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The Influence Of Estrogen And Glucocorticoid On Th2 Cell Survival And Transcriptional Activation Of CRTh2

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Abstract

Women are more likely to have severe asthma than men. Recent data suggest this could be due to women having more circulating $CD4^{+}CRTh2^{+}$ T cells (Th2 cells). Glucocorticosteroids (GC)s are the main therapy for asthma as they inhibit cytokine production and eliminate inflammatory cells by apoptosis. The current study examined whether the female sex hormone estrogen influences the anti-inflammatory action of GC. Experiments show that ERα agonist reduced GC-induced apoptosis of primary Th2 cells and enhanced GC-mediated transcriptional activation of the proximal CRTh2 promoter. Genetic variation within CRTh2 is associated with asthma and allergic phenotypes. Using constructs representing the single nucleotide polymorphism *CRTh2*-6388G>A, ERα agonist enhanced GC-induced activity of the A but not the G allele. Collectively, these data suggest that *in vivo* Th2 cells may exhibit sex and genotype specific response to GC and that some of these effects are pro-inflammatory.

Keywords

Asthma, CRTh2, Estrogen signaling, Genetic variation, Glucocorticosteroid, Th2 inflammation

Summary for Lay Audience

Throughout the world, asthma places a large financial burden on the health care system. Genetic and environmental factors both play a role in the development of this disorder. Glucocorticosteroids (GC)s are the main treatment for asthma because of their anti-inflammatory action. Despite being effective in most patients, in severe asthmatics GC therapy fails to control asthma symptoms. Less understood is that GCs can also promote expression of some immune genes such as CRTh2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), which supports the survival of Th2 cells. Women are more likely to be diagnosed with severe asthma and were recently shown to have more circulating Th2 cells than men. This may be due to estrogen influencing Th2 differentiation or the effect of GC on Th2 cells. On this basis, it was hypothesized that estrogen *reduces* the ability of GC to trigger Th2 cell death, that this occurs by *enhancing* GC-mediated increase in CRTh2 expressing cells, and that the outcome of this cross-talk is altered by genetic variation in CRTh2. Results revealed that the ability of GC to induce Th2 cell death was dampened by co-treatment was estrogen. Though the mechanism of enhanced survival was not confirmed to be due to increased expression of CRTh2, results do show that GC activated CRTh2 transcription and this effect was enhanced by co-treatment with an estrogen receptor agonist. Analysis of the CRTh2 gene variant *CRTh2*-6388G>A showed that estrogen enhanced the ability of GC to induce transcriptional activation of the *CRTh2*-6388A allele. This study suggests that combined exposure to GC and estrogen, an environment found in women taking GC for their asthma, increases Th2 cell survival. If this occurs *in vivo*, this cross-talk may explain why women have more Th2 cells and asthma severity than men. Transcriptional activation of CRTh2 was induced by GC and estrogen in a genotype specific manner. Ultimately, these results suggest that precision medicine for asthma therapy should consider both sex and CRTh2 genetics.

Dedication

To Sophie,

for the everlasting love and support

To my family,

for always believing in me, even when I did not believe in myself

Co-Authorship Statement

Jenna Fortunato primarily performed all experiments and analyses presented in this thesis. The primary Th2 cell line used was previously generated and cryopreserved by Emily MacLean Scott. Meerah Vijeyakumaran conducted the initial Annexin V^+/7-AAD^- apoptosis assays (n=3, Figure 15 published in [1]). These raw data were combined with an additional 8 experiments conducted by Jenna Fortunato to generate Figure 3.2A/B of this dissertation. *CRTh2* promoter constructs (*CRTh2*pro-4513/Luc, *CRTh2*pro-6388G/Luc, *CRTh2*pro-6388A/Luc) were generated by a former member of the Cameron laboratory, Courtney Davidson. The data in Appendix 1.1, 1.2 and 4.1 was collected and analyzed by Dr. Lisa Cameron and Dr. Nami Shrestha Palikhe, and Appendix 1.3 data was generated by Meerah Vijeyakumaran and Dr. Lauren Solomon. These data are currently being prepared for publication (CV #1).

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
ANOVA	Analysis of variance
Ap	Activator protein
APAF	Apoptotic protease-activating factor
APC	Antigen presenting cell
AR	Androgen receptor
ARE	Adenylate-uridylate rich elements
ASMase	Acidic sphingomyelinase
ATP	Adenosine triphosphate
ATS	American Thoracic Society
AU	Adenylate-uridylate
BAD	B-cell lymphoma 2 associated death promoter
BAK	B-cell lymphoma homologous antagonist killer
BAL	Bronchoalveolar lavage
BAX	B-cell lymphoma 2 associated X protein
BCA	Bicinchoninic acid protein assay
Bcl-2	B-cell lymphoma 2

Bcl-xL	B-cell lymphoma-extra large
BH3	B-cell lymphoma 2 homology 3
BID	BH3 interacting-domain death agonist
BIM	B-cell lymphoma-2-like protein 11
Bp	Base pairs
Ca^{2+}	Calcium
Caspase	Cysteine-aspartic protease
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
ChIP-seq	Chromatin immunoprecipitation sequencing
cIAP	Cellular inhibitor of apoptosis
CO_2	Carbon dioxide
CRTh2	Chemoattractant receptor-homologous molecule expressed on T helper 2 cells
C_t	Cycle threshold
CXCR	C-X-C chemokine receptor
Cyt *c*	Cytochrome complex
DEX	Dexamethasone
DHS	DNase hypersensitive
DNA	Deoxyribonucleic acid
DHT	Dihydrotestosterone

DP	D-prostanoid
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ERS	European Respiratory Society
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FC	Fragment crystallizable
FEV_1	Forced expiratory volume in one second
FKBP5	FK506 binding protein-5
FLICA	Fluorochrome-labeled inhibitors of caspases
FOX	Forkhead box
GC	Glucocorticosteroid
GILZ	Glucocorticoid-induced leucine zipper
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GPER	G protein-coupled estrogen receptor
GR	Glucocorticosteroid receptor
GRE	Glucocorticosteroid response element
HDM	House dust mite
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC2	Group 2 innate lymphoid cells
iTreg	Induced regulatory T cell
LABA	Long-acting beta-agonist
LB	Lysogeny broth
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MKP-1	Mitogen-activated protein kinase phosphatase-1
mRNA	Messenger ribonucleic acid
MURF1	Muscle RING-finger protein-1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT	Nuclear factor of activated T-cells
nGRE	Negative glucocorticosteroid response element
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PARP	Poly (adenosine diphosphate-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PGD_2	Prostaglandin D_2
PGH	Prostaglandin endoperoxide
PI	Propidium Iodide
PI-PLC	Phosphoinositide-specific phospholipase C
PI3K	Phosphoinositide 3-kinase
PP5	Protein phosphatase 5
PPT	Propylpryazole-triol
pRL-TK	Thymidine kinase promoter-Renilla luciferase reporter plasmid
qRT-PCR	Quantitative real-time polymerase chain reaction
rATG	Relative to the transcription start site
rh	Recombinant human
RLU	Relative luciferase units
RNA	Ribonucleic acid

rs	Reference SNP
SABA	Short-acting beta agonist
SARS	Severe acute respiratory syndrome
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
Sp	Specificity protein
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor
TCR	T-cell receptor
TF	Transcription factor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAILS	Tracking Adolescents' Individual Lives Survey
TSLP	Thymic stromal lymphopoietin
UTR	Untranslated region
VDR	Vitamin D receptor

VitD	Vitamin D
WT	Wild type

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Chapter 1

1 Introduction

Sex differences have been reported in various autoimmune and infectious diseases, as well as inflammatory stress responses. Interestingly, it has been estimated that 78% of autoimmune patients are women [2, 3]. Additionally, the distribution of the autoimmune diseases Hashimoto's thyroiditis, Graves' disease, systemic lupus erythematosus, and Sjogren's syndrome is over 80% female [4]. The progression from the human immunodeficiency virus (HIV)-1 to acquired immunodeficiency syndrome (AIDS) has been shown to be faster in women than men despite equivalent blood viral load, an effect mediated by continuous immune activation [5].

There are also sex differences in the prevalence and severity of asthma [6], though the pathobiology is poorly understood. Interestingly, our laboratory has observed that women with severe asthma have significantly higher levels of pro-inflammatory $CD4^+$ T cells expressing CRTh2 (Th2 cells) than patients with mild or moderate asthma and severe asthmatic men (App. 1.1). Despite the use of glucocorticosteroids (GC) to alleviate the underlying inflammation causing asthma symptoms, many women are resistant to this treatment [7]. This may be due to altered responsiveness of their Th2 cells to the effects of GC since a positive association between the total daily dose of inhaled corticosteroids and the proportion of circulating Th2 cells was found in women but not men (App. 1.2). Activation of CRTh2 inhibits Th2 cell apoptosis [8] and so factors that increase its expression may enhance Th2 cell survival. To continue this line of investigation, the current study examined if estrogen could reduce GC-mediated apoptosis by increasing expression of CRTh2. Additionally, whether a CRTh2 genetic variant associated with asthma severity and increased CRTh2 influences this regulatory pathway was examined.

1.1 Development of Allergic Asthma

Asthma is a chronic inflammatory disease that affects 300 million people worldwide; it is thought to be one of the most common chronic diseases [9]. Understanding the origin of asthma is an active area of research, and crucial for understanding my study. Interactions between genes and environmental factors also mediate asthma susceptibility [10, 11]. Gene polymorphisms, such as that of a disintegrin and metalloproteinase 33 (*ADAM33*), have been associated with asthma pathogenesis [12]. Individuals have a 25% or 50% likelihood of developing asthma if one or both parents, respectively, have asthma [13]. Additionally, monozygotic twins have a higher risk compared to dizygotic twins of having asthma if one twin is affected, highlighting the important role of genetics in development of allergic asthma [14]. However, the development in monozygotic twins is only 75%, indicating that environmental factors indeed interact [13]. Certain genes associated with risk of developing allergic disease are only expressed upon certain environment exposures; filaggrin mutations related to eczema development are enhanced by cat exposure in children [15].

Asthma is characterized by bronchial constriction, mucus production and airway obstruction. Clinically, patients present with symptoms of wheezing, chest tightness, shortness of breath and coughing, all of which vary in intensity and severity [16]. These indications require costly acute care and have potential to cause death [17]. The development and expression of asthma are influenced by host factors, such as sex, genetic variation and obesity, as well as the environment, allergens, pollutants, viral infection, tobacco smoke, stress and exercise [18].

Asthma can be allergic or non-allergic contingent on the presence of allergic sensitization [19]. Allergic asthma often begins in childhood and is characterized by eosinophilic inflammation and an increase in T helper (Th) 2 cells and Th2 cytokine responses [20-22]. Conversely, non-allergic asthma is often characterized by neutrophilic inflammation [23], Th1 cells and Th1 responses [24]. Understanding the mechanisms underlying development of these different immune responses and their influence on asthma phenotype is an active area of research and relevant to this thesis.

1.1.1 Innate vs Adaptive Immunity

Asthma arises as a combined result of inhaling foreign particles from the environment (allergen, pollution, virus) and the individual's response to this exposure. The immune system uses both innate and adaptive immunity to combat against foreign entities. The innate component is the first line of defense to mount a response to pathogens, using pattern recognition receptors to initiate rapid inflammatory responses; dysfunction in airway epithelial cells may play a role in asthma [25, 26]. In contrast, the adaptive immune response is highly specific towards the directed antigen. Epithelial cells within the mucosal lining interact with dendritic cells to activate T lymphocytes and trigger the development of specialized subsets of memory T cells [26]. T cells in turn interact with B cells to mediate development of antigen-specific immunoglobulin (Ig) responses. Although innate immunity is important, the focus of my study is on T cells and their potential role on persistence of asthma.

1.1.2 Antigen Presentation

Antigen presenting cells (APC), such as dendritic cells, macrophages, and B cells, recognize, process and present antigens to T lymphocytes. This is an initial process in adaptive immunity of asthma. Dendritic cells are professional APC and are responsible for sensitization and stimulation of naïve T cells [27]. Macrophages, the most abundant leukocyte in the conducting airways [28], have also been shown to be antigen-presenting [29], but function in a variety of other capacities, such as in innate defense mechanisms and facilitating T-lymphocyte proliferation [30]. Dendritic cells use phagocytosis or receptor mediated endocytosis to take up the allergen (antigen) [31] and exposure to pathogen-associated molecular pattern (PAMP)s activates their maturation. This involves upregulation of cluster of differentiation (CD) 80 and CD86 [32] and C-C chemokine receptor type 7 (CCR7) [33], which induce the dendritic cells to migrate to lymph nodes [34]. Maturation of dendritic cells can also be promoted by interleukin (IL) -33, thymic stromal lymphopoietin (TSLP) and IL-25 secreted by the epithelium [35-37]. Following maturation, these dendritic cells will process the allergen into a peptide and present it on

major histocompatibility complexes (MHC) class II molecules to the T-cell receptor (TCR) on naïve $CD4^+$ T cells in the lymph nodes [38, 39]. This binding along with co-stimulation of CD80 and/or CD86 binding to CD28, fully activates the naïve $CD4^+$ T cell [40, 41].

1.1.3 $CD4^+$ T Lymphocytes

$CD4^+$ T lymphocytes have numerous functions including producing cytokines and activating innate immune cells. It is now appreciated that $CD4^+$ T cells can develop into a number of subsets including, but not limited to: induced regulatory T (iTreg) cells, T follicular helper cells (Tfh), Th1, Th2, and Th17 cells. Although the focus of my study is on Th2 cells, the asthmatic response does involve numerous other cells. Differentiation depends on the antigen concentration and cytokine microenvironment: transforming growth factor (TGF)-β and IL-2 for iTregs, IL-6 and IL-21 for Tfh, interferon (IFN)-γ and IL-12 for Th1, IL-2 and IL-4 for Th2 and IL-23 and IL-17 for Th17 cells [41-46].

1.1.4 Th1:Th2 Dichotomy

Naïve $CD4^+$ T cells making direct contact with dendritic cells causes tubulin fibers to deliver antigen specific MHC class II into the centre of the contact synapse [47]. The strength of the TCR cross-linking will direct differentiation; moderate stimulation will lead to Th2 cells, whereas strong stimulation will favor Th1 cell development [48].

Th1 cells are responsible for intracellular microorganism immunity, whereas Th2 cells provide extracellular pathogen immunity [49]. Originally, mature $CD4^+$ T cells were discovered to be made up of two distinct Th populations with unique functions [49]. They were categorized by their ability to produce IL-4, IL-2 and/or IFN-γ; Th2 cells produce IL-4, whereas Th1 cells cannot [50], but unlike Th2 cells, Th1 cells can produce IL-2/IFN-γ [49]. Later it was determined that differentiation into the subtypes was largely dependent on activated transcription factors. T-box transcription factor (T-bet) enhances the production of IFN-γ leading to the differentiation into the Th1 subset, and also suppresses

the development of Th2 and Th17 [51, 52]. Th1 differentiation is also dependent on the transcription factor signal transducer and activator of transcription (STAT)1 and STAT4, transducers of the IFN-γ and IL-12 receptors [53, 54].

In contrast to Th1 cells, Th2 differentiation is mediated by STAT6 and GATA3 [55-57]. Both differentiation pathways use positive feedback mechanisms, and are mutually inhibitory. Th1 cells secrete IFN-γ which further promotes Th1 and inhibits Th2 differentiation [58]. Similarly, Th2 cells secrete IL-4, which inhibits Th1 development but promotes Th2 [58]. Microenvironments that drive Th1 cells primarily include the cytokines IL-2 and IFN-γ, acting to activate macrophages, increasing their microbial activity [59]; whereas, Th2 microenvironments primarily include IL-4, promoting Th2 cell differentiation and mediating IgE switching in B cells [45, 60].

1.1.5 Th2 Cell Differentiation

Th2 cell differentiation is initiated by CD3 and CD28 crosslinking, which induces the downstream signaling cascade that will induce the activation of nuclear factor of activated T cells (NFAT) and activator protein (AP)-1 [61], the production of IL-2 and low levels of IL-4 [62]. IL-4 will then bind to the IL-4R, activating STAT6, which increases GATA3 and leads to higher IL-4 production and Th2 differentiation [55, 63]. TCR activation will also upregulate GATA3; however, the binding of GATA3 to the *Il4/Il13* loci is not enough to induce alone, rather IL-2 activated STAT5 must be present to maintain the accessibility of the *Il4* locus [64, 65]. The combined presence of GATA3 and STAT6 will also induce chromatin remodeling, increasing the accessibility of the IL-4 enhancer and genes that regulate maintenance of the Th2 phenotype [66] such as CRTh2 [67, 68].

1.1.6 Type 2 Inflammation

In addition to allergens, pollutants, such as cigarette smoke, diesel exhaust, and viral exposure can prompt the production of IL-25, IL-33 and TSLP, further inducing IL-13 production, IL-4 and -5 production, and Th2 cell differentiation, respectively [69-72]. Additionally, IL-33 stimulates group 2 innate lymphoid cells (ILC2) to secrete IL-13, promoting dendritic cell migration to the lymph nodes for Th2 differentiation [73, 74]. Therefore, type 2 inflammatory responses can be orchestrated through allergic sensitization or other environmental exposures, carried out by both Th2 and ILC2 cells [75].

Figure 1.1 provides a schematic representation of the type 2 cytokines IL-4, -5, and -13 play and their role in driving asthmatic symptoms. IL-5, produced by Th2 cells [76], increases the production of eosinophils in the bone marrow, as well as heightens airway sensitivity [77, 78]. The cytokine IL-13 plays a large role in asthma, activating eosinophils, increasing mucus secretion, inducing hyper-responsiveness in the airway and causing bronchial constriction [79, 80]. The alpha subunit of the IL-4 receptor (IL-4Rα) is part of both IL-4R and IL-13R complexes, implicating their shared roles [81]. IL-4 induces IgE secretion by inducing B cells to initiate isotype switching to IgE production [82]. IgE can then bind to both low-affinity IgE receptor (fragment crystallizable (Fc)εRI) on B cells and high affinity IgE receptor (FcεRII) on mast cells and basophils; both receptors are upregulated by IL-4 [83]. Upon allergen exposure, mast cells primed with IgE will degranulate, releasing mediators such as histamines and prostaglandins [84, 85]. Both IL-5 and IL-13 are important mediators in driving features of asthma such as tissue eosinophilia, airway remodeling and increased mucous production [80]. Due to the role these cytokines play in asthma, IL-4, -5 and -13 targeted treatments have been recently developed and shown to relieve symptoms associated with asthma [86-88].

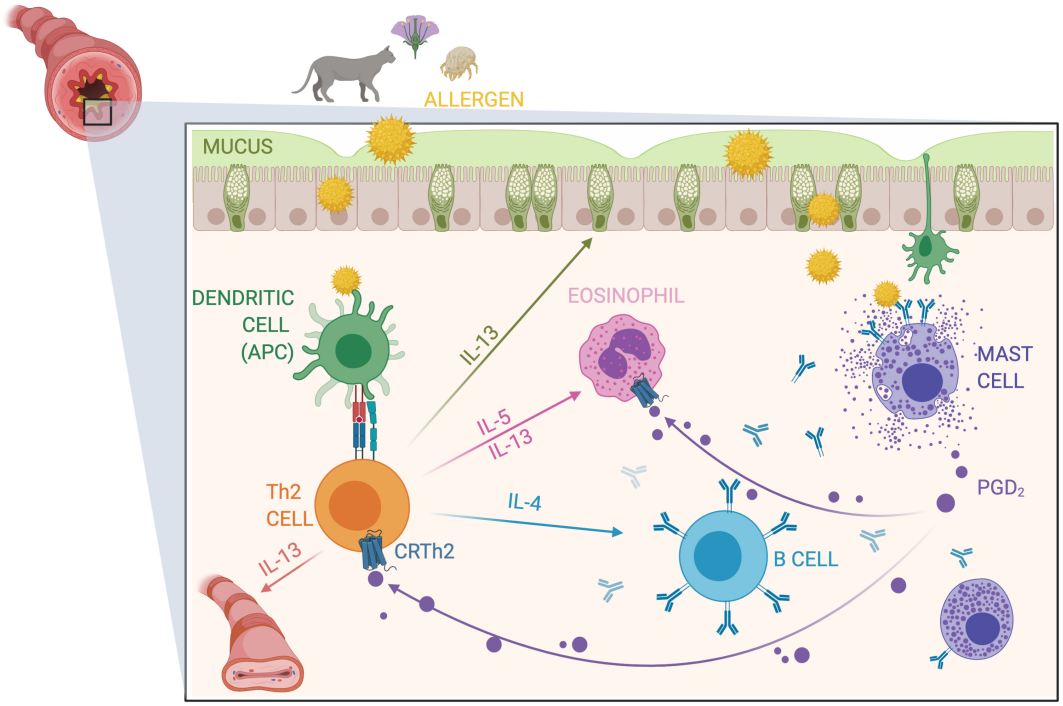

Figure 1.1: Type 2 Inflammatory Response to Allergen

Allergen presentation by dendritic cells to Th2 cells induces production of the type 2 cytokines IL-4, -5, and -13. Type 2 cytokines mediate allergic inflammation. IL-13 induces bronchial constriction, excess mucus production and in combination with IL-5 activates eosinophils. IL-4 induces B-cells to produce IgE that will bind and prime mast cells. Upon allergen exposure, primed mast cells will degranulate and release mediators such as PGD_2 which binds to the receptor CRTh2 expressed on Th2 cells and eosinophils. Abbreviations are as follows: antigen presenting cells, APC; chemoattractant receptor-homologous molecule expressed on Th2 cell, CRTh2; interleukin, IL; prostaglandin D_2, PGD_2; T helper cell type 2, Th2. Created with *BioRender.com*.

