduction of FKBP5 and GR mRNA indicate that there could be a reduction in GR effect. This makes the IL-13 results more significant because even with reduced activity, the GR was still able to inhibit IL-13 mRNA expression. If GR activity is reduced we wanted to assess whether this would affect the dexamethasone-mediated upregulation of CRTh2. However we found that the downregulation of by T cell activation, could not be reversed by

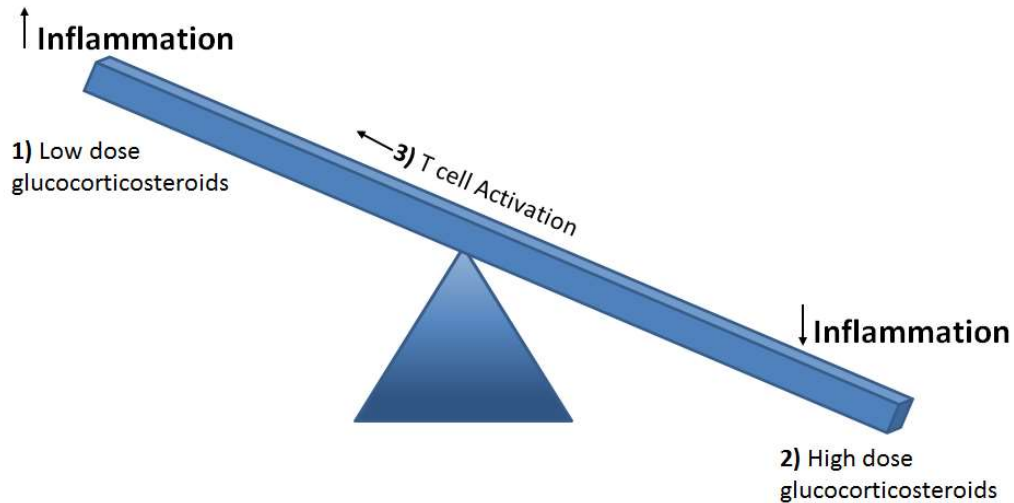
dexamethasone treatment [80]. However, whether this is due to effects on CRTh2 transcription rather than reduced GR activity, is possible. Work in our lab has identified that NFAT binds to the CRTh2 locus upon activation to downregulate mRNA. NFAT-AP-1 sites have been shown to overlap with GREs, thus preventing the GR from regulating transcription (Maclean, unpublished).

Previous research has identified that GR signaling can interfere with factors up-regulated in T cell activation and vice versa [53, 172]. The level of T cell activation versus GR activation becomes important when trying to understand two competing pathways. With FKBP5 and GR levels reduced during T cell activation this indicates that the level of activation outweighs GR signaling. This again becomes evident with the inability of GR to regulate CRTh2 expression as previously described. Potentially, this points to the fact that GR resistance may occur from constant T cell activation which is a feature of chronic allergen exposure. Thus, low dose GCs in patients may not be able to surmount the level of allergen induced T cell activation, thereby rendering GCs ineffective.

## 4.5 Conclusion

The data demonstrate that the effect of GCs on Th2 cells is largely dose dependent. Whether cells are at rest or activated, to promote anti-inflammatory effects of the GR a competitive dose of GCs is necessary. Clinically, this may translate to prescribing more GCs since insufficient doses of GCs enhance the potential for Th2 mediated inflammation. Side effects from GCs are a concern, as long-term treatment can lead to developing cataracts, osteoporosis and adrenal insufficiency. Interestingly, this work suggests that there may be side effects from taking low doses that suppress cytokines but are insufficient to induce Th2 cell apoptosis. Insufficient dose of GCs may allow Th2 cells to survive and thrive due to CRTh2 upregulation. The balance between GCs and Th2 inflammation forces us to rethink asthma therapy strategies based on patient characteristics (Figure 20). One approach of asthma therapy could be to measure the drop in Th2 cells as they undergo GC induced apoptosis. This approach should be coupled with improvements in GC selectivity and potency due to the side effects many patients experience when taking high doses of the currently available GCs. Collectively, this study highlights the importance of further work to

develop methods for determining a personalized approach for achieving maximum anti-inflammatory effects.



**Figure 20: The effect of glucocorticosteroids is dose dependent**

Our data suggest that the ability of GCs to promote or reduce inflammation is dependent on dose of GCs. 1) The ability of GCs to increase CRTh2 expression and reduce IL-13 shows that GCs can inhibit immediate inflammatory effects while enhancing Th2 inflammatory potential. 2) High dose GCs induces apoptosis in Th2 cells and thereby reduce Th2 mediated inflammation. 3) Activation of T cells inhibits GC response and thereby shifts the balance towards increased inflammation.