1.2 Regulation and Function of Chemoattractant Receptor-Homologous Molecule Expressed on Th2 Cells

In 1999, Nagata and colleagues performed a subtractive hybridization screen for differentially expressed genes between Th1 and Th2 cells. They found a G protein-coupled receptor expressed by Th2 cells, but not Th1 cells [89]. They named this gene chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2), due to its homology to other receptors with chemotactic activity [89, 90]. Since this discovery CRTh2 has also been shown to be expressed by eosinophils, basophils, ILC2 and mast cells [91-94]. However, expression of CRTh2 within the $CD4^+$ T cell subset is considered the most reliable marker of Th2 cells [95]. GATA3, increased through the IL-4 dependent STAT6 induction, increases *CRTh2* promoter activity [96], however, little else is known regarding the transcriptional regulation of this important Th2 gene.

1.2.1 CRTh2 and Allergic Disease

$CD4^+$ T cells expressing CRTh2 are considered Th2 cells and studies show they are increased in individuals with atopic dermatitis as well as allergic asthma [97, 98]. Elevated levels of *CRTH2* transcripts in bronchoalveolar lavage (BAL) fluid were found in severe asthmatics compared to those with mild/moderate asthma [98]. Moreover, a positive association was found between asthma exacerbations, poor control or Th2 cytokines, and *CRTH2* levels [98]. Our laboratory has also reported an increased proportion of circulating $CD4^+CRTh2^+$ T cells in severe asthmatics compared to those with mild or moderate asthma [99].

1.2.2 Genetic Variation within CRTh2

The *CRTH2* gene is located on chromosome 11q12-13 [90]. Numerous single nucleotide polymorphisms (SNPs) within the *CRTH2* gene locus have been associated with allergic asthma and sensitization, as well as higher bronchial hyper-responsiveness, in ethnically distinct populations (ie. Chinese and African American) [100, 101]. Genetic variation

within a German population of children has also been associated with the development of allergic sensitization and asthma [102]. A specific example is *CRTh2* -6388G>A (rs533116); our group has shown that individuals with the AA genotype had increased CRTh2 expression, higher levels of circulating eosinophils, enhanced Th2 differentiation and cytokine production compared to those of the GG genotype [103].

1.2.3 Functional Outcomes of PGD_2 Activation of CRTh2

Prostaglandin D_2 (PGD_2) is a ligand for CRTh2 [89, 104]. It is considered the second D-prostanoid receptor (DP2) for PGD_2 [105]. PGD_2 is a metabolite derived from the conversion of arachidonic acid to prostaglandin endoperoxide H_2 (PGH_2) by cyclooxygenases, where it acts as a substrate to PGD synthase, a catalyst to the conversion to PGD_2 [106, 107]. Following exposure, allergen crosslinking of two IgE molecules activates mast cells to degranulate and release the lipid mediator, PGD_2 [84, 85]. Indeed, it has been shown that CRTh2 activation by PGD_2 mediates the mobilization of intracellular calcium [108], phosphatidylinositol 3-kinase (PI3K) phosphorylates protein kinase B (Akt), nuclear factor of activated T-cells (NFAT) translocate into the nucleus and drives type 2 cytokine gene expression [61, 109]. PGD_2 activation of CRTh2 is known to mediate pro-inflammatory response in allergic disease, as supported by *in-vivo* mouse models of atopic dermatitis and allergic asthma, demonstrating that ovalbumin (OVA) challenged mice that were nebulized with a CRTh2 specific agonist (DK-PGD_2) had increased eosinophilia compared to vehicle-treated mice [110]. In human cells, many of the mechanisms have been worked out in-vitro [111].

One functional outcome of PGD_2 activation of CRTh2 is the mediation of chemotaxis of Th2 cells, eosinophils and basophils [104]. Mechanistically, this interaction activates PI3Kγ, leading to Akt phosphorylation, and further actin polymerization [109]. Studies have found that antagonizing CRTh2 inhibited chemotaxis [112], as did phosphoinositide 3-kinase (PI3K) inhibition, confirming the signaling pathway [109].

Studies have shown that activation of CRTh2 increased the type 2 pro-inflammatory cytokines, IL-4, -5, and -13 [68, 113]. This effect is dependent on calcium activated calcineurin, further dephosphorylating and activating NFAT to allow for translocation into the nucleus and cytokine gene transcription [109]. Therefore, blocking PI3K and calcium (Ca^{2+}) pathways has been shown to reduce cytokine production, as does the CRTh2 antagonist Ramatroban [68, 109].

IL-2 is an important survival and growth factor for T cells [114] and so deprivation of this cytokine triggers T cell apoptosis [115]. Interestingly, the binding of PGD_2 to CRTh2 has been shown to inhibit Th2 apoptosis. Indeed, the addition of PGD_2 significantly reduced the percentage of Th2 cells containing fragments of cleaved cysteine-aspartic protease (caspase) -3 and poly adenosine diphosphate polymerase (PARP), signatures of death by apoptosis [8].

1.3 Asthma: A Clinical Perspective

Th2 cells, type 2 inflammation and genetic variation collectively influence the persistence of asthma symptoms. My study examines how Th2 cells respond to various asthma related factors and therefore this section provides an overview of asthma diagnosis, subsets of severity and treatment.

1.3.1 Diagnosis

Diagnosis of asthma is based on clinical symptoms as well as lung function tests. Forced expiratory volume in the first second (FEV_1) is the maximum volume of air expelled from the lungs in one second of forced expiration, measured through a spirometer [116]. Measurements are taken prior to the administration of 200 to 400 μg of the bronchodilator albuterol, and 10-15 min post bronchodilator [17]. As asthma is classified as reversible airflow obstruction, an increase in FEV_1 greater than 12% and 200 mL indicates reversibility, which is consistent with a positive diagnosis [117]. The administration of an anti-inflammatory controller for four weeks resulting in an improved FEV_1 greater than 12% can further confirm this diagnosis [118]. If an increase is not achieved with this

medication, but the patient presents with characteristics of asthma, a bronchial provocation test may be used. This test involves administering increasing doses of methacholine to induce bronchoconstriction; a reduction in FEV_1 by 20% with a dose less than 8 mg/mL indicates hyper-responsiveness characteristic of a positive diagnosis of asthma, compared to the minimum 16 mg/mL required for a non-asthmatic [119]. Other targeted approaches are also be used to further characterize the type of asthma, such as airway or sputum eosinophilia or neutrophilia [120].

1.3.2 Asthma Severity

The Global Initiative for Asthma (GINA) uses symptoms, airflow limitation and lung function to divide asthma into the categories of intermittent, mild, moderate and severe persistent [121]. Intermittent asthmatics have symptoms less than 2 days per week with no activity interference and normal lung function [122]. In contrast, mild, moderate and severe asthmatics are symptomatic more than 2 days per week, daily, and numerous times throughout the day, respectively [122]. Exacerbations increase in frequency along the diagnosis scale; intermittent asthmatics have no exacerbations, whereas severe asthmatics may have as many as one exacerbation per week [122]. Lung function also deteriorates along the same scale [122]. The European Respiratory Society (ERS) and American Thoracic Society (ATS) defines severe asthma as that in which a patient is treated with a high dose of inhaled glucocorticosteroid as well as a secondary controller, such as a β_2-adrenergic agonist, to prevent worsening symptoms, but remains uncontrolled despite this treatment [123]. It is estimated that 5-10% of all asthmatics are classified to have severe asthma [124]. This phenotype is also predictive of increased hospitalizations [125]. Certain symptoms associated with severe asthma, such as wheezing and shortness of breath, have a strong genetic component [126].

1.3.3 Asthma Treatment

Strategies to reduce the chronic inflammation of asthma and relieve symptoms include controllers and relievers. Controllers are a long-term therapy, acting to control symptoms through reducing airway inflammation and preventing exacerbations; inhaled corticosteroids (ICS), such as budesonide, are a common controller first prescribed upon diagnosis [127, 128]. Relievers act to immediately relieve symptoms that are worsening during exacerbation. Both long- and short-acting inhaled β_2-adrenergic agonists (LABA, SABA, respectively), such as formoterol, provide relaxation of bronchospasm [129]. They are not effective as a monotherapy; rather, most patients with persistent symptoms have found combination LABA and ICS therapy to be beneficial in improving symptoms, reducing exacerbations and achieving control of lung function [127, 130-132]. Severe asthmatics do not experience major benefit from ICS, like mild and moderate asthmatics, or even long-term oral or parenteral systemic corticosteroid therapy [133]. As such, severe asthmatics are considered to be less corticosteroid responsive. The mechanism(s) driving this resistance are not well understood but may be related to Th2 cells and endogenous factors influencing their function.

1.4 Glucocorticosteroid

Glucocorticoid (GC) and mineralocorticoid are two classes of corticosteroids, responsible for suppression of inflammation [134] and electrolyte balance [135], respectively. The term corticosteroid is often used synonymously with GC. The GC, cortisol, is a steroid hormone synthesized in the adrenal cortex and secreted in the blood in response to stress [136]. Cortisol is the precursor to cortisone, first extracted from the adrenal gland in 1936, and in 1950, found to be beneficial in treating asthma [137, 138]. Since then, synthetic GCs with higher potencies have been developed and used to treat inflammatory diseases, such as asthma. The systemic GC prednisolone was originally used in clinical practice for treating asthma; however, long term use is associated with serious adverse effects such as cardiovascular disease, osteoporosis, and diabetes [139-141]. This led to the transition of asthma therapy to inhaled GC [142].

1.4.1 Mechanism of Action

GCs bind to their cytoplasmic glucocorticoid receptor (GR), a 777 amino acid protein expressed by nearly all cells in the human body [143]. As a member of the ligand regulated nuclear receptor family [144], the GR structure consists of three domains: the C-terminal domain, responsible for steroid (ligand) binding and repressing transcription-activation until such binding occurs; the conserved central deoxyribonucleic acid (DNA) binding domain, responsible for dimerization, direct binding to the major groove of DNA, and transcription-activation through zinc clusters [145-147]; and the N-terminal domain, required for transcriptional activation [148, 149]. Between the DNA and ligand binding domains is the hinge-region, which aids in nuclear translocation [150].

Unbound, GR remains inactive within the cytoplasm as a hetero-oligomer, consisting of chaperone proteins such as heat shock protein (hsp) 90 and FK506 binding protein-5 (FKBP5) [151, 152]. Through passive diffusion, GCs pass through the cytoplasm and bind directly to the ligand binding domain, inducing a conformational change of the GR and the dissociation of hsp 90 and FKBP5 [153]. This activation allows translocation of the GC-GR complex into the nucleus, where it forms homodimers [153]. This complex is now capable of regulating gene transcription through a variety of mechanisms (Fig. 1.2).

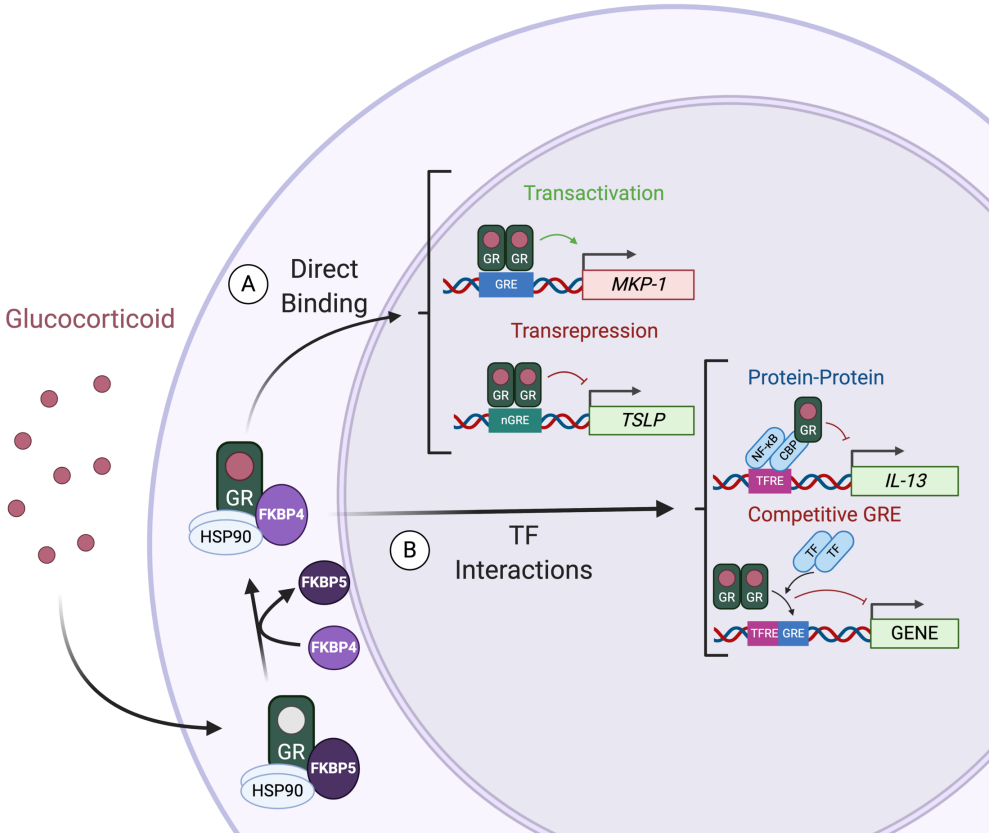

Figure 1.2: Glucocorticoid Mechanisms of Action

GC passively diffuses through the cytoplasm and binds to GR, inducing the dissociation of the chaperone proteins hsp90 and FKBP5 previously bound. The GC-GR complex then translocates into the nucleus and forms a homodimer. The GC-GR complex can directly bind to a GRE or nGRE and activate (transactivation) or inhibit (transrepression) gene transcription, respectively (A). Alternatively, the homodimer can interact with transcription factors (B) and inhibit their ability to activate transcription through protein-protein interactions. Competitive interactions can also occur when there are overlapping glucocorticoid and transcription factor response element sites, therefore the binding of GR inhibits the binding of the TF. Abbreviations are as follows: CREB binding protein, CBP; FK506 binding protein-5, FKBP5; glucocorticoid, GC; glucocorticoid receptor, GR; glucocorticoid response element, GRE; heat shock protein, HSP; interleukin, IL; mitogen-activated protein kinase-1, MKP-1; negative glucocorticoid response element, nGRE; thymic stromal lymphopoietin, TSLP; transcription factor, TF; transcription factor response element, TFRE. Created with *BioRender.com*.

GC-GR Direct Binding to DNA Sequences

GCs can directly bind to DNA sequences within promoter and regulatory regions entitled glucocorticoid response elements (GREs); the consensus sequence of a GRE is composed of the half site AGAACA, followed by 3 nucleotides and the other half site TGTTCT [154]. Direct binding to GREs can induce either transactivation or transrepression (Fig. 1.2A) [155, 156]. Transactivation is the activation of gene transcription such as the arachidonic acid inhibitor, p11 [157]. GC can also act through transrepression, the inhibition of transcription, through interaction with negative GRE sites (nGREs) [158]. It has also been shown that binding of the GR complex to GRE may alter the conformation of DNA or chromatin [159]. Activated mitogen-activated protein kinase (MAPK) cascades are key regulators in the production of pro-inflammatory mediators [160]; however, MAPK phosphatase 1 (MKP-1) dephosphorylates conserved threonine and tyrosine residues, thus terminating the MAPK pathway [161]. A GRE is present in the *MKP-1* promoter, and GC binding to this GRE induces this gene, further acting in an anti-inflammatory capacity [162, 163].

GC-GR Interactions with Transcription Factors

GR can also regulate transcription through interactions with transcription factors (TF) (Fig. 1.2B). Many pro-inflammatory genes, such as cytokines, do not have nGREs. Rather, the GC-mediated effect is through GR interacting with transcription factor binding [164]. For instance, GR binding to the TFs, nuclear-factor-κB (NF-κB) and activator protein-1 (AP-1), represses their ability to activate transcription [165-167]. This can result in the inhibition of cytokine gene transcription, such as IL-5 and IL-13 [168]. Interestingly, protein-protein direct interactions between GR and a TF can induce mutual repression of the protein dependent genes [169]. A competitive GRE occurs where the transcription factor has an overlapping site for GR, thus GR binding would prevent the positive TF from activating the gene, as seen in the osteocalcin promoter [170].

GC-GR Modulation of mRNA Stability

The GC-GR complex is also capable of altering the stability of messenger ribonucleic acid (mRNA). Shaw et al., found that a conserved region in the 3' untranslated region (UTR) contained adenylate-uridylate (AU) rich elements (AREs) implicated in mRNA instability in human granulocyte-macrophage-colony-stimulating-factor (GM-CSF) [171]. mRNA is often degraded through shortening of the poly(A) tail by ribonucleases, an effect mediated by AREs [172]. Moreover, it has been observed that GC enhances the transcription of ribonucleases capable of degrading AU-rich elements, and selectively decreases the stability of mRNA for cytokine genes such as IFN-β, and IL-1β [173, 174]. It was also noted that the 3' UTR of inflammatory cytokine, lymphokine and growth factor genes also contain AU-rich elements, indicating a potential mechanism in which GC acts on to suppress inflammation [175]. Interestingly, GCs have been shown to reduce IL-4Rα mRNA through destabilization [176], however further studies are required to validate this.

1.4.2 Pro-Inflammatory Effects of GC

While GC are primarily considered anti-inflammatory, there is a growing body of evidence suggesting they also have pro-inflammatory effects. Indeed, GC has been shown to increase expression of immune receptors such as IL-2R [177], C-X-C chemokine receptor (CXCR) 4 [178], and toll like receptor (TLR) 2 and TLR4 [179]. Whether GC regulates these genes by GRE mediated transcription, translation or cooperation with other transcription factors is not clear and needs further study.

1.4.3 GR Interactions

Research has shown that GC and its receptor can interact with other hormone receptors and proteins. This interaction is termed cross-talk and can lead to enhanced or reduced transcriptional effects, depending on the gene, as well as to chromatin remodeling and altered accessibility of regulatory elements. For instance, cross-talk between vitamin D (Vit D) and GC signaling pathways has been shown to improve the effectiveness and anti-

inflammatory effects of GC [180]. Peripheral blood mononuclear cells (PBMC) from asthmatic children were treated with the GC dexamethasone (DEX) in the presence or absence of Vit D. A significant suppression of T-cell proliferation was found with co-treatment, compared to no inhibition with DEX alone, mimicking a steroid resistant model [181]. Zhang and colleagues showed that that Vit D receptor (VDR) is recruited to the GRE site in the *MKP-1* promoter and enhances GR binding, inactivating the pro-inflammatory MAPK cascade [182]. Similarly, synergistic interactions between GR and forkhead box O (FOXO) have also been shown in muscle RING-finger protein-1 (MuRF1) [183]. Collectively, these data show that cross-talk between GR and other transcription factors, such as VDR and FOXO may enhance GR-mediated transcription.

1.4.4 Apoptosis Mechanism of Action

All cells will inevitably die through manners such as pyroptosis, autophagy, apoptosis or conversely, oncosis leading to necrosis. [184, 185]. The two contrasting mechanisms, apoptosis and necrosis, differ based on the death signal [184]. Necrosis is passive cell death, uncontrolled and toxic; it is induced by direct damage and energy supply reduction [186]. A key feature in this process is the disruption of the cell membrane, releasing cytoplasmic and chemotactic signals [186]. In contrast, apoptosis is an active process, requiring energy [187]. Apoptosis, or self-induced cell death, features caspase activation, resulting in plasma membrane blebbing, apoptotic body formation, DNA cleavage and condensation, and phosphatidylserine expression on the outer membrane [188]. No inflammatory reaction is produced with apoptosis, as the membrane remains intact and the constituents of the cell are contained within apoptotic bodies phagocytosed by macrophages [189]. If caspase and adenosine triphosphate (ATP) availability decrease during apoptosis, the process of cell death will convert to necrosis [190].

Apoptosis can be induced by extrinsic factors, such as the cell surface death pathway, or intrinsic causes, initiated by the mitochondria. Cytochrome complex (cyt *c*) is essential for mitochondrial respiration, acting as part of the electron transport chain, and when thereby released, creates dysfunction, and leads to apoptosome complex formation, containing

apoptotic protease-activating factor 1 (Apaf)-1 that binds and activates pro-caspase 9, leading to caspase-3 activation [191, 192]. Extrinsic stimuli can bind to receptors, such as tumor necrosis factor receptor (TNFR), on the surface of the cell, and initiate the cascading responses that activate caspase and induce apoptosis [193]. Intrinsically, apoptosis is regulated by the B cell lymphoma (Bcl-2) protein family of pro- and anti-apoptotic factors that engage or block the development of the pore that would allow cyt *c* to leak out of the mitochondria and initiate cell death. Bcl-2 and Bcl2-extra large (Bcl-xL), are anti-apoptotic members, acting to increase resistance to death stimuli and pro-apoptotic members such as: Bcl-2-associated X protein (BAX), Bcl-2 homologous antagonist killer (BAK), Bcl-2-like protein 11 (BIM), and BCL-2-associated death promoter (BAD) inducing apoptosis [194]. The activation of CD95 (Fas) or TNFR1 death receptors induces the cleavage of BH3 interacting-domain death agonist (BID), acting as a ligand that induces BAK and BAX oligomerization to form the mitochondrial pore leaking cyt *c* [195-197]. Bcl-2 and Bcl-xL prevent the oligomerization of BAK and BAX, thereby inhibiting mitochondrial pore formation, caspase activation, and further, block apoptosis [198]. In contrast, BIM and BAD displace anti-apoptotic Bcl-2 and Bcl-xL, preventing their inhibition of apoptosis and allowing for mitochondrial pore formation [198].

1.4.5 GC Induced Apoptosis

GCs are anti-inflammatory, functioning to induce apoptosis of inflammatory cells. This is mediated through a variety of ways. Chromatin immunoprecipitation-sequencing (ChIP-seq), used to detect genome wide binding of a factor to DNA, has identified a GR binding site in BIM regulatory elements, with GC treatment detectably upregulating BIM and inducing apoptosis [199, 200]. In human pre-B-cell lines, varying levels of *BCL-2* altered the ability of GC to induce apoptosis, with higher expression resulting in less apoptosis, further proving the anti-apoptotic effect Bcl-2 has [201]. GC bound to GR induces activation of phosphatidylinositol-specific phospholipase C (PI-PLC), inducing the activation of acidic sphingomyelinase (ASMase), and further caspase [202, 203]. The bound complex can also repress both Ap-1 and IL-2 [204]. Glucocorticoid-induced leucine zipper (*GILZ*) transcription is also modulated through GC [205]. In transgenic mice,

overexpression of GILZ induced apoptosis, as detected through reduced Bcl-xL expression and elevated caspase-3 activation [206].

1.4.6 GC Resistance and Severe Asthma

Despite being an effective treatment for most, since 1968 it was known that GCs are ineffective to a small proportion of asthmatics with severe disease and that these patients place a large financial burden on society [207, 208]. There is evidence that some patients respond to a higher dose of GC; however, that is not the case for all GC resistant asthmatics [209]. Numerous studies have been conducted to try and identify markers indicating resistance to GC treatment. T lymphocytes from both GC resistant and sensitive asthmatics have been cultured in the GCs methylprednisolone or DEX, and cells from resistant asthmatics showed less inhibition of proliferation compared to GC sensitive patients [210, 211]. Mechanistically, in peripheral blood T cells from severe uncontrolled asthmatics, reduced GC binding to GR was observed compared to controlled asthmatics [212]. Furthermore, multiple distinct binding abnormalities were identified in steroid resistant asthmatics, associated with reduced binding affinity [213]. Deficit in other mechanisms such as GR nuclear translocation and histone acetylation have been suggested to mediate GC resistance [214]. Indeed, the expression of GRβ, the negative regulator of immunosuppressive GRα, has been associated with steroid resistance [215-217]. Polymorphisms in the GR gene, such as R477H and G678S mutations have been shown in-vivo to alter the capacity for ligand binding and transactivation [218]. Although cross-talk between GR and endogenous factors such as other hormone receptors has been shown [180, 183, 219], whether these interactions play a role in GC resistance in these patients is not clear.

1.5 Sex Differences in Asthma

A birth cohort analysis in New Zealand identified male sex as a risk factor for asthma in children under the age of 14 years, with the prevalence being twice as high compared to girls [220]. The Tracking Adolescents' Individual Lives Survey (TRAILS) study found that at mean age 11.1 years, prevalence rates were equal between sexes; however, by the age of 16.3, females had significantly higher rates than males [221]. Furthermore, the Isle of Wright Birth Cohort revealed that male sex is associated with remission of asthma in adolescence [222]. This may be related to lung development, since it has been shown to vary based on sex, starting from a very young age. Phosphatidylglcerol, a main component in pulmonary surfactant, was found earlier in gestation in female neonates than males, potentially explaining female's elevated airflow rate and minimal resistance compared to males [223, 224]. That being said, male lungs have been found to be larger than those of females in children under 14 years [225]. As a major component of the physiological differences between males and females, sex hormones have been shown to be involved in lung development, such as androgens inhibiting versus estrogens stimulating lung maturation in animal models, as well as lung disease like asthma [226, 227].

In addition to development of asthma, sex also appears to influence the severity of asthma. A multi-center randomized control trial in New Zealand found that female sex was associated with future severe exacerbations [228]. A national Canadian study found an association between asthma and obesity in women [229], and further cluster analyses identified that the late onset development of asthma in obese women was more often of the severe phenotype requiring oral corticosteroid [230, 231]. Interestingly, obesity is characterized by increased adipose tissue that increases the hormones estrogen and leptin [232], and positive associations have been made between elevated leptin levels and overweight female asthmatics [233]. Moreover, women are more likely to be diagnosed with asthma, severe asthma, and to have more frequent exacerbations, hospitalizations and relapses following treatment [6, 234, 235]. Collectively, these observations have led to the realization that research focused on understanding sex differences in asthma is greatly needed.

1.5.1 Effects of Male Sex Hormones on Type 2 Inflammation

The male sex hormone, testosterone, is believed to have a protective effect against allergic airway inflammation, as elevated levels in the serum of adult males has been associated with higher FEV_1 [236]. Testosterone has also been shown to decrease IL-4 production in mouse model [237]. Additionally, castrated mice being given androgens show reduced lung inflammation [238] and androgen signaling inhibited the differentiation of ILC2 progenitors into ILC2s, further preventing type 2 responses [239]. Indeed, androgens-androgen receptor (AR) signaling reduced both the number ILC2 progenitor cells and the inflammation the cells mediate [240]. Cephus and colleagues [241] found that gonadectomized male mice had reduced ILC2 cells within the lung when given the testosterone derivative, 5α-dihydrotestosterone (DHT), versus control. Interestingly, no change in ILC2 cell numbers were seen when mice were given estradiol. Additionally, when stimulated ILC2 cells from female mice were treated with DHT they produced less IL-5 and -13. Despite studies showing that testosterone suppresses type 2 the inflammation, there is also evidence indicating that sex differences in asthma are not merely due to low levels of this hormone in females. For instance in a house dust mite (HDM) model of asthma, androgen signaling *reduced* but estrogen signaling *increased* type 2 allergic inflammation, suggesting this inflammatory pathway may be differentially regulated in males and females [242].

1.5.2 Female Sex Hormones and Asthma

In contrast to testosterone, the female sex hormones estrogen and progesterone have been shown to have pro-inflammatory effects and to be associated with reduced respiratory function at the times these hormones fluctuate within the menstrual cycle [243, 244]. As such, the prevalence switch of asthma between sexes with less male and more female cases around the time of puberty and the commencement of the menstrual cycle suggests that female sex hormones play a role in the development of asthma in girls and women [221].

The Menstrual Cycle

The menstrual cycle involves cyclical fluctuations in sex hormones and consists of two phases: follicular and luteal [245]. The follicular phase occurs at the start of menstruation, with estradiol levels rising until the luteal phase begins at the start of ovulation [245]. At this point, estrogen levels gradually decrease and progesterone levels increase until the end of the menstrual cycle, where they rapidly plummet [245]. The negative effects these hormone fluctuations have on respiratory function have been shown in a variety of studies. One assessed pulmonary function before and after exercise, and the reduction in FEV_1 from exercise was significantly greater on day 21 of the subjects' menstrual cycle, when salivary progesterone was at its highest concentration, compared to day 5, when it was at its lowest [243]. Moreover, women experienced more respiratory disturbance during their sleep in the follicular phase, at the point of high estrogen, compared to the luteal phase [244]. Specifically, the fluctuations in estrogen and progesterone at the end of the luteal phase have been shown to worsen or exacerbate asthmatic symptoms, a phenotype entitled premenstrual asthma that affect approximately 40% of asthmatic females [246, 247]. Tan and colleagues showed that the provocative concentration of methacholine to reduce FEV_1 by 20% was reduced by 2.5-fold in asthmatic female adults with well-controlled asthma during peak estrogen and progesterone levels of their menstrual cycles [248]. Furthermore, asthmatic women taking oral contraceptives, thereby reducing luteinizing hormonal surges and progesterone levels, had reduced risk of wheeze, and significantly reduced cyclical airway reactivity changes compared to those not taking oral contraceptives [249, 250].

Pregnancy

Other times of hormonal variation, such as during pregnancy, have also been associated with exacerbation of symptoms to the extent that they require medical intervention or hospitalization in 20% or 6%, respectively, of asthmatic patients [251]. This has been suggested to be related to the increased serum concentrations of progesterone that accompanies pregnancy [252]. Additionally, pregnancy has been described as a Th2 phenomenon; the Th2 cytokine, IL-4, produced at the maternal-fetal interface was elevated

in comparison to the Th1 cytokine, IFN-γ, and was associated with more successful pregnancies [253, 254]. In 1984, a prospective study identified that 35% of women experienced worsening asthma during their pregnancy [255]. Moreover, approximately 40% of asthmatic pregnant females experienced no change, while 30% had increased severity of asthmatic symptoms despite bronchodilator therapy [256]. Females with severe asthma prior to pregnancy have been identified as prime candidates to develop worsening asthma during pregnancy [251]. Interestingly, severe life threatening asthma has been reported in a small population of females during the first trimester of their pregnancy, with dramatic improvements in asthma and airflow obstruction within 24 hours of termination of the pregnancy [257, 258]. Together, these findings suggest that the increase in female sex hormones associated with pregnancy may be contributing to increased Th2 inflammation and asthma severity often experienced [253, 255].

Menopause

Menopause is associated with the drop of estrogen and progesterone levels and is considered protective against asthma if the female has never used hormone replacement therapy; however, this effect is reversed if hormone replacement therapy has been used [259]. Specifically, studies show that if a female had never taken external estrogen, the menopause-induced reduction in hormones was associated with reduced risk of asthma [259]. However, a positive association between dose of conjugated estrogen, commonly used to treat menopausal symptoms, and risk of asthma has been reported [259, 260]. Together, these studies suggest that estrogen may also play a role in late onset asthma.

1.5.2.1 Female Sex Hormones and Th2 Inflammation

Estrogen has been shown to induce the production of the Th2 cytokines IL-4, IL-5 and IL-13 in a mouse model [261]. In women with peri-menstrual asthma increased serum estradiol during the luteal phase was associated with increased sputum IL-5 [262]. Another mouse model found that phytoestrogens promote isotype switching in B cells, producing

increased allergen-specific IgE [263]. Moreover, in the presence of TNFα, estradiol caused dendritic cells to mature rapidly, resulting in the increased ability to induce Th2 responses [264]. Larger quantities of mast cells, as well as higher concentrations of histamine released by mast cells and basophils, were observed in rats when estrogen levels were at their highest [265-267]. These quantities were reduced with ovariectomy, indicating estrogen can also promote mast cell degranulation [267]. An increase in mucus producing goblet cells has been found when cells from female donors were treated with estradiol [268]. Although all these effects induced by estrogen are characteristic of the Th2 response that occurs in allergic asthma, little is known regarding whether estrogen mediates these effects by directly acting on Th2 cells.

1.5.2.2 Female Sex Hormones and Cell Survival

One way that estrogen may influence Th2 cells is through apoptosis. Indeed, Th2 cells treated with estradiol had elevated surface staining for the anti-apoptotic factor, Bcl-2 [269]. Estrogen signaling has been shown to stimulate cell proliferation in a mouse cell model and increase the anti-apoptotic protein Bcl-xL [270]. A global gene expression profiling analysis revealed that in a breast cancer cell line (MCF-7), treatment with estradiol significantly up-regulated proliferative genes such as growth factors and cell cycle progression regulators, as well as down-regulated pro-apoptotic, anti-proliferative and transcriptional regulators such as Ap-1 members [271]. Additional gene profiling studies have shown that estrogen works synergistically with NFκB, a pro-survival transcription factor, to upregulate anti-apoptotic genes such as cellular inhibitor of apoptosis (cIAP) 2 [272]. Since an important aspect of the anti-inflammatory capacity of GC is induction of apoptosis, these studies suggest that estrogen may reduce Th2 cell sensitivity to GC and in this way play a role in asthma severity.

1.5.3 Estrogen Receptors and Signaling Pathways

The term estrogen refers to a group of hormones: estrone (E1), estradiol (17β-estradiol; E2), estriol (16-hydroxyestradiol; E3), and estretrol (15α-hydroxyestriol; E4). E2 is produced by the ovaries and is the most predominant estrogen in the human body during the reproductive years; however, all estrogens are produced at some point within the life of a female [273]. During pregnancy, E3, produced by the placenta, is the major estrogen [274]; however, after menopause, E1, derived from androgen conversions, is the largest estrogen in the female body [273]. E4 is synthesized by the liver of the fetus during pregnancy, and circulates through the placenta to the mother [275].

All four estrogens are capable of binding to nuclear and membrane receptors [276]. The estrogen receptor (ER) was first discovered in 1958 by Jensen and colleagues, and since then three ERs have been characterized: ERα, ERβ, and most recently G Protein-Coupled ER (GPER)1 [277-279]. ERα and ERβ are nuclear receptors and ligand-activation transcription factors encoded by the gene ESR1 and ESR2 on chromosome 6 and 14, respectively [280, 281]. The membrane receptor, GPER1 gene is encoded on chromosome 7 [282].

Despite being completely separate genes, ERα and ERβ signal similarly through mechanisms such as the classical direct genomic pathway. Estrogen diffuses through the plasma membrane and binds to ERα and/or ERβ, inducing a conformational change and either homo- or hetero-dimerization [283]. The complex then translocates into the nucleus where it binds to estrogen response elements (EREs) and regulatory elements within promoters [284]. It is thought that ERα interactions with EREs typically enhance gene expression, whereas ERβ repress expression at AP1 sites [285]. That being said, approximately one third of genes regulated by ERs do not have ERE sequences [286]. Therefore, they act through indirect genomic signaling or cross-talk/protein-protein interactions with other response elements and transcription factors like AP-1[219, 286, 287]. Additionally, ER can act on specificity protein (Sp)-1, enhancing the binding of the protein to its site [288]. Lastly, non-genomic estrogen signaling occurs through the membrane-associated GPER1, indirectly influencing gene expression through activating

signal transduction pathways that on act downstream transcription factors [279]. For example, estrogen binding to GPER1 rapidly activates extracellular signal-regulated kinases (ERKs) [289].

Both ERα and ERβ are known to be expressed in human lung tissue [290]. Furthermore, it is thought that ERα is more relevant than ERβ in inducing allergic inflammation. On the genomic level, SNPs in the ESR1 gene have been associated with decreased FEV_1 in asthmatics [291]. On a cellular level, both eosinophils and mast cells express ERα and not ERβ, and mice challenged with an allergen and then treated with ERα antagonists had reduced eosinophils as well as Th2 cytokines [261, 292, 293]. ERβ has also been found to have an anti-inflammatory role in asthma; airway smooth muscle hyperplasia, characteristic of allergic asthma, depends on stimulation by platelet-derived growth factor for proliferation, and an ERβ agonist was shown to suppress this proliferation [294]. Further, ERβ signaling has been associated with increased apoptosis, as well as an increase in pro-apoptotic BAX expression [270]. A mouse model demonstrated that E2 treatment reduced tidal volume and airflow rate, increased eosinophils, and induced alveolar and muscular layer destruction in asthmatic mice compared to control asthmatic and non-asthmatic groups. The group receiving E2 as well as a selective ERα antagonist resulted in no difference in any of the measurements compared to control asthmatic mice, indicating the pro-inflammatory effect was primarily through ERα [295].

1.5.4 Cross-talk between Estrogen and Glucocorticosteroid

Estrogen receptors participate in cross-talk, interacting with other proteins and steroids to modulate gene transcription through competition or cooperation (Fig. 1.3). Importantly for this thesis, crosstalk been ER and GR occurs, and has been suggested to play a role in the increased disease severity and reduced sensitivity to treatment many asthmatic women experience [296].

Competition

In certain circumstances, GR and ER can interact in a competitive manner and have opposing effects on inflammation (Fig. 1.3B). Breast cancer cell lines treated with estradiol had downregulated GR expression and responses to GC [297]. More so, an inverse relationship between ER and GR expression has been identified [298]. Estradiol induces protein phosphatase 5 (PP5), which inhibits GR phosphorylation and further prevents GC-induced transcription of anti-inflammatory *MKP-1* [299, 300]. This is an example of an in-direct interaction between the two steroid receptors. The synthetic GC DEX inhibits proliferation of a human breast cancer cell line, whereas E2 stimulates proliferation [301]. Interestingly, the effects are opposite in bone; GC induces resorption whereas E2 inhibits resorption [302, 303]. It has been shown that ER expression can inhibit the activation of GR, and vice versa due to the competition for functionally limiting transcription factors [304]. A ChIP study used to identify protein interactions with DNA, discovered that GR occupied multiple EREs when treated with both DEX and E2; however, if DEX or E2 treatment was administered alone, minimal GR binding at the EREs occurred [305]. Additionally, the GR recruitment was associated with ERα displacement, but also required ERα, FOXA1 and AP1 binding, thus uncovering a mechanism for GC inhibition of ERα activation [305]. Yang and colleges found that GR repressed ERα activated transcription through binding to EREs already occupied by ERα, halting the effective activation [306]. On the other hand, estradiol has been shown to inhibit GILZ expression in uterine epithelial cells through reduced binding of GR to a GRE within the GILZ promoter in the presence of ERα [307]. These studies highlight that in some circumstances GR and ER can act in a competitive manner, with ER inhibiting GR mediated transcription.

Cooperation

GR and ER cross-talk has also been shown to be cooperative, increasing the accessibility to regulatory elements (Fig. 1.3C). The use of indirect genomic estrogen signaling can allow for transcription factors, such as Sp-1, to have enhanced binding at GC-rich sites [308]. These steroids can work together in a rat model of lung inflammation since the ERα

antagonist reversed the anti-inflammatory effect of DEX [309]. Molecularly, Hager and colleagues discovered that when both estradiol and GC are present and their respective receptors are activated, receptor binding is redistributed [310]. Overlapping ER and GR binding sites were found in cells treated with E2 or DEX alone compared to treatment with both DEX and E2, respectively, as well as unique binding sites specific to E2, DEX, and E2 and DEX co-treatment [310]. Nine major binding clusters were identified between ER and GR combined, the majority representing regulatory regions; however, GR and ER individual binding clusters were found and activated by their own receptor, and for some, activated by the other receptor, indicating assisted-loading regions [310]. Moreover, DNase I digestions showed that activation of the receptor promoter by the opposite receptor induced unique chromatin structural changes, facilitating this new binding [310]. Furthermore, a GR ChIP-seq identified GR binding regions, and that co-treatment with DEX and E2 increased GR enrichment at sites through chromatin remodeling [311]. Dual receptor activation also resulted in GR showing increased binding at GR, ER, FOXO and AP1 regulatory elements [311]. Cumulatively, the research suggests that within some cell types ER may act to stabilize or increase accessibility, enhancing GR binding.

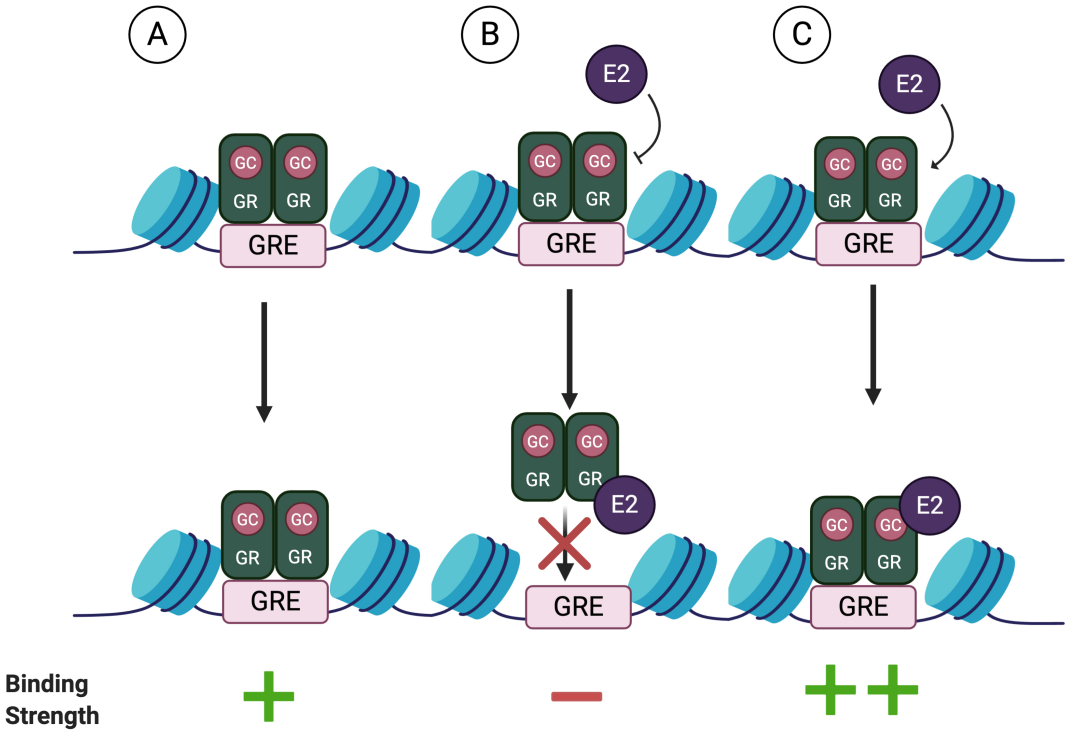

Figure 1.3: Glucocorticoid and Estrogen Cross-talk

Unperturbed, the GC-GR homodimer complex binds to the GRE (A). In the presence of E2, cross-talk between the GC and E2 can occur and result in B) the inhibition of GC binding to GRE, or C) increased stability and accessibility allowing for more binding of GC to the GRE. Abbreviations are as follows: estradiol, E2; glucocorticoid, GC; glucocorticoid receptor, GR; glucocorticoid response element, GRE. Created with *BioRender.com.*

1.6 Rationale

Despite the abundance of evidence revealing sex differences in the development and severity of asthma [6, 312], the pathobiology driving these differences is still not well understood. In a study performed by my colleagues, significantly higher levels of circulating Th2 cells were found in women compared to men with severe asthma (App. 1.1). This may be due to differential response to GC, since the level of Th2 cells was positively correlated with daily dose of inhaled GC in women, but not men (App. 1.2).

There are two main mechanisms by which GCs reduce inflammation - suppression of cytokine production and induction of apoptosis [134]. The Cameron laboratory recently demonstrated that estrogen, in concert with GC, increases the level of CRTh2 mRNA and protein (App. 1.3) in primary human Th2 cells. This could suggest that Th2 cell sensitivity to PGD_2 and its ability to inhibit apoptosis differs between men and women.