## 4.6 Future Direction

GCs function is dependent on dose, which means the strength of GCs is important in determining the effect. To validate these findings, it would be interesting to compare GCs of different potencies with their ability to regulate transcription and apoptosis. This will be directly applicable clinically as although there are guidelines for prescribing doses of GCs, there are no current guidelines for which inhaled GCs to prescribe.

For this study, we focused on CRTh2 surface expression rather than total protein expression because surface CRTh2 is the functional protein. One of the downfalls is that because we assessed surface expression we are still unclear whether mRNA changes at concentrations



above  $0.01\mu\text{M}$  are reflected by total CRTh2 protein. Determining total protein will also help identify whether the limiting factor is the ability of cells to transport CRTh2 to the surface.

One major limitation of this study is the lack of functional readout for the increase in CRTh2 at low dose GCs. This increase could have implications in responses following allergen exposure leading to enhanced cytokines, chemotaxis and/or Th2 cell survival. Pre-treating Th2 cells with low dose GCs prior to PGD<sub>2</sub> treatment may provide evidence of the deleterious effect of low dose GC.

CRTh2 activation is considered to play a significant role in asthma pathogenesis. The obvious thought would be to inhibit PGD<sub>2</sub> production to reduce inflammation. It may not be that simple since there is an asthma phenotype called aspirin sensitive asthma that is mediated primarily by leukotrienes [179]. Since arachidonic acid is a precursor for both PGD<sub>2</sub> and leukotrienes [180], when aspirin inhibits the COX enzymes the production of PGD<sub>2</sub> is reduced leading to increased production of leukotrienes [180]. Therefore, inhibition of PGD<sub>2</sub> production may be an effective approach for reducing inflammation, but would have to be tested in patients that do not exhibit aspirin sensitivity.

This study focuses on the balance between GC signaling and Th2 inflammation. Although severity of asthma has been correlated with PGD<sub>2</sub> and percentage of Th2 cells, this does not address why severe asthmatics have elevated inflammation in the first place. This could be related to GC effect on Th2 cell differentiation. We have seen that GCs promote differentiation of T<sub>regs</sub>, whether this holds true for Th2 cells remains to be seen. Previous research has shown that CD4<sup>+</sup> T cells pretreated with GCs under differentiating conditions enhances type 2 cytokine release. Thus, although we have shown GCs can reduce type 2 cytokines, GC treatment prior to the production of cytokines may help prime the cells for an enhanced cytokine release. This could further provide evidence of the effect of low dose GCs in not only sustaining but developing Th2 cells.

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## Curriculum Vitae

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### Peer Reviewed Publications:

Frank W. Schmitges, Ernest Radovani, Hamed S. Najafabadi, Marjan Barazandeh, Laura F. Campitelli, Guoqing Zhong, Hongbo Guo, **Tharsan Kanagalingam**, Wei F. Dai, Andrew Emili, Jack F. Greenblatt, and Timothy R. Hughes. 2016. "Multiparameter functional diversity of human C2H2 zinc finger proteins." *Genome Res.* Dec ;26(12): 1742-1752.

### Regional Abstract Publications:

**Tharsan Kanagalingam**, Zahra Naghdi, Asma Yaghi, Teah Jazey, Lisa Cameron. "High Dose Glucocorticosteroids Induce Apoptosis in Th2 Cells." Pathology Research Day, Western University, London, Ontario (*March 30, 2017*)

**Tharsan Kanagalingam**, Zahra Naghdi, Asma Yaghi, Teah Jazey, Lisa Cameron. "The Differential Effect of Glucocorticosteroids on Th2 cells." London Health Research Day, Western University, London, Ontario (*March 22, 2017*)

**Tharsan Kanagalingam**, Zahra Naghdi, Asma Yaghi, Teah Jazey, Lisa Cameron. "The Effect of Glucocorticosteroids on Th2 cells." Pathology Research Day, Western University, London, Ontario (*April 7, 2016*)

**Tharsan Kanagalingam**, Zahra Naghdi, Asma Yaghi, Lisa Cameron. "Glucocorticosteroids Increase Expression of CRTH2 and sustain inflammatory function in Th2 Cells." Infection and Immunity Research Forum, Western University, London, Ontario (*Sep 23, 2016*)

**Tharsan Kanagalingam**, Zahra Naghdi, Asma Yaghi, Mary Lu, Sindy Ha, Lisa Cameron. "Glucocorticosteroids Increase Expression of CRTH2 by Th2 Cells." Infection and Immunity Research Forum, University of Western Ontario, London, Ontario (*Nov 6, 2015*)

**Kanagalingam T**, Cameron L. "Determining the Effect of Glucocorticosteroid Treatment on CRTh2 Expression in Th2 cells." Pathology and Laboratory Medicine Research Day, University of Western Ontario, London, Ontario (*March 30, 2015*)