The finding that GC and estrogen increase CRTh2 expression is intriguing, but whether this increase occurs at the level of transcriptional activation is not known. This seems possible since the CRTh2 promoter contains a number of GREs and estradiol has been shown to cooperate with GR to enhance gene expression [311, 313, 314]. Transcriptional activation of CRTh2 may also be influenced by genetic variation, since the CRTh2 promoter SNP, *CRTh2*-6388G>A, was previously shown to associate with asthma risk [102] and increased CRTh2 expression [103].

1.7 Hypothesis and Objectives

1.7.1 Hypothesis

Estrogen inhibits glucocorticoid-induced apoptosis by increasing CRTh2 expression and this effect occurs at the level of CRTh2 promoter activation.

1.7.2 Objectives

Objective 1: To determine how estrogen receptor signaling influences GC-induced apoptosis

PGD_2 activation of CRTh2 inhibits Th2 cell apoptosis [68] and so whether an increase in CRTh2 expression, mediated by GC and estrogen, enhances Th2 cell survival was examined.

Sub-hypothesis: Estrogen signaling reduces GC-induced apoptosis by increasing expression of CRTh2 and sensitivity to PGD_2.

Objective 2: To determine whether GC and estrogen receptor signaling activate the CRTh2 promoter and if this response is altered by a *CRTh2* promoter SNP

Mapping of putative transcription factor binding sites within CRTh2 revealed a number of GRE sites, including one 6 bp away from the *CRTh2*-6388G>A SNP. Estradiol can enhance GR binding [311, 313, 314] and so whether concomitant exposure to these hormones would increase transcriptional activation of *CRTh2* in an allele specific manner was investigated.

Sub-hypothesis: GC and estrogen signaling induce CRTh2 promoter activation and the magnitude of this effect differs based on the *CRTh2*-6388G>A SNP.

Chapter 2

2 Materials and Methods

2.1 Cell Culture

2.1.1 *In Vitro* Differentiated Primary Th2 Cells

A number of attempts were made to differentiate Th2 cells for this study. Blood was drawn from healthy non-asthmatic donors (Western University Ethics Review Board; approval number 106770) and peripheral blood mononuclear cells (PBMC)s were obtained by centrifuging blood over Ficoll Histopaque PLUS (GE Healthcare, Sweden) and isolating the mononuclear cells. The naïve $CD4^+$ T cell Isolation Kit II (Miltenyi Biotech, CA, USA) was used to obtain untouched naïve $CD4^+$ T cells. With this kit all cell types other than naïve $CD4^+$ T cells are labeled with biotin-conjugated antibodies/microbeads and are retained within the MACs column. Cells were activated for 3 days on plate-bound antibodies (anti-) to CD3ε (1 μg/mL; Clone UCHT1, R&D Systems Bio-Techne, MN, USA) and CD28 (1 μg/mL; Clone 37407, R&D Systems Bio-Techne, MN, USA) in X-VIVO 15 medium (Lonza, USA) with 10% Premium Fetal Bovine Serum (FBS) (Wisent, QC, Canada) and Penicillin, Streptomycin, and L-glutamine (1%, Gibco, Canada), as well as Th2 polarizing conditions: recombinant human (rh) IL-2 (5 ng/mL; R&D Systems Bio-Techne, MN, USA), rhIL-4 (10-20 ng/mL; R&D Systems Bio-Techne, MN, USA), antibodies against IFN-γ (1 μg/mL; polyclonal, R&D Systems Bio-Techne), and IL-12 (1 μg/mL; Clone C8.6; Invitrogen, CA, USA). The cells were then reseeded in fresh media and polarizing conditions in the absence of anti-CD3/CD28 for 4 days. On day 14, cells were incubated with magnetic beads conjugated with antibody against CRTh2, loaded onto MACS columns and cells expressing CRTh2 were obtained using the $CRTh2^+$ cell selection kit (Miltenyi Biotech, CA, USA).

Despite these attempts to generate newly differentiated Th2 cells, the studies within this thesis were carried out with Th2 cells previously differentiated by a former laboratory member. This Th2 cell line was generated by isolating PBMCs from blood donated by a healthy, non-asthmatic female (University of Alberta Ethics Review Board; approval

number PRO1784) and centrifugation over Ficoll Histopaque PLUS (GE Healthcare, Sweden). The starter population was total $CD4^+$ T cells using a $CD4^+$ T cell Isolation Kit II (Miltenyi Biotech, CA, USA). These cells were cultured in identical differentiation conditions, except that the X-VIVO 15 media was supplemented with 10% FBS from HyClone and cells were differentiated for 14 days prior to CRTh2 isolation ($CRTh2^+$ cell selection kit, Miltenyi Biotech, CA, USA). These $CD4^+CRTh2^+$ T cells (*i.e.* Th2 cells) were expanded by activation on plate-bound anti-CD3/CD28 and multiple vials frozen down. For this thesis, these Th2 cells were thawed and re-cultured in X-VIVO 15 (Lonza, MD, USA), supplemented with 10% Premium FBS (Wisent, QC, Canada) and the antibiotics Penicillin, Streptomycin, and L-glutamine (1X; Gibco, ON, Canada) and cultured as above on cycles of activation (plate-bound anti-CD3/CD28, 1 μg/mL; 3 days) and proliferation (rhIL-2, 5 ng/mL; 4 days) at a concentration of 2.0×10^6 cells/mL and incubated at 37°C, in 85% humidity and 5% carbon dioxide (CO_2).

2.1.2 CCRF-CEM Cells

CCRF-CEM [CCRF CEM] (ATCC ® CCL-119 TM) cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Sigma Aldrich, ON, Canada) supplemented with 10% Performance FBS (Wisent, QC, Canada) and the antibiotics Penicillin, Streptomycin, and L-glutamine (1X; Gibco, ON, Canada). The cells were seeded every second day at 0.2×10^6 cells/mL and maintained at 37°C, 5% CO_2, and 85% humidity. CCRF-CEM cells are an immortalized $CD4^+$ T lymphoblastic cell line, derived from a 4-year-old Caucasian female.

2.2 GC and Estrogen Exposure of Th2 Cells

Experiments with primary Th2 cells (1.3×10^6 cells/mL) were performed after the proliferation phase in media supplemented with rhIL-2 (1.25 ng/mL). Experiments with CCRF-CEM cells (0.2×10^6 cells/mL) were performed on the day after split, during logarithmic growth. Cells were treated with varying concentrations and combinations of

dexamethasone (0.01-1.0 μM; Sigma Aldrich, ON, Canada), Propylpyrazole-triol (1.0 μM PPT; Tochris, ON, Canada), 17 β-estradiol (0.1 μM; Sigma Aldrich, ON, Canada), and Prostaglandin D_2 (1.0 μL; Caymen Chemicals, Czech Republic), all reconstituted in ethanol. Vehicle control was 0.03% ethanol.

2.3 Flow Cytometry

2.3.1 CRTh2 Surface Levels

Surface level of CRTh2 on primary Th2 cells and CCRF-CEM cells was demonstrated using a similar staining protocol. Cells were harvested 24 hours following treatment, washed with phosphate buffered saline (PBS) fluorescence-activated cell sorting (FACS) (3% FBS, 0.5% bovine serum albumin, 1% sodium azide), centrifuged (900 rpm for 10 minutes at 4°C), then re-suspended in fresh PBS FACS and re-washed. Cells were blocked with rat IgG antibody (3 mg/mL; Invitrogen, CA, USA) in PBS FACS (0.5×10^6 cells/mL) for 45 minutes at room temperature. Following this step, anti-CRTh2 AlexaFluor 647 antibody (75 ng/mL; clone BM16; Rat IgG2a, BioLegend, CA, USA) or rat IgG2a κ Alexa647 control (75 ng/mL; clone RTK2758; BioLegend, CA, USA) was incubated with the cells (30 minutes, at 4°C). After two washes with PBS FACS, cells were fixed with 4% paraformaldehyde. Data was obtained using the LSR II Flow Cytometer (BD, CA, USA) with FACSDiva Software (BD, CA, USA) at the London Regional Flow Cytometer Facility. The data was then analyzed using the FlowJo (Treestar, Ashland, USA) computer application depicting total percent of cells positive for CRTh2.

2.3.2 Apoptosis

Annexin V and 7-Aminoactinomycin D

To assess apoptosis and cell death, cells were stained with Annexin V and 7-Aminoactinomycin D (7-AAD). Annexin V stains phosphatidylserines translocated from the inner membrane to the outer membrane (characteristic of apoptosis) and 7-AAD stains

DNA and thereby identifies cells with compromised plasma membranes. For these experiments, Th2 cells and CCRF-CEM cells were treated similarly with various concentrations of dexamethasone and estrogen receptor agonists for 48 hours. After this exposure, cells were washed with PBS FACS (0.5×10^6/tube), centrifuged (900 rpm for 10 minutes at 4°C), and re-suspended in 100 μL of Annexin V binding buffer (10mM HEPES, 140mM NaOH, 2.5mM $CaCl_2$, and 15.2 mL of H_2O) before staining with Annexin V-Alexa Fluor 647 reagent (1 μL; BioLegend, CA, USA) and 7-AAD Viability Staining Solution (5 μL; BioLegend, CA, USA). The tubes were incubated at room temperature for 15 minutes in the dark before diluting with Annexin V binding buffer (400 μL). At the London Regional Flow cytometer facility, using the data obtained through the LSR II Flow Cytometer (BD, CA, USA) and FACSDiva Software (BD, CA, USA), total percent of Annexin V^+ and 7-AAD $^+$ cells was acquired and presented through FlowJo (Treestar, Ashland, USA).

Fluorochrome-Labeled Inhibitor of Caspases Assay

To confirm apoptosis, caspase activity was assessed using a fluorochrome-labeled inhibitor of caspases assay (FLICA). Primary Th2 cells (0.5×10^6 cells/condition) treated with dexamethasone (0.1-1.0 μM) and/or PPT (1.0 μM) for 48 hours were harvested and washed in PBS FACS and then re-suspended in PBS FACS and 30X FAM-FLICA poly caspase inhibitor reagent (ImmunoChemistry Technologies, LLC; MN USA). Following an incubation period (30 min at room temperature) in absence of light, cells were washed twice with 1X Apoptosis Wash Buffer (ImmunoChemistry Technologies, LLC; MN USA) and centrifuged (1000 rpm for 5 minutes at room temperature). The cells were then re-suspended in 400 μL 1X Apoptosis Wash buffer, and 2 μL Propidium Iodide (PI) to exclude the dead cells, before data was acquired on the BD LSR II Flow Cytometer (BD, CA, USA) and analyzed on FlowJo (Treestar, Ashland, USA).

2.4 Transcriptional Activity

2.4.1 Plasmid Generation

Reporter constructs of varying lengths of the *CRTh2* promoter were previously generated by my colleagues. Nomenclature is relative to the translation start site (rATG) using human genome build GRCh8/hg38. *CRTh2*pro-4513/Luc contains the 1836 base pairs (bp) upstream of the transcription start site and was designed to study core transcriptional activation of *CRTh2*. Longer *CRTh2* promoter constructs (-7013, rATG) were generated to study the *CRTh2* SNP rs533116G>A located at -6388 and were named *CRTh2*pro-6388G/Luc and *CRTh2*pro-6388A/Luc. *CRTh2* promoter fragments were cloned into pGL3-Basic reporter vector (Promega, Madison, WI) using KpnI and NheI sites, and sub-cloned into DH5α *E. coli.*

2.4.2 Plasmid Growth

These *CRTh2* promoter constructs are high copy plasmids and were grown for 8 hours by inoculating 5 mL of Lysogeny broth (LB) supplemented with Ampicillin (100 μg/mL), agitated (300 rpm at 37°C), before being transferred into 100 mL of fresh LB (100 μg/mL Ampicillin) for 16 hours (300 rpm at 37°C). An EndoFree Plasmid Maxi Kit (Qiagen, Netherlands) was used to purify plasmids and DNA was eluted in 100 μL of endotoxin-free Tris EDTA buffer. The yield and purity were assessed using the Epoch Microplate Spectrophotometer (Biotek, VT, USA) and the software Gen 5 Analysis Software (Biotek, VT, USA) prior to dilution to 1 μg/μL.

Single and double digests (1 μg DNA) were performed with Kpn I and/or NheI in NEBuffer™ 1.1 (1X, New England Biolabs, ON, Canada), incubated at 37°C for 1 hour. Digests were electrophoresed on a 1% agarose gel to the separate the fragments based on size and confirm the integrity of each plasmid.

2.4.3 Transient Transfection by Electroporation

Transcriptional activity of CRTh2 was determined by transiently transfecting the CRTh2 promoter constructs into CCRF-CEM cells by electroporation. Transfection efficiency was assessed by co-transfection of thymidine kinase promoter-Renilla luciferase plasmid (pRL-TK; 11.5 ng) and *CRTh2*pro/Luc (20 μg or molar equivalent) with 10-minute incubations on ice for each plasmid. A square wave electroporator (ECM 830, Harvard Apparatus, MA, USA) was used with settings of 1 pulse 50 msec at 240 V. Cells (5.0×10^6 cells/condition) were then treated with dexamethasone (0.01-0.1 μM) in the presence or absence of PPT (1-10 μM) for 22 hours prior to being harvested and lysed in 250 μL of passive lysis buffer (1X PLB, Promega, WI, USA) and frozen.

2.4.4 Dual-Luciferase Promoter Assay

Frozen lysate was thawed and 10 μL of each sample was incubated with 50 μl of the Firefly luciferase substrate, luciferin, and repeatedly mixed before reading on the GloMax 20/20 Luminometer (Promega, WI, USA) with an integration time of 10 seconds. Renilla luciferase substrate, coelenterazine, (50 μL) was then added, mixed and read on the Luminometer. Total lysate protein was measured by standard curve generated using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, IL, USA). Results were expressed as relative luciferase units (RLU), normalized for transfection efficiency and protein concentrations using the following formula:

$$\text{RLU} = \text{Firefly Luciferase Count} \times \frac{\text{Mean Baseline Renilla}}{\text{Renilla Count}} \times \frac{\text{Mean Protein}}{\text{Protein Count}}$$

2.5 Statistical Analysis

Results are presented as mean ± standard error of mean (SEM), with the exception of results with only two experiments which were presented as deviation of individual data from the mean. One-way analysis of variance (ANOVA) or one-way repeated measures ANOVA were performed to compare independent and related groups, respectively. The one-way repeated measures ANOVA, alternatively referred as a randomized block design ANOVA, is similar to a paired *t*-test for multiple comparisons [315]. This statistical test accounts for day-to-day variation of the cell line and has been used by others [316]. Each experimental run, referred to as a block [315], had multiple measurements made in parallel, rationalizing the choice of statistical test. Multiple comparison analyses were performed using either Tukey's test, to calculate the confidence interval and identify statistical differences between means that are larger than the standard error, and/or Holm-Sidak test, to accept or reject the null hypotheses. A *p*-value less than 0.05 was indicative of a significant difference. Significance was assessed with SigmaPlot (Version 12.5; Systat Software Inc, CA, USA).

Chapter 3

3 Results

3.1 Th2 Cell Differentiation

The Cameron laboratory has a long history of differentiating human primary Th2 cells [317, 318] and so I began my project with the goal of creating newly differentiated primary Th2 cell lines. Despite a number of attempts (n=9), viable Th2 cells could not be generated. Naïve $CD4^+$ T cells were cultured in IL-2, IL-4, antibody against IFNγ and IL-12 and alternating cycles of activation (3 days on or 4 days off plates coated with antibody against CD3 and CD28). On day 14, if the amount of cells and predicted yield was feasible, $CRTh2^+$ cells were isolated and the enriched Th2 cells cultured in similar conditions, without IL-4 or neutralizing antibody. This protocol was previously shown to result in a doubling of cells (*i.e.*, growth rate = 2) [317, 318], however, only a few of the lines I differentiated maintained that growth rate and most cell lines died prior to day 14 (Fig. 3.1). Cells from donors 1 through 6 had reduced/negligible growth prior to isolating cells expressing CRTh2. Despite maintaining viability, there were not enough Th2 cells from donor 9 to complete a CRTh2 isolation with a suitable yield. Two sets of Th2 cells from donor 7 and 8 maintained steady growth until day 14, when Th2 cells were isolated. Unfortunately, these Th2 cells failed to expand following isolation. As such, experiments within this thesis were performed with a Th2 line differentiated by Emily MacLean Scott (Dr. Cameron's former M.Sc. student, University of Alberta) [318] or with the immortalized $CD4^+$ T cell line CCRF-CEM, an immortalized cell line previously shown to have features similar to Th2 cells [319].

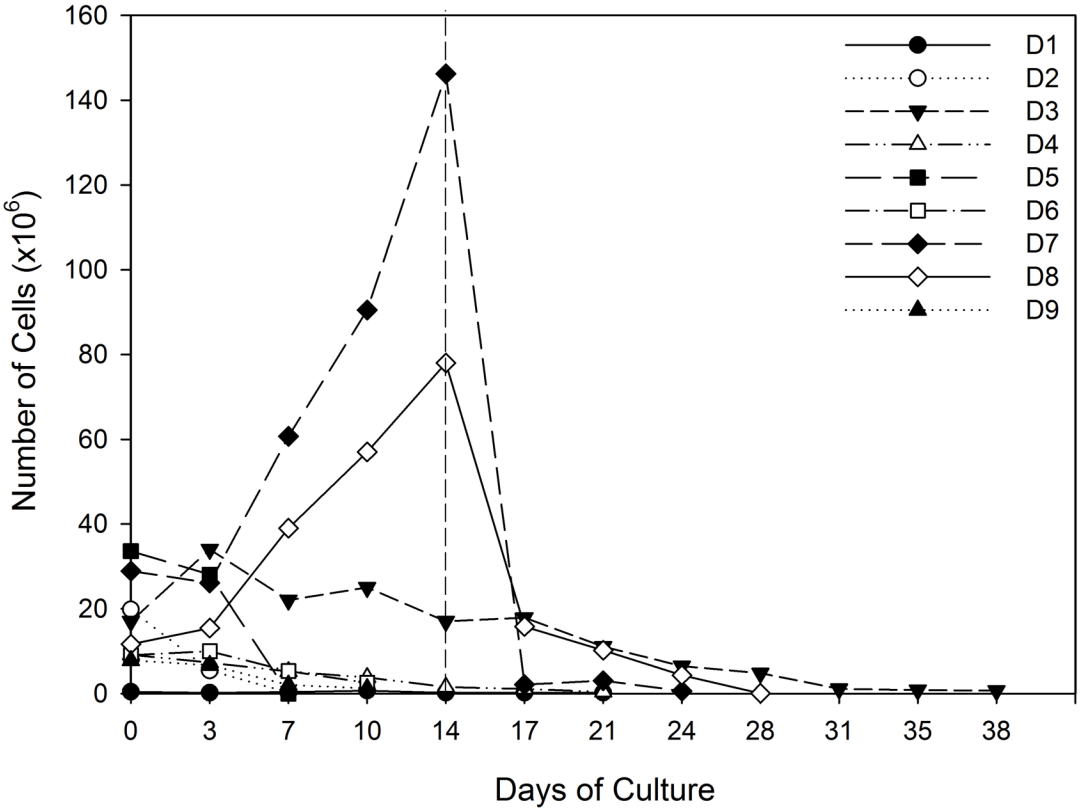

Figure 3.1: Growth of CD4$^+$ T Cells Culture in Th2 Differentiating Conditions

Primary naïve CD4$^+$ T cell were isolated from healthy donors (D) and cultured on alternating cycles of activation (3 days on plates coated with αCD3 and αCD28) followed by proliferation (IL-2, 4 days). Cell counts were performed using a microscopic hemocytometer and extrapolated to determine cell population quantity on days of reseeding (alternating on and off αCD3/28 coated plates). Isolation of CRTh2+ cells was performed on day 14 of culture (indicated by dotted line), if the number of cells was predicted to provide a feasible yield of Th2 cells. n=9.

3.2 ERα Agonist Dampens GC-Induced Apoptosis of Primary Th2 Cells

My colleagues have previously shown that treating primary Th2 cells with the synthetic GC dexamethasone (DEX) induces cell death through apoptosis [320]. These experiments were performed by quantifying the proportion of intact cells exhibiting positive staining for phosphatidylserine residues, which flip from the inner to the outer leaflet of the plasma membrane during apoptosis [188]. My research question centered on whether estrogen receptor signaling could influence GC-induced apoptosis. To test this, Th2 cells were cultured with DEX (0.1 – 1.0 μM) in the presence or absence of a selective ERα agonist (PPT, 1.0 μM) for 48 hours and cells stained with fluorochrome-conjugated Annexin V. This approach identifies phosphatidylserines on the outer and inner plasma membrane that occurs during the early stages of apoptosis and late stages of cell death, respectively. To exclude necrotic cells with ruptured plasma membranes, the cells were also stained with the nucleic acid stain, 7-AAD conjugated to a fluorophore. Positive staining was assessed by flow cytometry and the proportion of Annexin V^+/7-AAD^- cells were reported as apoptotic Th2 cells, gating as shown in Appendix 3.1.

Similar to previous reports [319, 320], I found that DEX treatment induced Th2 cell apoptosis. Phosphatidylserine levels measured by Annexin positivity were significantly increased with all DEX treatments alone, and significantly lower with PPT co-treatment (Fig. 3.2A). Approximately 10% of the cells stained positive for Annexin V when treated with vehicle and this was similar with PPT alone. Treatment with DEX induced apoptosis to approximately 44%, however, cells co-treated with PPT showed a reduction in Annexin V^+ cells to approximately 38%, on average 12% lower (6.7-17.9% dependent on DEX concentration) ($P<0.05$; Fig. 3.2A). A limiting factor of this analysis is the lack of specificity differentiating apoptotic and necrotic cells; therefore, apoptosis levels were also assessed by identifying Annexin V^+ cells and excluding 7-AAD necrotic cells (Annexin V^+/7-AAD) (Fig. 3.2B). Th2 cells treated with DEX had an increase in apoptosis from ~10% of non-treated cells to 30% ($P<0.05$; Fig. 3.2B). A reduction in apoptosis in DEX and PPT co-treated cells was apparent at all concentrations of DEX by a mean of 10% (6.1-13.3%) ($P<0.05$; Fig. 3.2B).

Since this is an indirect method of measuring apoptosis, these findings were confirmed by staining with a fluorochrome-labeled inhibitor of caspases (FLICA). This reagent binds and inhibits active caspase 1, 3, 4, 5, 6, 7, 8 and 9, the enzyme responsible for apoptosis [203]. The inhibitor is conjugated to a fluorophore detectable by flow cytometry. The conformational FLICA stain proved that DEX treatment significantly increased the quantity of activated caspases and that co-treatment of 0.1 μM DEX and 1.0 μM PPT showed significantly less caspase activation (Fig. 3.2C). Control and PPT alone had ~5% active caspase positive cells, while DEX treatment induced approximately 30% that was reduced to 12% with co-treatment with 1.0 μM PPT, a 48.3% reduction ($P<0.05$; Fig. 3.2C). In all analyses, PPT alone had no effect on caspase activation, suggesting the reduction was due to the combination of GC and ERα.

Analysis of $FLICA^+$ cells and Annexin $V^+/7\text{-}AAD^-$ showed comparable results. Th2 cells treated with DEX were 40% Annexin V^+ (Fig. 3.2A), however, both Annexin $V^+/7\text{-}AAD^-$ and $FLICA^+$ identified ~30% of the cells were apoptotic (Fig. 3.2B/C). These results suggest that the Annexin $V^+/7\text{-}AAD^-$ method has similar sensitivity as FLICA for detecting apoptosis.

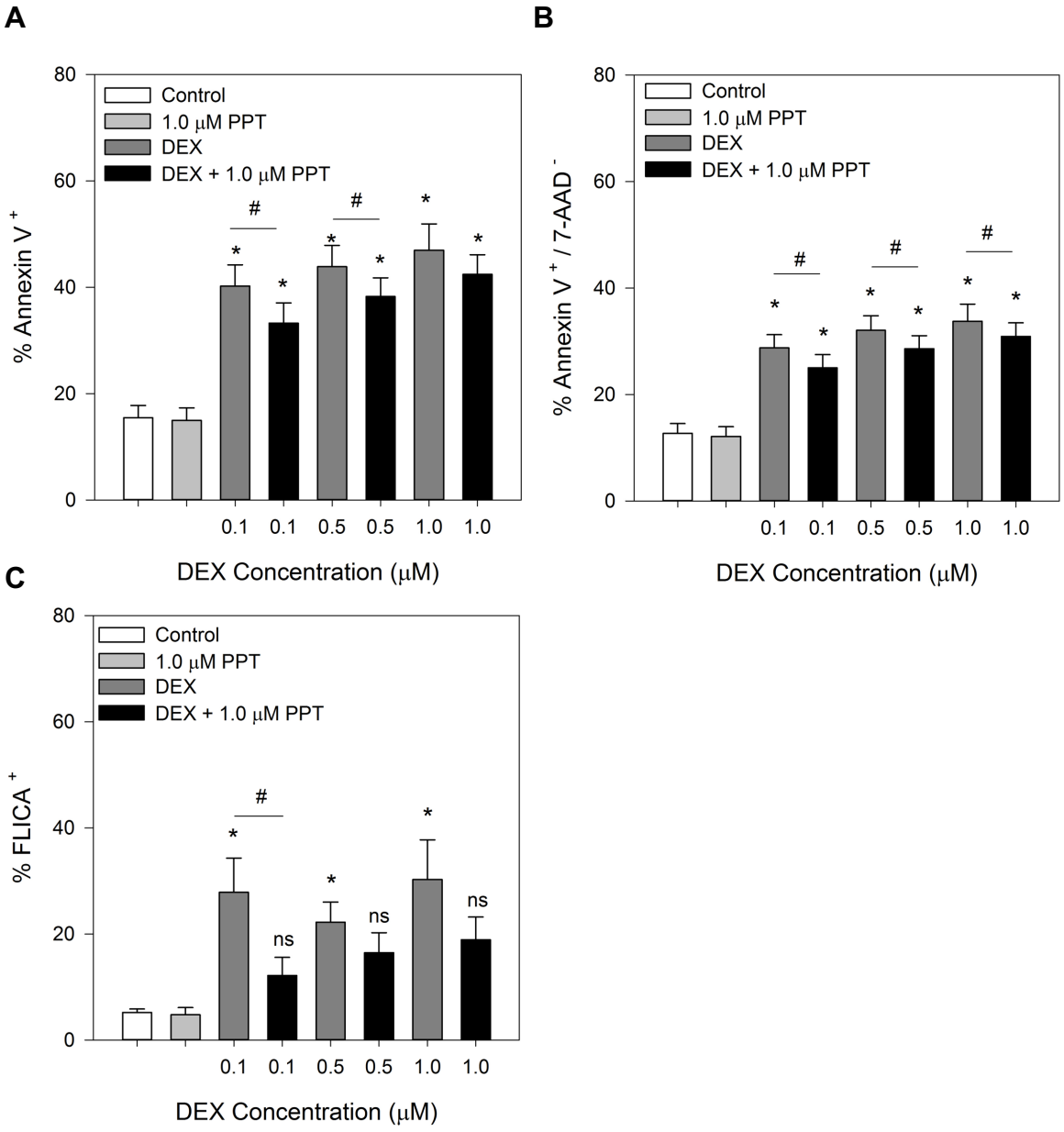

Figure 3.2: The Effect of PPT on DEX-Induced Apoptosis in Primary Th2 Cells

Measurement of apoptotic primary Th2 cells by flow cytometry after 48 hour exposure to DEX in the presence and absence of PPT. Percentage of Th2 cells that were Annexin V+ (A; n=11) or Annexin V positive and 7-AAD negative (B; n=11). Percentage of Th2 cells that were FLICA positive as assessed by flow cytometry under the same conditions was also assessed (C) (n=5). All data represents mean ± SEM. One-way repeated measures ANOVA were used to determine statistical differences. Asterisk (*) represent significance (P<0.05) *vs* control, number sign (#) represents statistical differences between conditions, and ns represents condition not significant *vs* control.

3.3 The Effect of E2 on GC-Induced Apoptosis is Similar to that of ERα Agonist in Primary Th2 Cells

My hypothesis was that estrogen reduces GC-induced apoptosis by increasing CRTh2 levels and sensitivity to PGD_2-mediated inhibition of apoptosis. Therefore, I next set out to confirm that the influence of the physiologically relevant sex hormone, estradiol (E2), was similar to the effect of the selective ERα agonist (PPT). Preliminary experiments were performed to determine the concentration of estradiol that cooperates best with DEX to induce CRTh2 levels. Primary Th2 cells were treated with DEX in the presence and absence of E2 for 24 hours and then stained for CRTh2 by flow cytometry (Fig. 3.3). Control Th2 cells were approximately 30% CRTh2 positive, and this was not significantly changed with treatment with either DEX or E2 alone. Interestingly, co-treatment with either 0.1 or 1.0 μM E2 and DEX increased surface CRTh2 levels to approximately 60%, a 2 fold increase ($P<0.05$; Fig. 3.3A). Normalization to control to eliminate variability within experiments indicated similar findings (Fig. 3.3B). Co-treatment with 0.1 μM DEX and 0.1 μM E2 increased surface levels by over 1.5 fold, and DEX with the higher 1.0 μM E2 had increased CRTh2 surface levels just under 1.5 fold. Based on these findings, 0.1 μM E2 was used for apoptosis assays.

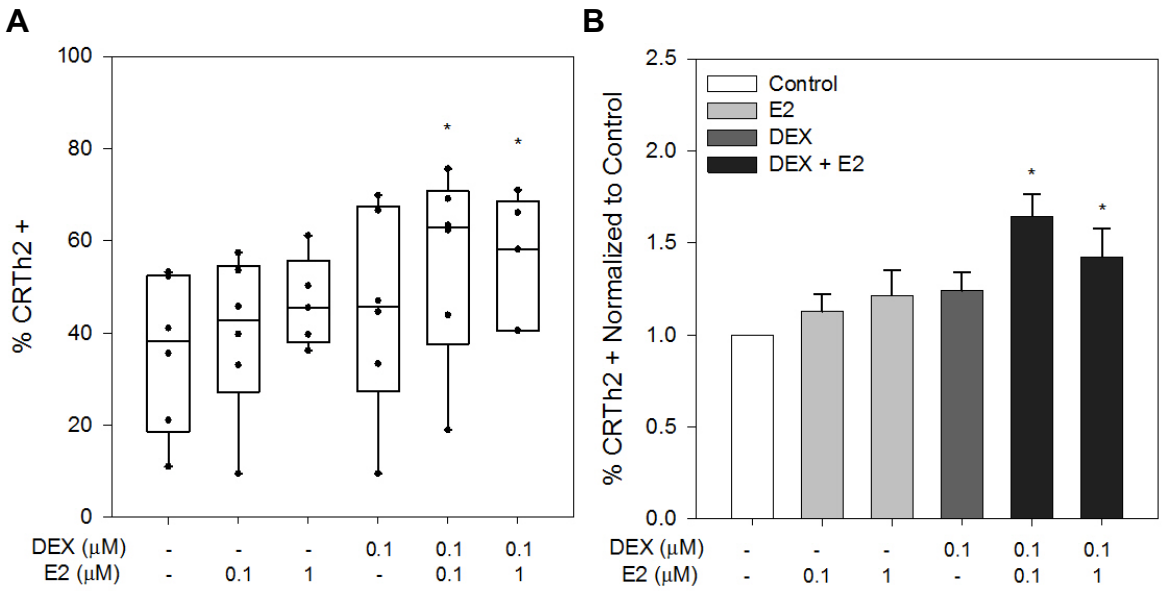

Figure 3.3: CRTh2 Surface Levels in Primary Th2 Cells in Response to E2

Primary Th2 were treated for 24 hours with DEX and/or E2. CRTh2 surface staining was then performed and assessed by flow cytometry. The proportion of the entire population that is CRTh2 positive (A) and proportion normalized to control (B) was identified and represented as mean ± SEM. Statistical differences were determined through one-way repeated measures ANOVA. Asterisk (*) represent significance (P<0.05) *vs* control. n=6.

Primary Th2 cells were then treated with DEX (0.1-1.0 μM) in the presence and absence of E2 (0.1 μM) for 48 hours. Cells were stained and the proportion of Annexin V^+/7-AAD- cells, identifying apoptotic cells, was assessed by flow cytometry. The outcome of this experiment was that E2 treatment alone had no effect on apoptosis; 15% of the Th2 cells were Annexin V^+/7-AAD^- with either vehicle control or E2 treatment. DEX treatment at all concentrations resulted in approximately 35% apoptotic cells and co-treatment with 0.1 μM DEX and 0.1 μM E2 significantly reduced the apoptosis achieved by DEX alone by 38.2 % to 20% (P<0.05; Fig. 3.4).

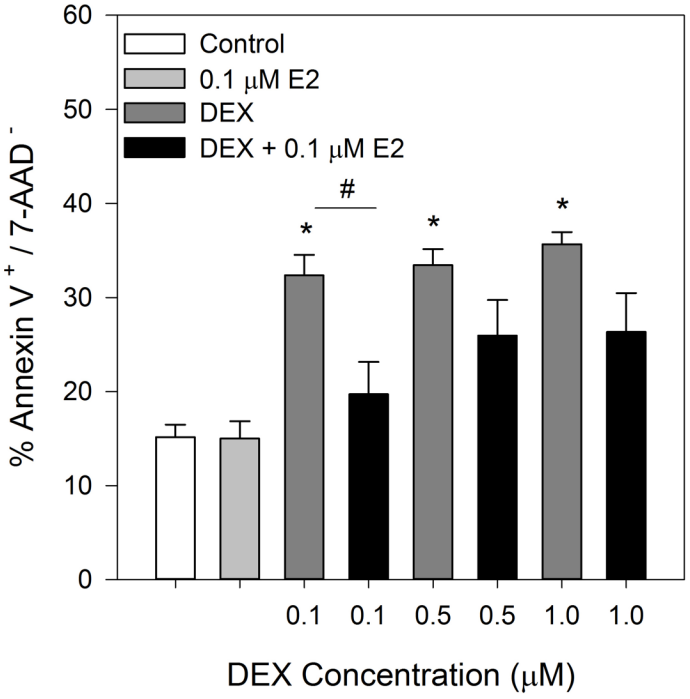

Figure 3.4: Apoptosis of Primary Th2 Cells in Response to E2 and DEX

Primary Th2 cells were treated with DEX and E2 for 48 hours to determine apoptosis and cell death using flow cytometry. Percentage of Annexin V positive 7-AAD negative cells were assessed. Data were represented as mean ± SEM. Statistical differences were determined by using one way ANOVA. Asterisk (*) represents statistical differences (P<0.001) *vs* control, and number sign (#) represents statistical differences (P<0.05) between conditions. n=4.

3.4 Identifying the Mechanism behind Reduced Apoptosis with GC and ERα Agonist Co-treatment

ERα Selective Agonist, but not E2, increases CRTh2 Surface Levels in CCRF-CEM Cells

CRTh2 surface levels were found to be elevated in primary Th2 cells after exposure to a combination of the E2 and DEX (Fig. 3.3). We required more Th2 cells to continue these experiments and so differentiation of new primary Th2 cell lines was attempted, but without success (Fig. 3.1). Therefore, experiments were transition to the immortalized lymphoblast T cell line, CCRF-CEM cells. The effects of DEX and E2 on CRTh2 surface levels were first examined, but were dissimilar to primary Th2 cells (Fig. 3.3). While control cells were ~60% CRTh2 positive, following 24 hour treatment with DEX (0.1-0.01 μM) and/or E2 (0.01-10 μM), no significant increases were found (Fig. 3.5). Although treatment of cells with 10 μM E2 did result in more CRTh2 positive cells compared to baseline levels (Fig. 3.5C), normalization of results to vehicle eliminated this effect (Fig. 3.5D).

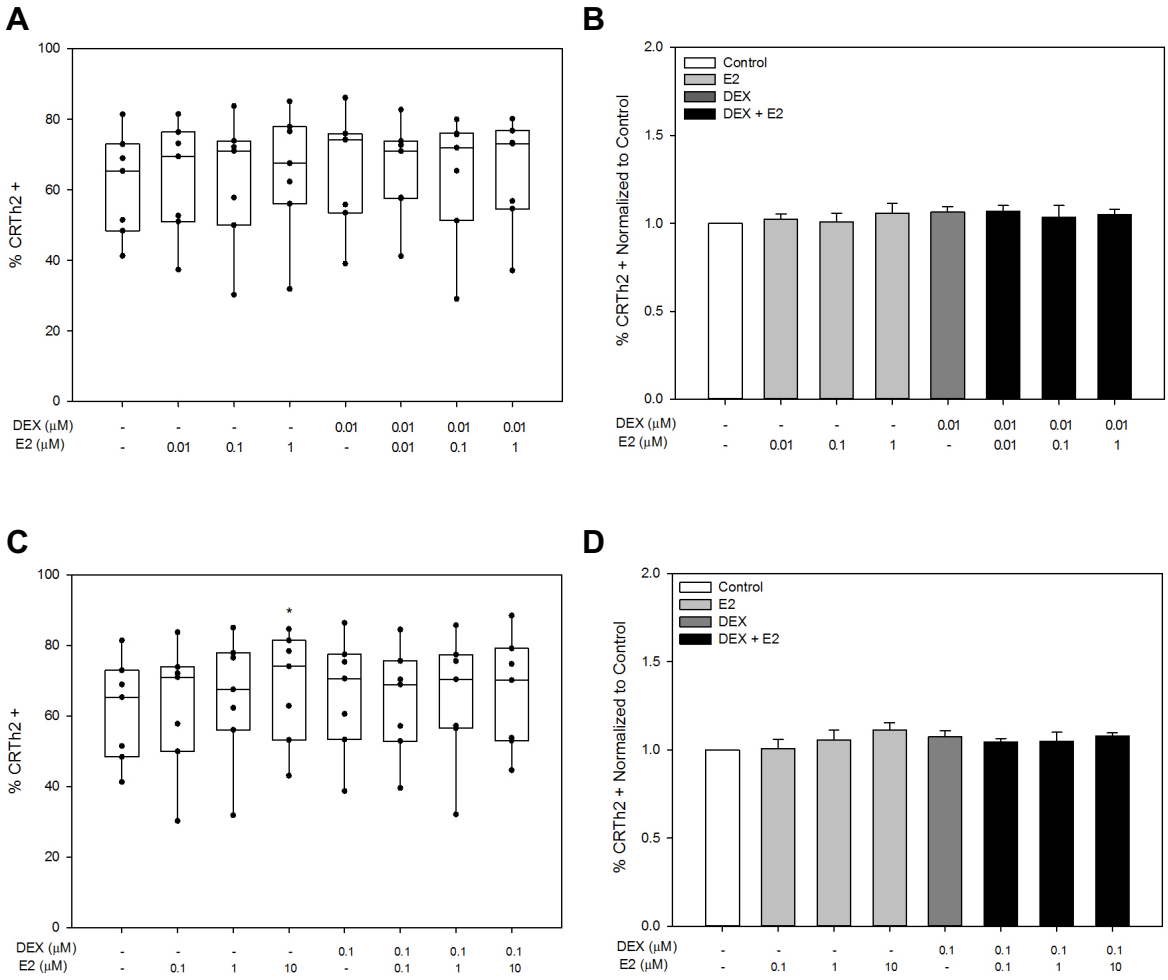

Figure 3.5: The Effect of DEX and E2 on CRTh2 Surface Levels in CCRF-CEM Cells

CCRF-CEM cells were treated with DEX and/or E2 for 24 hours. CRTh2 surface level was measured by flow cytometric analysis. DEX concentrations of 0.01 μM (A/B) and 0.1 μM (C/D) were co-treated with varying concentrations of E2 (0.01-10 μM). Data is represented as mean ± SEM of percentage of cell population expressing CRTh2 (A/C) and data normalized to control (B/D). Significance was determined through one-way repeated measures ANOVA. Asterisk (*) represents significance versus control. n=7.

The Cameron laboratory had previously shown that co-treatment with GC and a selective agonist for ERα (PPT) also increased CRTh2 levels on primary Th2 cells [1]. Therefore, I concurrently performed a dose response to determine if directly activating ERα with PPT induces CRTh2 surface levels on CCRF-CEM. These experiments showed that cells treated with DEX (0.01 or 0.1 μM) and/or PPT (1 or 10 μM) for 24 hours did exhibit increased surface levels of CRTh2. The total percentage of CRTh2 positive cells (Fig. 3.6A/C) was found to be significantly increased compared to vehicle control. Normalization to vehicle control further demonstrated these same findings; with significant increases in surface levels observed present in all DEX and PPT co-treatments (Fig. 3.6B/D).

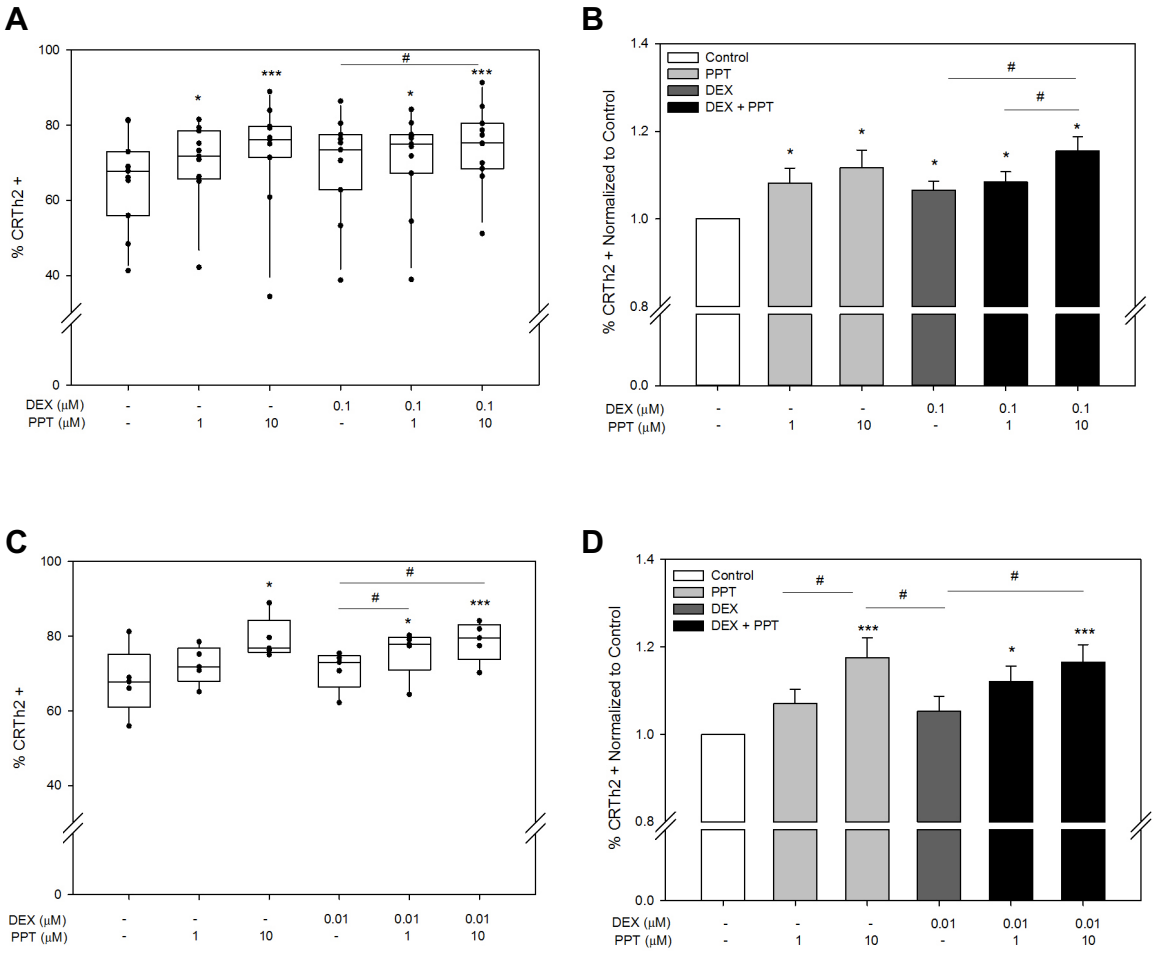

Figure 3.6: CRTh2 Surface Levels in Response to DEX and PPT Co-Treatment in CCRF-CEM Cells

CRTh2 surface levels on CCRF-CEM cells following treatment with DEX and/or PPT for 24 hours was determined by flow cytometry. DEX concentrations of 0.1 μM (A/B; n=11) and 0.01 μM (C/D; n=5) were used in combination with 1μM and 10 μM PPT. The data represents mean ± SEM of percentage of the entire cell population expressing CRTh2 (A/C), or the percentage normalized to control (B/D). Statistical differences were measured using one-way repeated measures ANOVA. Asterisks (* and ***) represent significance (P<0.05 and P<0.001, respectively) *vs* control, and number sign (#) represents significance between conditions.

CRTh2 Activation by PGD_2 does not Reduce Apoptosis in CCRF-CEM Cells

Since increased CRTh2 surface levels were observed following DEX and PPT co-treatment in CCRF-CEM cells and the activation of CRTh2 has been shown to mediate anti-apoptotic effects [8], I next examined if this was the mechanism behind the reduced apoptosis we found in primary Th2 cells with this co-treatment. CCRF-CEM cells were treated with various combinations of DEX (0.1-0.5 μM), PPT (1-10 μM) and PGD_2 (0.1-1.0 μM) for 48 hours before being stained for phosphatidylserine and nucleic acid with Annexin V, 7-AAD, respectively. Flow cytometric analysis of Annexin V^+/7-AAD^- cells identified the proportion undergoing apoptosis. At baseline conditions, 5% of the CCRF-CEM cell population were apoptotic; treatment with PPT or PGD_2 did not alter the proportion of Annexin V^+/7-AAD^- cells. DEX (0.1-0.5 μM) induced apoptosis of the CCRF-CEM cells (~20%), but PPT did not reduce DEX induced apoptosis in CCRF-CEM cells (Fig. 3.7), as it did in primary Th2 cells (Fig. 3.2). The ligand for CRTh2, PGD_2, was added in combination with DEX to activate the CRTh2 on CCRF-CEM cells (Fig. 3.7). Despite the study suggesting activation of CRTh2 inhibits apoptosis [8], there was no difference in the proportion of apoptotic cells in this co-treatment compared to cells treated with DEX alone. Similarly, CCRF-CEM cells treated with DEX and PPT as well as PGD_2 also showed no reduction in the levels of apoptosis compared to DEX treatment alone or DEX and PPT.

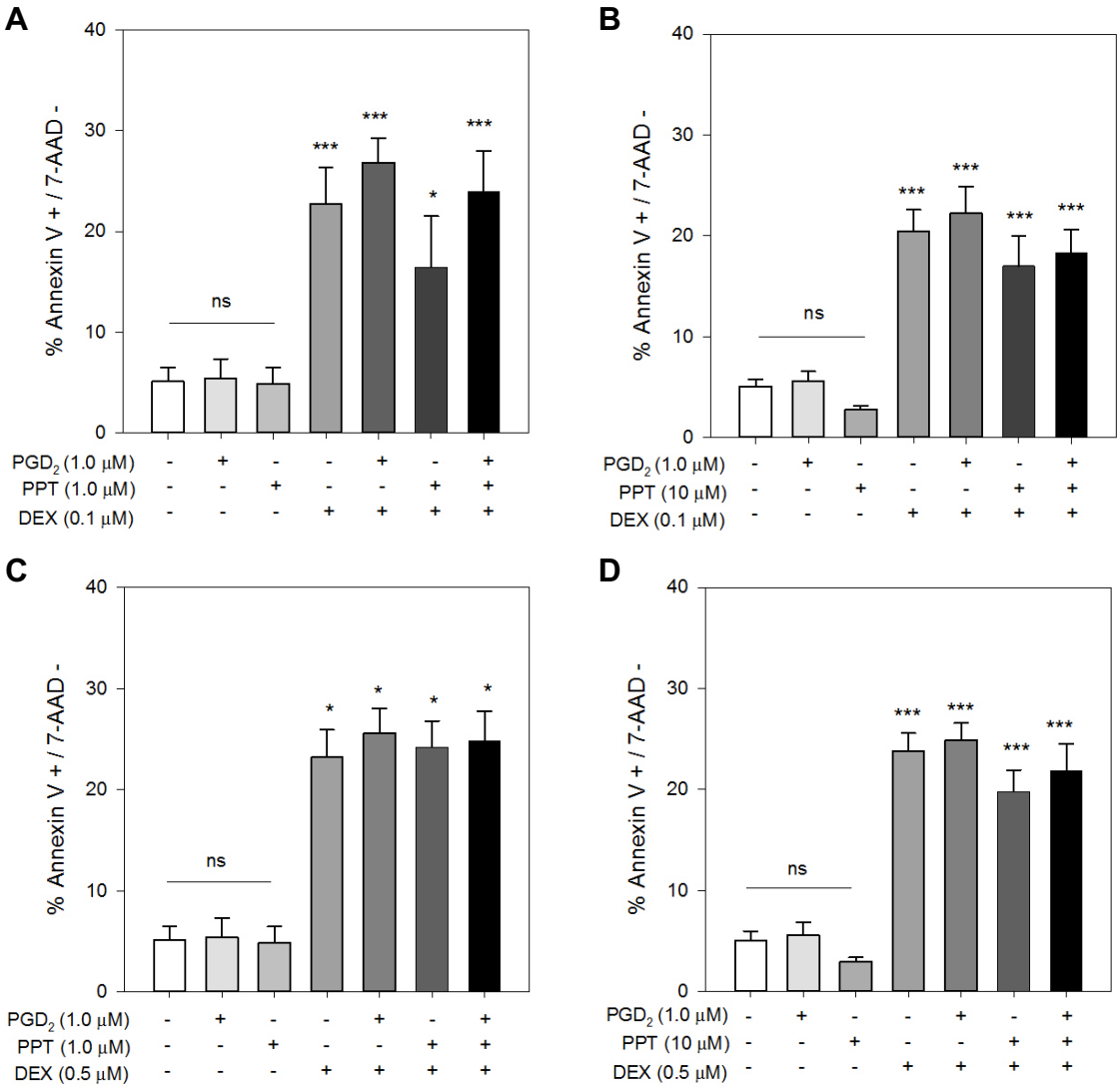

Figure 3.7: The Effect of CRTh2 Activation by PGD_2 on Apoptosis in CCRF-CEM Cells

CCRF-CEM cells were treated with PGD_2, DEX (0.1 µM), either 1.0 µM PPT (A; n=4) or 10 µM PPT (B; n=8) and various combinations of the three conditions for 48 hours to determine apoptosis and cell death using flow cytometry. Additionally, these experiments were completed with the higher concentration DEX (0.5 µM) and 1.0 µM PPT (C; n=4) or 10 µM PPT (D; n=6). Percentage of Annexin V positive 7-AAD negative cells were assessed for single treatments and co-treatments. All data were represented as mean ± SEM. Statistical differences were determined by using one-way repeated measures ANOVA. Asterisks (* and ***) represent significance (P<0.05 and P<0.001, respectively) *vs* control, and ns represents condition not significantly different *vs* control.

My colleagues previously found that *pre-treating* primary Th2 cells with DEX and PPT for 24 hours and then activating with PGD_2 increased type 2 cytokine production [1]. Therefore, I also tested a similar setup. Cells were pre-treated with DEX and PPT for 24 hours to upregulate CRTh2 and then PGD_2 was added with DEX for 48 hours and apoptosis measured with Annexin V^+/7-AAD^- staining. Pre-treatment was performed using 0.01 μM DEX and 10 μM PPT, as shown in Figure 3.6, the treatment seen to induce the largest upregulation of CRTh2 surface levels. Stronger apoptosis inducing concentrations of DEX (0.1-0.5 μM) as in (Fig. 3.2 and 3.7) and various PGD_2 (0.1 – 1μM) concentrations were chosen based on previous studies [1, 8]. The proportion of the cell population that was Annexin V^+/7-AAD^-, *i.e.* early apoptosis, increased from 2% in non-treated cells to 20% in those pre-treated but not activated (treated with 0.1-0.5 μM DEX) (Fig. 3.8). However, the addition of PGD_2 to activate the upregulated CRTh2 did not change the amount of apoptosis induced by DEX alone.

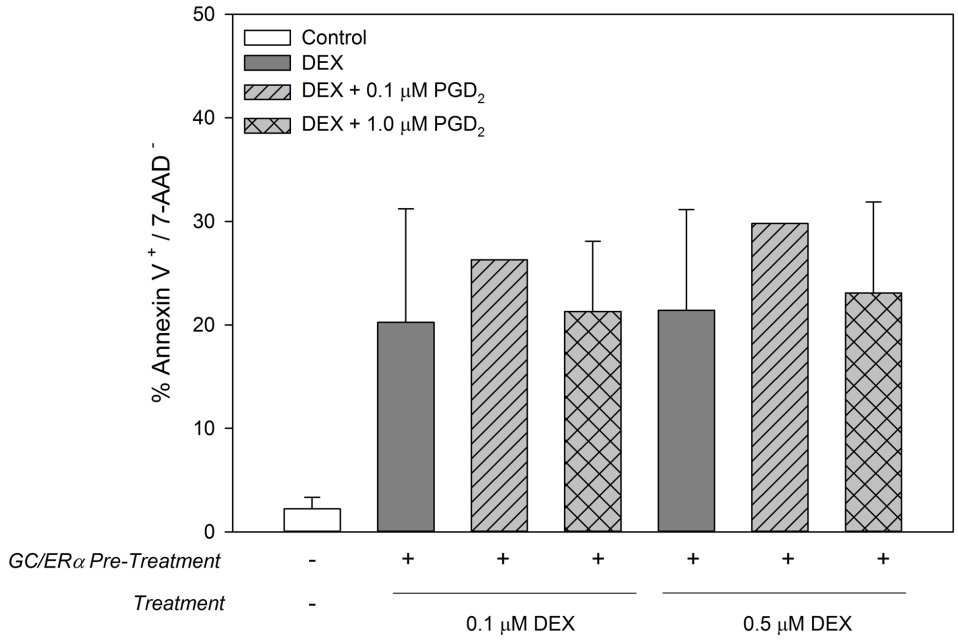

Figure 3.8: The Effect of DEX and PPT Pre-Treatment and CRTh2 Activation by PGD_2 on CCRF-CEM Cells

CCRF-CEM cells were pre-treated with 0.01 μM DEX and 10 μM PPT for 24 hours followed by treatment with a higher concentration of DEX (0.1-0.5 μM; n=2) and PGD_2 (0.1 μM, n=1; 1.0 μM; n=2). Apoptosis was assessed by flow cytometry staining and analysis of proportion of Annexin V^+ and 7-AAD^- cells. Data represented as mean ± standard deviation.

3.5 GC and ERα Agonist Regulate CRTh2 at the Level of Transcription

CRTh2 expression was initially shown to be a characteristic of Th2 but not Th1 cells [89] and later demonstrated to be regulated by GATA3 during Th2 cell differentiation [321, 322]. More recently, CRTh2 expression within ILC2 was also associated with GATA3 expression [323]. Despite the importance of the CRTh2 pathway to type 2 inflammation, relatively little is known regarding the molecular regulation of this gene.

The Cameron laboratory has shown that the CRTh2 promoter is transcriptionally activated by GATA3 [96] and this activity is modified by TCR activation and NFAT inhibition of GATA3 binding [318]. CRTh2 has also been shown to be regulated by genetic variation. Indeed, a number of SNPs have been shown to associate with increased risk of asthma and asthma severity [100] as well as other allergic phenotypes [102]. My colleagues have shown that a SNP within the *CRTh2* promoter 6388 bp upstream of the ATG (-6388G>A), is associated with the development of asthma and that the A allele increased CRTh2 expression and response to PGD_2 [103]. *In silico* analyses show that the CRTh2 (*PTGDR2)* promoter contains conserved regions containing a number of putative transcription factor binding sites as well as GREs, *i.e.* binding sites for GR, including one in the vicinity of *CRTh2*-6388G>A (Fig. 3.9). Therefore, this aim examined whether the GC and estrogen increase in CRTh2 expression occurs at the level of transcriptional activation and if the CRTh2 promoter SNP -6388G>A alters this activity.

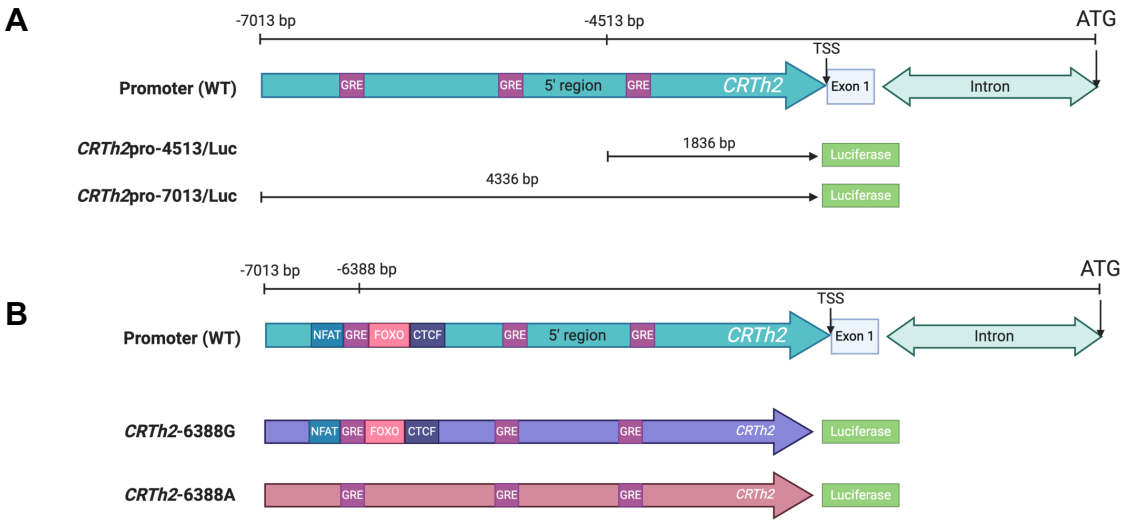

Figure 3.9: *CRTh2* Promoter Constructs

Mapping of the *CRTh2* promoter. Putative GRE sites identified using MatInspector analysis within the upstream region of CRTh2. *CRTh2*pro/Luc constructs generated of short (1836 bp) and long (4336 bp) length containing 1 and 3 putative GREs, respectively (A). Additional putative transcription factor binding sites and rs533116G>A location labeled on *CRTh2* promoter (B). Constructs representing the G (wild type) and A allele of the CRTh2 promoter (*CRTh2*pro-6388G/Luc and *CRTh2*pro-6388A/Luc) were generated and schematics identify variation between predicted transcription factor binding sites. Abbreviations are as followed: base pairs, bp; chemoattractant receptor-homologous molecule expressed on T helper 2 cell, CRTh2; conserved region, CR; forkhead box transcription factors, FOXO; glucocorticoid response element, GRE; luciferase, Luc; nuclear factor of activated T-cells, NFAT; promoter, pro; transcription start site, TSS; wild type, WT. *Created with Biorender.com.*

Transcriptional regulation of CRTh2 was studied using CRTh2 promoter reporter constructs previously generated by our laboratory. The *CRTh2*pro-4513/Luc contains the 1836 bps immediately upstream of the transcription start site (TSS), 4513 bps relative to the ATG (rATG) and has 1 putative GRE. The *CRTh2*pro-7013/Luc contains the 4336 bps upstream of the TSS, 7013 bp rATG and contains 3 putative GRE sites (Fig. 3.9A).

To test my hypothesis that GC and ERα agonist induce CRTh2 promoter activation, constructs were transiently transfected into CCRF-CEM cells treated with DEX (0.1 μM) in the presence and absence of PPT (1.0 μM) for 22 hours and luciferase activity measured. Promoter activity of both constructs was found to be significantly increased when control was compared to DEX alone ($P<0.05$), and even more significantly increased with DEX and PPT co-treatment ($P<0.05$; Fig. 3.10). Specifically, promoter activity at baseline level was approximately 750 RLU and treatment with DEX increased promoter activity to ~1500 RLU. Promoter activity of the shorter construct (*CRTh2*pro-4513/Luc) was increased to ~1750 RLU with DEX and PPT co-treatment (Fig. 3.10A), and the longer construct (*CRTh2*pro-7013/Luc) to ~2000 RLU (Fig. 3.10B); however, no significant difference was found between the different CRTh2 promoter constructs.

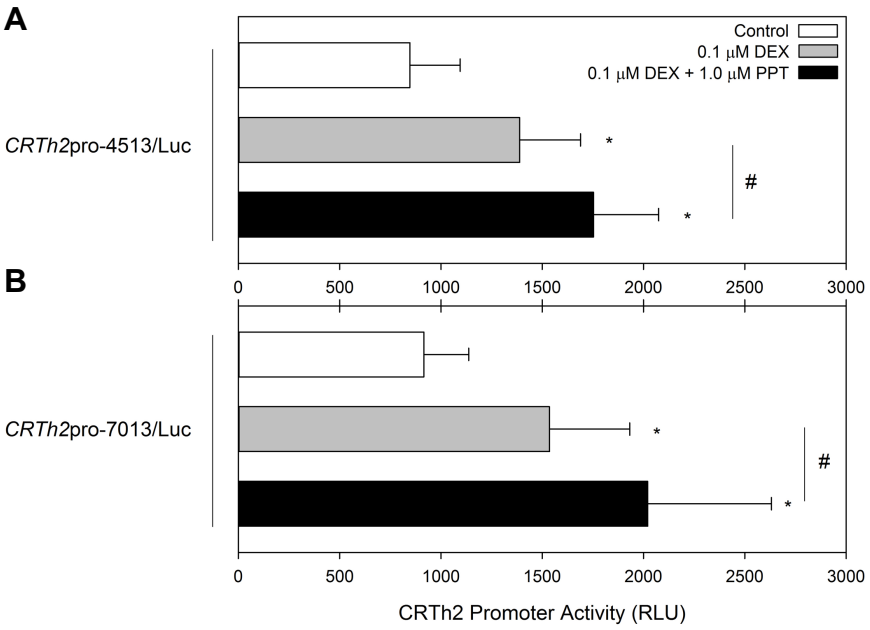

Figure 3.10: *CRTh2* Promoter Activity in Response to DEX and PPT Co-Treatment in CCRF-CEM Cells

CCRF-CEM cells were transiently transfected with CRTh2 luciferase reporter constructs (*CRTh2*pro/Luc) and treated with DEX and PPT for 24 hours. Transcriptional activity of the *CRTh2* promoter was measured by fold increase of relative luciferase activity, corrected for total protein abundance (BCA assay) and normalized to Renilla. Data for the shorter construct (*CRTh2*pro-4513/Luc; A; n=5) and longer construct (*CRTh2*pro-7013/Luc; B; n=10) were recorded as relative luciferase units (RLU) and represented as mean ± SEM. Statistical differences were determined by using one-way repeated measures ANOVA. Asterisk (*) represents significance ($P<0.05$) *vs* vehicle and number sign (#) represents significance ($P<0.05$) between conditions.

3.6 Genetic Variation Influences CRTh2 Transcriptional Activation in Response to GC and ERα Agonist

The SNP, *CRTh2*-6388G>A, has been associated with increased CRTh2 expression and allergic asthma [103]. Here, the promoter activity of the *CRTh2*pro-7013/Luc construct with either allele, *CRTh2*pro-6388G/Luc and *CRTh2*pro-6388A/Luc (Fig. 3.9B), were compared. Mapping of putative transcription factor binding sites identified an unperturbed GRE site 6 bp from *CRTh2*-6388A; however, loss of a variety of other transcription factor binding sites was predicted, including a site that binds NFAT, a transcription factor capable of inhibiting CRTh2 expression [318]. This would indicate less competition for GR binding in the A allele compared to the G allele.

To test if there are allele specific differences in the effect of GC and ERα signaling on CRTh2 transcription, CCRF-CEM cells were transiently transfected with the *CRTh2*pro-6388G/Luc or *CRTh2*pro-6388A/Luc construct and treated with DEX (0.1 μM) and/or PPT (1.0 μM). *CRTh2*pro-6388G/Luc showed increased luciferase activity after DEX treatment, from 1000 RLU of control to 1500 RLU (Fig. 3.11A). The addition of PPT (1.0 μM) co-treatment did not alter promoter activity from that induced by DEX alone (~1.5-fold). Similar trends were found with analysis normalized to control; both DEX alone, and in the presence of PPT resulted in ~2 fold induction of promoter activity (Fig. 3.11B). This analysis minimized experimental variation. Similar to the G allele, cells transfected with *CRTh2*pro-6388A/Luc promoter had a transcriptional activity of 1000 RLU in control cells, and 1500 RLU with DEX treatment alone, though this was not a significant increase (Fig. 3.11A/C). Conversely, cells transfected with the *CRTh2*pro-6388A/Luc promoter did exhibit a significant increase in transcriptional activity in cells treated with both DEX (0.1 μM) and PPT (1.0 μM), compared to DEX alone (2.5-fold, normalized to control (Fig. 3.11D).

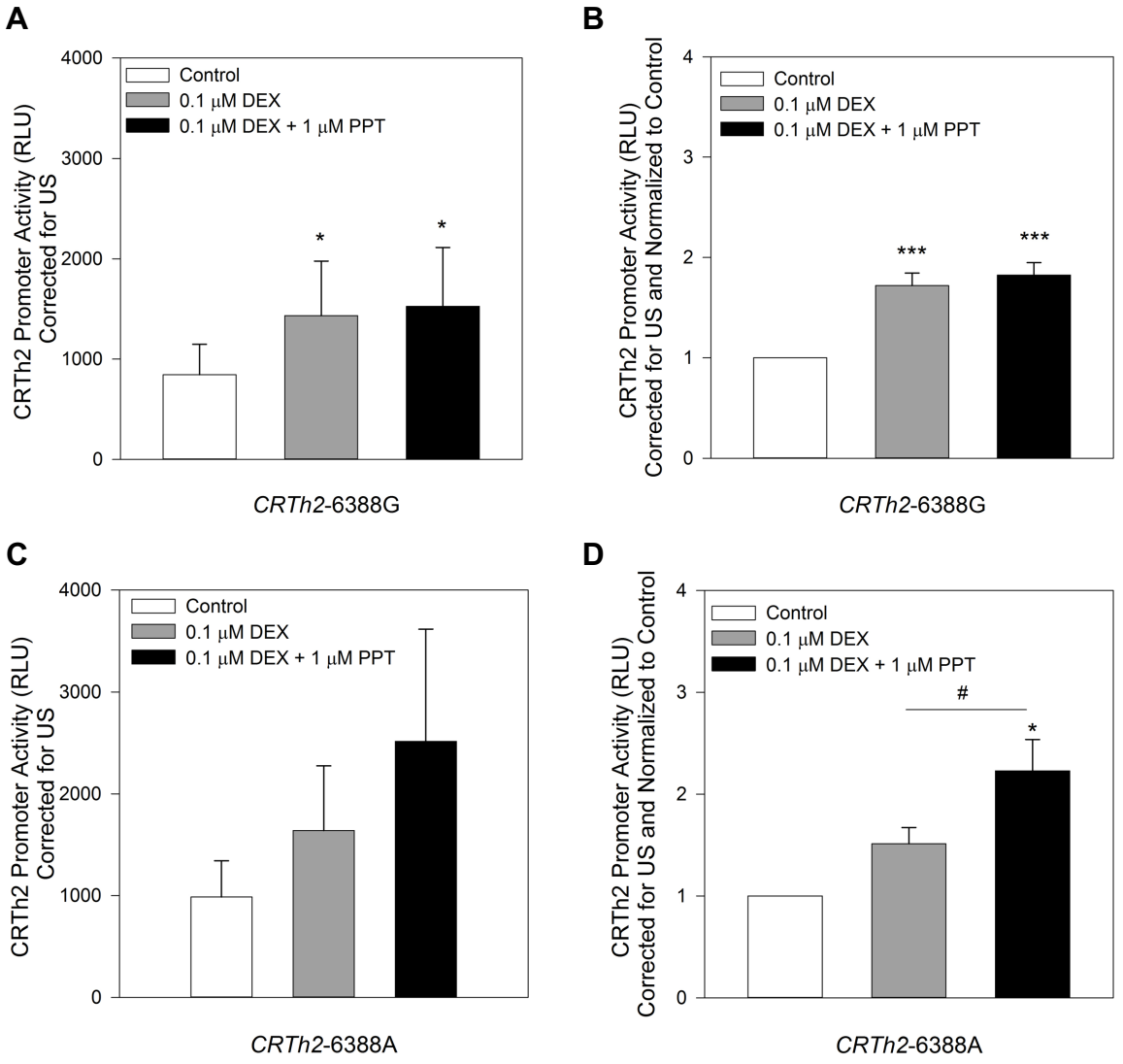

Figure 3.11: Transcriptional Responses to DEX and PPT in *CRTh2*-6388G and A Alleles

CCRF-CEM cells were transiently transfected with *CRTh2* promoter luciferase reporter constructs (*CRTh2*pro-6388/Luc) of either *CRTh2*-6388G (A/B) or *CRTh2*-6388A (C/D) allele. They were then treated with DEX in the presence and absence of PPT for 24 hours. CRTh2 promoter activity was corrected for total protein abundance (BCA assay) and normalized to Renilla. Data, recorded as relative luciferase units (RLU), was measured by fold increase (A/C) and normalized to control (B/D). All data were represented as mean ± SEM. Significance was determined by one-way repeated measures ANOVA; asterisks (* and ***) represents statistical differences ($P<0.05$ and $P<0.001$, respectively) *vs* control and number sign (#) represents differences between conditions. n=5.

ERα Agonist Enhances GC Mediated Activity of CRTh2-6388A, but not CRTh2-6388G

To further assess the influence of ERα agonist on GC induced activity of the CRTh2 genetic variants, DEX and PPT condition was normalized to DEX alone. Figure 3.12 shows that while maximum activity of cells transfected with the *CRTh2*pro-6388G/Luc promoter was seen with DEX (0.1 μM) and no added activity was shown with PPT (1.0 μM) co-treatment, maximum activity of the *CRTh2*pro-6388A/Luc promoter required both DEX and PPT (Fig. 3.12A).

Experiments examining surface expression of CRTh2 on CCRF-CEM cells identified that 0.01 μM DEX and 10 μM PPT were the optimal concentrations to induce the largest increased in CRTh2 (Fig. 3.6C/D). Preliminary experiments show that CCRF-CEM cells treated with these concentrations of DEX and PPT compared to DEX alone exhibited greater increases in promoter activity for the *CRTh2*pro-6388A/Luc (~2-fold) compared to G (~1-fold) (Fig. 3.12B).

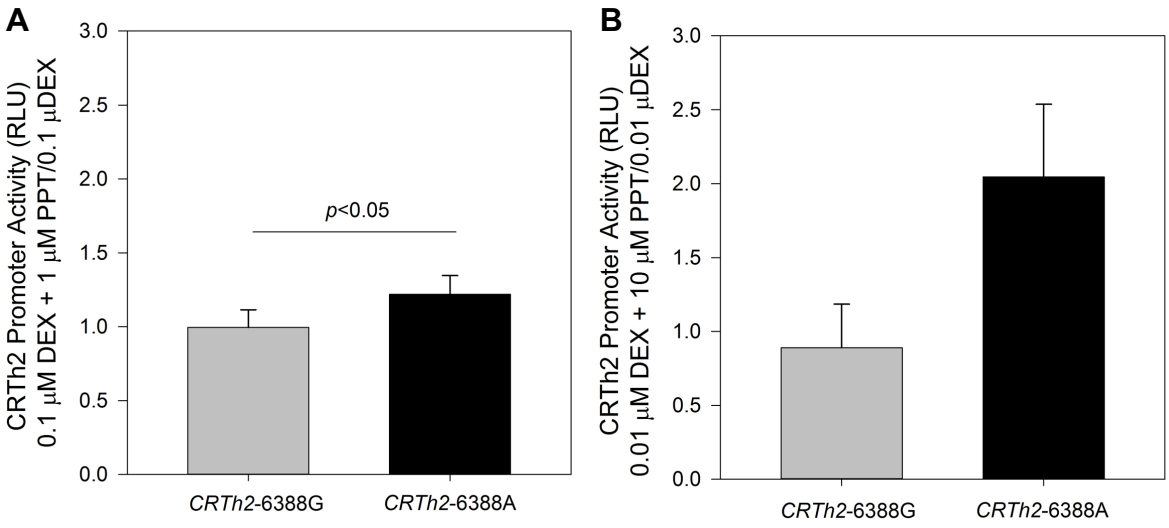

Figure 3.12: *CRTh2*-6388G>A Comparison of Increase in *CRTh2* Promoter Activity with PPT Addition from DEX

CCRF-CEM cells were transiently transfected with *CRTh2* luciferase reporter constructs (*CRTh2*pro-6388/Luc) of G or A allele and then treated with DEX and/or PPT for 24 hours. Promoter activity was corrected for total protein abundance (BCA assay) and normalized to Renilla. Data, recorded as relative luciferase units (RLU), was measured by fold increase and promoter activity induced by DEX+PPT was normalized to DEX. Co-treatment of 0.1 μM DEX and 1.0 μM PPT (A; n=5) was represented as mean ± SEM, and co-treatment with 0.01 μM DEX and 10 μM PPT (B; n=2) was represented as mean ± standard deviation. The statistical difference (A) were determined by a t-test.

Chapter 4

4 Discussion

Women are more likely to have severe asthma than men [6]. A growing body of evidence suggests this sex difference is associated with Th2 cell response to GC, both its effect on apoptosis as well as gene regulation. Differential expression of CRTh2 may play a central role, since activation of this receptor supports Th2 cell survival and type 2 cytokine expression. This thesis investigated whether Th2 cell response to GC was altered by the presence of estrogen and if this combined exposure increased CRTh2 expression. Collectively, the data suggest that GR and ER crosstalk in Th2 cells affects their function and may differ based on *CRTh2* genotype.

4.1 Estrogen Receptor Signaling Reduce GC-Induced Apoptosis

Previously, an ERα selective agonist was reported to reduce DEX induced cell death, though whether the effect was due to apoptosis was unclear [1]. My thesis continued this line of investigation and showed that ERα agonist reduces GC mediated cell death by attenuating apoptosis (Fig. 3.2). Multiple methods were used to assess ERα agonist effects. Examining Annexin V^+ alone showed approximately 10% more positive cells than with Annexin V^+/7-AAD^- and FLICA staining, indicating the latter analyses were more sensitive and specific measurements of early apoptosis. Both stains were performed within the same experiment, as a head-to-head comparison. Despite the similarities between the percentage of Annexin V^+/7-AAD^- and FLICA following GC treatment, differences were observed. A larger reduction was seen with the ERα agonist (PPT) and 0.1 μM DEX with the FLICA experiments, however, no reduction was found with co-treatment of all other concentrations of DEX. This may be due to FLICA being an activated caspase assay with a short window of opportunity for detection, suggesting that apoptosis induced by higher concentrations of GC needed to be measured earlier than lower concentrations to ensure the cell had not yet died. Moreover, a poly caspase FLICA was used, the reagent binding covalently to caspase-1, -3, -4, -5, -6, -7, -8, and -9 [324]. It is important to note that

caspase-1, -4, and -5 are responsible for pyroptosis [325]; therefore, we are only able to confirm the induction and reduction of programmed cell death, and not differentiate between apoptosis and pyroptosis. Future studies should consider the use of apoptotic specific caspase-3, -6, -7, -8, and/or -9 FLICAs [325]. These results suggests that Th2 cells exposed to both GC and ER agonist, as would occur within a female, may be less steroid responsive to the apoptotic effects of GC than Th2 cells exposed to GC alone, similarly to what would occur in males.

These results build upon previous studies regarding the pro-inflammatory effects of ERα, as they suggest that ERα is in fact reducing the anti-inflammatory effect of GC. ERα antagonists are used to treat breast cancer, as ERα signaling has been found to stimulate proliferation [270, 326]. Moreover, in estrogen free environments, treatment with a neurotoxin reduces the ratio of pro-apoptotic (BAD) and anti-apoptotic (Bcl-2) proteins, thereby inducing apoptosis [327]. However, ERα but not ERβ signaling was found to maintain the ratio to levels as if no neurotoxin was present, implying a protection against apoptosis effect [327]. This is an example of the Bcl-2 rheostat in action, the balance of pro- and anti-apoptotic proteins [328, 329]. There may be a similar disequilibrium caused by GC and ERα; GCs increase pro-apoptotic proteins [330], whereas ERα signaling increase anti-apoptotic proteins [331]. The results observed herein suggest the anti-apoptotic effects of ERα signaling are dampening the impact of GC.

The use of a selective agonist (PPT) allowed examination specifically of the effect of ERα, however, the relevance of the data is limited by the chemical nature of the compound. A more physiologically relevant approach would be to examine the effect of the sex hormone, β-estradiol (E2), which signals through both ERα and ERβ and exhibits differential effects on cell growth and apoptosis, depending on the receptor isoform [332]. E2-ERα activate PI3K/Akt and ERK/MAPK, responsible for preventing the apoptotic cascade and progressing the cell cycle, while E2-ERβ phosphorylates pathways involved in activating caspase-3 and inducing apoptosis [332]. Additionally, E2 has been found to increase the anti-apoptotic protein Bcl-2 [331]. Therefore, the effect of E2 was also examined and revealed that CRTh2 surface expression on primary Th2 cells was increased by co-

treatment with DEX and E2 treatment (Fig. 3.3) similar to DEX and PPT [1]. These findings are similar to previous work showing increased CRTh2 expression on primary Th2 cells following treatment with GC and the ERα agonist by flow cytometry as well as mRNA and total protein abundance [1].

Examining E2 and GC on CRTh2 levels also acted as a dose-finding experiment to assess the concentrations of E2 needed to impact GC-induced apoptosis. Analysis confirmed that E2 co-treatment with GC significantly reduced apoptosis compared to GC alone (Fig. 3.4), similar to findings with the selective ERα agonist PPT (Fig. 3.2). E2 treatment alone did not alter level of apoptosis, though co-treating with GC and E2 resulted in significantly fewer apoptotic cells than GC alone. Again, these results suggest Th2 cells in an estrogen-rich environment are less responsive to the apoptotic effects of GC than those in an estrogen-free/low environment. Contrary to the findings with ERα agonist, co-treatment with GC and E2 resulted in apoptosis levels no different than control, suggesting the effect of E2 was stronger than the selective ERα agonist.

These data highlight and build upon existing evidence of the pro-inflammatory and anti-apoptotic nature of estrogen. In fact, Huber et al. found that E2 treatment of Th2 cells increased Bcl-2, an anti-apoptotic factor, and suggested this may be the cause of the enhanced survival of Th2 cells in females and not males during viral myocarditis [269]. Although the apoptotic trigger differed, these findings show the anti-apoptotic effects of E2. It is important to note, however, that these studies identified a protective effect with estrogen alone, in contrast to my study which suggests the effect was due to interfering with GC (Fig. 3.4). Not only has the female sex hormone been shown to have an anti-apoptotic effect, but E2 has also been shown to promote proliferation of epithelial cells in mammary glands through modification of cell cycle genes [333]. In fact, estrogen has long been shown to play a strong role in the development and growth of breast cancer [334, 335], and is the basis for the use of the anti-estrogen tamoxifen as a treatment for breast cancer [336]. Additionally, global gene expression profiling identified that in ER positive breast cancer cells, in response to E2, growth factor genes were up-regulated and pro-apoptotic genes were down-regulated [271]. Combined, these studies illustrate the proliferative effects of estrogen, an area of research that needs to be further investigated in

relation to GC. Collectively, this builds upon the knowledge that estrogen acts to inhibit apoptosis of cells as well as promote growth. Further research needs to be conducted to determine the specific effects of GC and E2 on pro- and anti-apoptotic genes in order to understand the effects that the co-treatment has on the apoptotic cascade.

A limitation of this study is that the data showing that estrogen reduces GC-induced apoptosis of primary Th2 cells could only be performed on a single cell line. Multiple attempts were made to substantiate these findings in primary Th2 cells, using protocols developed and previously employed by the Cameron laboratory [317, 318]. T cells were isolated from peripheral blood and cultured in Th2 polarizing conditions (n=9). Contrary to positive differentiations of preceding studies [317, 318], none of the cell lines exhibited robust growth (Fig. 3.1). Unanticipated obstacles were faced and possibly the cause of the cell death. These include incubator contamination and CO_2 set-point errors resulting in reduced CO_2. Mammalian cell culture requires CO_2 to provide nutrients for cell growth and pH homeostasis [337]; therefore the cells may have been at a nutritional deficit. As such, further experiments with Th2 cells differentiated from a number of donors would help to validate the findings that estrogen reduces GC-induced apoptosis. It would be particularly interesting to perform head-to-head comparisons of Th2 cells derived from male and female donors, which would reveal differences related to the sex of the cells.

4.2 The Effects of Estrogen Signaling on CRTh2 in CCRF-CEM Cells

Previously, Xue et al. found that activation of CRTh2 by PGD_2 inhibits apoptosis induced by IL-2 deprivation [8]. This thesis examined if CRTh2 activation could inhibit GC-induced apoptosis and whether the upregulation of CRTh2 (induced by GC and ERα agonist) could enhance this effect. Due to difficulties with differentiating primary Th2 cell lines (Fig. 3.1), these experiments were performed on the immortalized lymphoblast T cell line, CCRF-CEM, with the expectation of being repeated in the future on primary Th2 cells. Unlike primary cells, however, CRTh2 surface levels were not increased by co-treatment with DEX and E2 (Fig. 3.5).

CRTh2 surface levels were previously seen to be increased on primary Th2 cells by GC and the selective ERα agonist (PPT) [1]. Since E2 can act on both ERα and ERβ, we also examined the influence of PPT and found that co-treatment of CCRF-CEM cells with GC and this ERα agonist significantly increased CRTh2 surface levels (Fig. 3.6). While lower concentrations clearly showed an additive effect of these two agents (Fig. 3.6C/D), higher concentrations of either GC alone (0.1 μM DEX) or ERα agonist alone (10 μM PPT) had increased CRTh2 levels compared to baseline (Fig. 3.6B). This could suggest that each agent plays a role in inducing CRTh2. Interestingly, the finding that the higher concentration of ERα agonist increased CRTh2 expression builds upon existing evidence that estrogen drives and inhibits both Th2 and Th1 cytokines and mediated diseases, respectively [338], as CRTh2 is a marker for Th2 cells [95].

These experiments revealed the surprising result that in CCRF-CEM cells, E2 and ERα agonist had different effects. This may be related to differential expression of ERα and ERβ in primary Th2 and CCRF-CEM cells. E2 binds to ERα and ERβ homo- or heterodimers [283]. Both receptors are encoded by different genes and have different properties [280, 281]; therefore not only will expression vary, but the functional responses do as well. Indeed, ERα and ERβ appear to have different effects on inflammatory responses in the context of asthma; ERα signaling was shown to be pro-inflammatory, compared to the anti-inflammatory effects of ERβ signaling. Experiments using selective agonists and antagonists for the specific receptors have validated that ERα is involved in increasing type 2 cytokines as well as eosinophils [261, 293] and ERβ relaxes airway smooth muscle as well as induces apoptosis though increasing pro-apoptotic factors [270, 294]. Specifically, one study found that treatment with E2 increased inflammatory effects of $CD4^+$ cells from male mice, and testing indicated these cells expressed ERα, thereby demonstrating that when the sex hormone E2 binds to ERα, pro-inflammatory responses are the result [261]. Based on these studies and my results showing that ERα activation increases CRTh2, a receptor with inflammatory capabilities [8, 68, 109], treatment with a selective ERβ agonist may in fact reduce CRTh2 expression. The data from primary Th2 cells suggests they may have a higher ratio of ERα to ERβ than CCRF-CEM cells. This

could have resulted in E2 binding more to ERβ dimers than ERα in CCRF-CEM cells, leading to the differing outcome in the two cell lines. This is an area for future research: to assess the effects of ERβ agonist treatment alone, and in combination with GC. Ideally, measurement of *ESR1* and *ESR2* levels in both primary Th2 cells and CCRF-CEM cells and comparison of the ratio between the two cell types.

Baseline CRTh2 surface levels for CCRF-CEM were higher than those seen in primary Th2 cells; non-treated CCRF-CEM cells were approximately 70% CRTh2 positive (Fig. 3.5), compared to the 40% positivity of primary Th2 cells (Fig. 3.3). Differences between immortalized and primary cells have been shown elsewhere. The immortalized Sertoli cell line (MSC-1) is morphologically and genetically similar to primary Sertoli cells, however, inconsistent results were found between MSC-1 cell line and primary Sertoli cells [339, 340]. Therefore, while the goal was to use CCRF-CEM cells as a model Th2 cell line, these experiments reveal they have limitations that may include differences in expression of ERα, ERβ or GPER.

4.3 PGD_2 does not Reduce GC-Induced Apoptosis

Building on the existing evidence that CRTh2 activation is anti-apoptotic [8], this thesis proposed that the increased expression of CRTh2 following GC and/or ERα agonist may be protective against GC-induced apoptosis. The experiments performed in CCRF-CEM cells, however, did not validate this hypothesis. Firstly, in this cell line the ERα agonist did affect the level of apoptosis induced by GC treatment (Fig. 3.7). Secondly, when PGD_2 was added to GC-alone or GC and ERα agonist co-treatment there was no reduction in apoptosis (Fig. 3.7).

A possible explanation for ERα agonist reducing apoptosis in primary Th2 cells, but not CCRF-CEM cells, may be that Th2 cells produce PGD_2 [98] and may be capable of autocrine/paracrine activation of CRTh2. Whether the immortalized CCRF-CEM cell line produces PGD synthase and releases PGD_2 is not known and should be examined in the future. To assess whether the different outcomes between the two cell lines were related to

this, PGD_2 was added to the CCRF-CEM cells. Contrary to the suggestion made by Xue et al. that CRTh2 activation by the ligand reduces apoptosis [8], this result was not observed with GC and PGD_2 co-treatment; no difference in apoptosis was seen in cells that received GC and PGD_2 compared to those treated with GC alone. Previous experiments revealed that pre-treatment with GC and ERα agonist was required to see increased production of type 2 cytokines following PGD_2 stimulation [1]. Therefore, preliminary experiments were performed pre-treating with GC and ERα agonist (to increase CRTh2) prior to PGD_2 stimulation. However, still no difference in GC-induced apoptosis was observed (Fig. 3.8). Combined, the findings illustrate that in CCRF-CEM cells activation of CRTh2 by PGD_2 does not reduce GC-induced apoptosis.

Contrary to my hypothesis, cells treated with PGD_2 and GC appeared to show no difference in levels of apoptosis compared to GC alone (Fig. 3.7). Although this finding directly contradicts the results of Xue et al., it is important to note that that study examined apoptosis following IL-2 deprivation [8]. Specifically, it was suggested that activation of the PI3K survival pathway was mediating the inhibition of apoptosis as CRTh2-PGD_2 influxes calcium and stimulates the PI3K pathway, catalyzing BAD phosphorylation, sequestering it in the cytosol and rendering it unable to inhibit Bcl-xl, leading to efflux of cyt *c* and cleavage of caspase-3 to induce apoptosis [8]. However, through gene set enrichment analyses, mutations have been identified upstream of the PI3K/Akt pathway in CCRF-CEM cells, resulting in reduced ERK signaling [341]. These mutations in combination with the differences observed between primary Th2 cells and CCRF-CEM cells following DEX and PPT co-treatment indicate that these cell lines may not function similarly.

Despite these conflicting results regarding the apoptotic influence of CRTh2-PGD_2, if CRTh2 activation increases GC-induced apoptosis this would build upon other evidence reporting a pro-apoptotic effect. For instance, treatment of human osteoclasts differentiated from PBMCs with PGD_2 and a CRTh2 specific agonist induced apoptosis [342]. Moreover, the mechanism behind this was through caspase-9 activation, identifying that CRTh2-PGD_2 activation in these cells intrinsically induces apoptosis [342]. Increased DNA fragmentation and apoptotic bodies have also been observed following treatment of

leukemia cells with PGD_2 [343]. A study on lung adenocarcinoma-derived alveolar epithelial cells has also identified that treatment with PGD_2 induced apoptosis; however, the researchers concede that they used antagonists for both DP1 and CRTh2 [344]. Collectively, these results suggest that the CRTh2-PGD_2 pathway may have differential effects on apoptosis depending on the cell type and cell death pathway.

In effect, the differences between the CCRF-CEM and primary Th2 cells in CRTh2 expression and apoptosis in response to GC and ERα agonist indicate the need for future studies. Although the literature may appear conflicting in regard to the apoptotic nature of CRTh2 activation by PGD_2, further foundational experiments are needed to identify whether the receptor is function in immortalized CCRF-CEM cells. Such experiments could involve analysis of inflammatory cytokines in response to PGD_2 activation, as we have previously found this treatment to increase cytokines in primary Th2 cells [1]. Importantly, these studies need to be performed with primary Th2 cells.

The data show that Th2 cells treated with GC had more apoptosis than those exposed to both GC and estrogen. These experiments were performed *in vitro*, however, and so the strength of this conclusion is limited. Ideally, further studies examining Th2 cells exposed to GC *in vivo* as well as comparing GC sensitivity of Th2 cells obtained from women and men would help prove this mechanism.

4.4 Increase in *CRTh2* Promoter Activity by GC and ERα Agonist

Within the *CRTh2* promoter numerous putative GREs were found and so whether GC exposure influences transcriptional activation of CRTh2 was examined. Cooperative cross-talk between ER and GR has been shown [345] and so it was hypothesized that ERα agonist would enhance GC-induced activation of the *CRTh2* promoter. In line with this, the results confirmed that in response to GC (0.1 μM DEX), promoter activity was increased from baseline and cells treated with GC and ERα agonist (1 μM PPT) exhibited significantly higher promoter activity (Fig. 3.10). This was true for the shorter construct (*CRTh2*pro-4513/Luc), containing one GRE, as well as the longer construct (*CRTh2*pro-7013/Luc),

with three GREs. At baseline, both constructs exhibited activity ~1000 RLU (~3.5 fold higher than empty vector), corresponding with the finding that CCRF-CEM cells expressed CRTh2 on at the surface level with no treatment (Fig. 3.6). The fact that *CRTh2* promoter activity was increased with GC alone suggests that T cells exposed to GC undergo *CRTh2* transcription and coincide with the finding that GC alone increased surface level of CRTh2 (Fig. 3.6A/B). A caveat, however, is that a condition with the ERα agonist alone was not included here, since this effect was unexpected [1]. Therefore, we cannot rule out that exposure to ERα agonist alone may also increase *CRTh2* promoter activity. Activation of the *CRTh2* promoter is but one step in the process of a cell expressing CRTh2, however, they do coincide with the increase in endogenous CRTh2 mRNA and total protein abundance found with primary Th2 cells [1].

Transcriptional activation of *CRTh2* by GC is in line with many other studies showing that GC-GR complexes directly bind to GREs and increase transcription, a process called transactivation [154, 156]. This has been shown for many anti-inflammatory genes including p11 [157] and dual specificity phosphatase 1 [346]. Indeed, most studies showing GC transactivation is for genes that act in an anti-inflammatory capacity, reducing inflammation [347]. Whereas here, transactivation of the *CRTh2* promoter would result in a pro-inflammatory effect. This is not an original concept, rather increasing evidence has been building on the pro-inflammatory effects of GC. Often, depending on the concentration and chronicity of exposure, GC can elicit inflammatory effects in the central nervous system [348]. Cortisol, the endogenous GC, has 10 times higher affinity for mineralocorticoid receptors than GR; therefore basal levels of cortisol occupy most mineralocorticoid receptors [349]. In a period of stress when cortisol is elevated, as mineralocorticoid receptors are often already occupied, available cortisol binds to GR and can elicit inflammatory responses [349]. For example, during acute stress there are increased levels of cyclooxygenase-2 [350], NF-κB activity [351] and extracellular TNF-α [352]. Additionally, in rats, basal levels of corticosterone that are higher than average have been found to have increased pro-inflammatory mediators such as prostaglandin E_2 [353]. Therefore the finding that GC increased *CRTh2* promoter activity (Fig. 3.10) build on the existing evidence that GR activation can have pro-inflammatory effects. This thesis

used pharmacologically relevant concentrations of GC (0.01 - 1.0 μM). Indeed, oral administration of dexamethasone (12 mg) results in plasma concentrations in the range of 0.2 μM [354]. However, some physiologic situations may result in even higher exposure. For instance, blood cortisol concentrations in response to severe acute respiratory syndrome (SARS)-induced stress have been recorded as high as 7.22 μM in human patients [355], leading one to wonder about the repercussions of this medical scenario on *CRTh2* transcription and the ensuing inflammation.

Both *CRTh2*pro-4513 and -7013/Luc had significant increases in transcriptional activity with GC and ERα agonist co-treatment compared to baseline as well as treatment with GC alone (Fig. 3.10). This supports the hypothesis that the surface level increase observed with flow cytometry is due to a transcriptional effect. These experiments were performed *in vitro*, however, and so the strength of this conclusion is limited. Further studies examining Th2 cells from men and women exposed to GC *in vivo* would help to substantiate that these conditions truly have a differential effect on CRTh2 expression.

These results build on other research showing cooperative cross-talk between ER and GR and suggest that the enhanced effect on CRTh2 is due to ERα agonist and/or E2 increasing GR binding to GRE. This was previously shown by GR-ChIP-seq: enrichment of GR at GREs was observed with GC and E2 co-treatment [311]. The dynamic-assisted loading model demonstrated that for some genes GR and ER do not compete for binding sites; rather one receptor assists binding of the other [356]. Indeed, one study showed that repression of pro-inflammatory genes was reduced the most by DEX and E2 co-treatment (13/23 genes), compared to DEX alone (5/23 genes) and E2 alone (5/23 genes) [357]. Collectively, these studies support the conclusion that increased *CRTh2* promoter activity following DEX and PPT co-treatment is due to ERα enhancing the binding of GR, though quantitative GR-ChIP experiments and a PPT alone condition are needed to confirm this conclusion.

There was no additive effect when comparing the short (*CRTh2*pro-4513/Luc) and long (*CRTh2*pro-7013/Luc) constructs (Fig. 3.10). This was surprising since previous research has shown that genes with multiple GREs bound by GC have a significantly larger

induction than genes with one GRE [358]. Interestingly, they also observed that some genes, such as *PER1*, respond differently to lower vs higher concentrations of GC [358]. This may suggest that at lower GC concentrations (<0.1 μM), binding will only occur at regions with the strongest GR-GRE interactions. Previous research has also found that minute differences in sequence within and flanking the GRE, by even a single base pair, have been shown to alter the binding of GR [358]. Therefore, a titration curve of DEX should be explored, as 0.1 μM may have been an excess amount to observe differences, *i.e.* a difference in the effect of GC on the longer vs shorter construct (3 vs 1 GRE) may be observed using lower concentrations of DEX and/or *CRTh2* promoter construct.

4.5 Genetic Variation within the *CRTh2* Promoter Alters Response to GC and ERα Agonist

Contrary to my hypothesis, this thesis showed *CRTh2*-6388A was not more transcriptionally active than *CRTh2*-6388G at baseline or in response to GC alone or GC and ERα agonist (Fig. 3.11). With *CRTh2*-6388G, GC activation significantly increased promoter activity and this was not affected by the addition of ERα agonist. The *CRTh2*-6388A construct exhibited no significant increase in promoter activity with GC alone, but did show a significant increase with GC and ERα agonist co-treatment. This suggests that GC activates the G allele of the *CRTh2* promoter, whereas the A allele needs both GC and ERα agonist to significantly increase CRTh2 transcriptional activity.

Previously, *in silico* analysis (MatInspector) identified putative binding sites within the *CRTh2* promoter (Fig. 3.9). In the G allele, the presence of three GRE sites rationalizes the increased promoter activity with GC, as GC-GR binds to GRE and transactivates the gene [154]. At the location of the SNP, in addition to the GRE, the G allele has a number of sites predicated to bind other transcription factors such as NFAT, FOXO and the enhancer blocking insulator, CTCF [359]. While this may suggest there could be competition for GR binding to this GRE, there is also the potential that one or more of these factors could enhance GR binding, particularly the FOXO binding element. Indeed, in the promoter for MuRF1, an atrophy regulatory gene, a GRE and FOXO binding element were found to be

synergistically activated by GR and FOXO1 [183]. This was shown through a mutated GRE and intact FOXO site strongly inhibiting the GC induced promoter induction; however, when the GRE was intact and FOXO binding element was mutated, the induction was completely abolished [183]. This study suggests that FOXO may stabilize GR binding in the *CRTh2* promoter, resulting in increased promoter activity following GC treatment as seen in the *CRTh2*-6388G promoter. In contrast, analysis of transcription factor binding sites predicted with the *CRTh2*-6388A allele showed that with the exception of the GRE, at the location of the SNP, all of the previously listed putative binding sites were absent. In light of the fact that exposure to GC alone did not significantly increase *CRTh2*-6388A promoter activity, it is possible that the absence of FOXO failed to stabilize GR, as it may do within the G allele [183], leading to reduced/insufficient GR binding and transactivation.

The *CRTh2*-6388A allele had none of the other binding sites overlapping with the GRE. As such, it is possible that the stabilizing effect of ERα agonist may have been needed to increase GC-GR binding to the GRE, resulting in increased transcriptional activity seen between GC alone and GC and ERα agonist treatment. Previous research found that through differential CentriMo motif analysis, DEX and E2 treatment caused increased GR enrichment at both GREs and EREs compared to DEX treatment alone [311]. My results build upon this suggestion, however, further analyses are necessary to confirm this mechanism. For example, quantitative ChIP analysis could be performed to examine whether there is increased GR binding in the presence of ERα agonist. Since the ultimate question is whether there is increased GR binding at the *CRTh2*-6388A vs G allele, Th2 cells from donors of AA and GG genotype would need to be compared using ChIP sequencing, a technique with the potential to reveal site specific binding.

Examining the difference in promoter activity following GC and ERα agonist co-treatment compared to GC alone showed a significantly larger induction for the *CRTh2*-6388A than *CRTh2*-6388G allele (Fig. 3.12). Since the biggest increase in CRTh2 surface levels was found with 0.01 μM DEX in combination with 10 μM PPT (Fig. 3.6C/D), additional luciferase experiments were performed to investigate allelic differences with these concentrations. This analysis suggests that GC and ERα agonist may result in CRTh2-

6388A being more transcriptionally active than CRTh2-6388G (Fig. 3.12), though statistical analysis could not be performed due to few experiments (n=2). Further experiments are needed to confirm these results, but were not possible at this time.

A limitation of these transcriptional experiments is the absence of conditions examining the effect of ERα agonist alone and/or E2 on *CRTh2* transcriptional activity. Further, this assay does not consider the gene in the context of chromatin structure, as it would be within cells *in vivo*. Moreover, testing the effect of the sex hormones E2, as well as E1 and E3, which are prominent at different phases of life would also be informative. Further research should also include ChIP studies to identify the regions bound by GC-GR. A major limitation of this aspect of the project is that ideally promoter experiments should be followed up with assessing the response of Th2 cells from donors of GG and AA genotype. This work was planned, but could not be completed due to difficulty with primary cell culture and the inability to work in the laboratory due to pandemic precautions.

It is important to consider the physiological relevance of the GC and estrogen concentrations used. The daily dosage of prescribed dexamethasone is 12-16 mg/day for 2 days [360, 361], maximally observed as 0.26 μM in circulating blood [354]. Therefore, the concentration of 0.1 μM DEX used throughout all experiments in this thesis is a level that may occur *in vivo*. ERα concentrations were initially chosen based published concentrations that show the selectivity of PPT for ERα (Fig. 3.2) [362]. Concentrations of E2 for treatments in primary Th2 cells experiments (Fig. 3.3-3.4) were chosen based on papers studying GR-ER crosstalk (0.1 μM) [311]. However, we also wanted to consider physiologically relevant concentrations. A review of the literature uncovered that plasma levels of estrogen at the highest point during pregnancy can reach ~0.73 μM [363], those in females on oral contraceptives were ~ 0.01 μM [364] and during the menstrual cycle 0.0015 μM [365]. Therefore, the lower concentration of 0.01 μM in CCRF-CEM cells (Fig 3.6) was more physiologically relevant. Experiments examining differences in E2 concentration were not informative, since CCRF-CEM showed no increase CRTh2 at any concentration. Future experiments should include a positive control gene for E2 and focus on concentrations E2 that represent physiologically and/or pharmacologically relevant

concentrations. It is possible that these relatively high concentrations may exhibit hysteresis, a phenomenon in which high concentrations inhibit the effect of a drug or hormone and lower concentrations show effects [366]. As such, future experiments should use concentrations similar to the lowest ones used in this dissertation.

4.6 Significance and Conclusions

It is well known that women are more likely to have a severe asthma diagnosis [6] and severe symptoms requiring hospitalization [235]. This thesis shows that Th2 cells in an environment similar to that of a female taking GC treatment (GC and estrogen) have more CRTh2 compared to Th2 cells in a male-like environment (GC only). Additionally, estrogen was found to reduce GC-induced apoptosis, supporting Th2 cell survival. Indeed, this could suggest that when female asthmatics take GC, not only do CRTh2 levels increase but their Th2 cells resist GC-induced apoptosis. This protective effect could allow for proliferation of inflammatory Th2 cells, potentially explaining the positive association seen in women between daily dose of inhaled corticosteroid, and circulating Th2 cells (App 1.2). The ability of GC and estrogen to increase CRTh2 expression may prime Th2 cells to have an even larger PGD_2-mediated type 2 response, since these cytokines mediate many asthma symptoms, such as bronchial construction and excess mucous production [80]. This all suggests that GC may make asthmatic symptoms worse in some women, and supports the transition to biologic therapy for asthma.

Interestingly, a selective CRTh2 antagonist, fevipriprant (QAW039), has been investigated for therapeutic use [367]. In Phase II clinical trials, the daily administration of fevipriprant to uncontrolled allergic asthmatics on low dose ICS for 12 weeks resulted in significant improvement in pre-bronchodilator FEV_1 compared to placebo [368]. Additionally, moderate-to-severe asthmatics were also found to have reduced sputum eosinophilia and improvements in post-bronchodilator FEV_1 with the addition of fevipriprant to their treatment regimen [369]. Despite promising results, the population of asthmatics that would benefit most from this antagonist has yet to be discovered. Phase III trials have found that fevipriprant has no effect on clinical outcome, specifically exacerbations per year, in

moderate-to-severe asthmatics [370]. This may suggest that the benefits could be higher in milder allergic asthmatics. There are however other targeted biologic medications that have been approved for asthma including Dupilumab, an antagonist for IL4Rα [87], and Mepolizumab, an antagonist for IL-5 [371].

Recently our laboratory found an association between the *CRTh2*-6388AA genotype, severe asthma and increase in blood levels of CRTh2 mRNA in women, but not men (App. 4.1). The data generated here suggest this could be related to allele specific transcriptional effects of GC and estrogen exposure. Indeed, the promoter experiments imply there may be no difference in GC response across sexes if both have the G allele, while in those with the A allele women may experience more GC-mediated CRTh2 transcription than men. Overall, this study suggests *CRTh2*-6388G>A influences Th2 cell responses to GC in women and that studies characterizing asthma and allergic inflammation may need to adopt a sex specific analyses and approach to therapy.

In conclusion, this study shows that estrogen opposed the anti-inflammatory apoptotic effect of GC, cooperated with GC to induce transcriptional activation of CRTh2 and that this effect may differ based on *CRTh2* genotype. Collectively, these results suggest that women with severe asthma may have more Th2 cells due to estrogen reducing GC-induced apoptosis and that the deleterious pro-inflammatory effects of GC may have a greater impact on women than men.

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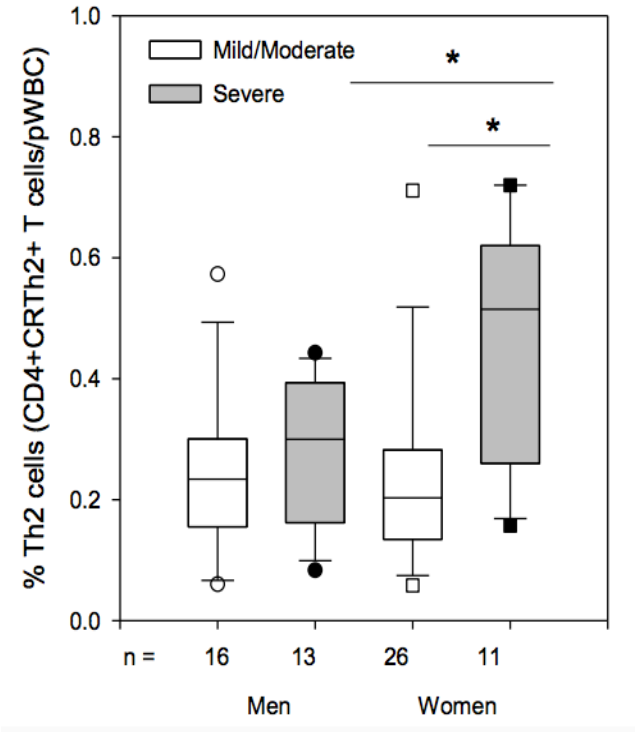
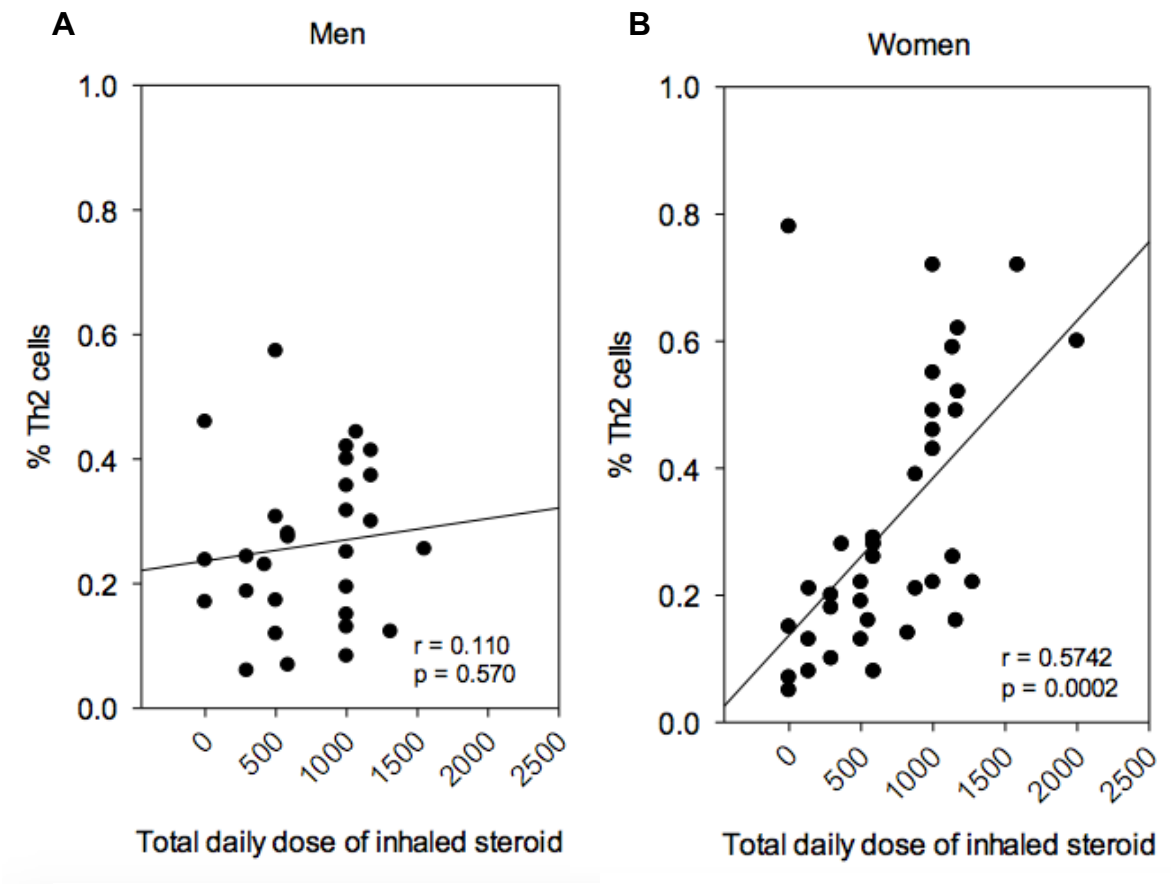
Appendices

Data generated by former members of Dr. Lisa Cameron's laboratory in collaboration with Dr. Shrestha Palikhe at University of Alberta. The following data in combination with my data is contributing to a manuscript in preparation.



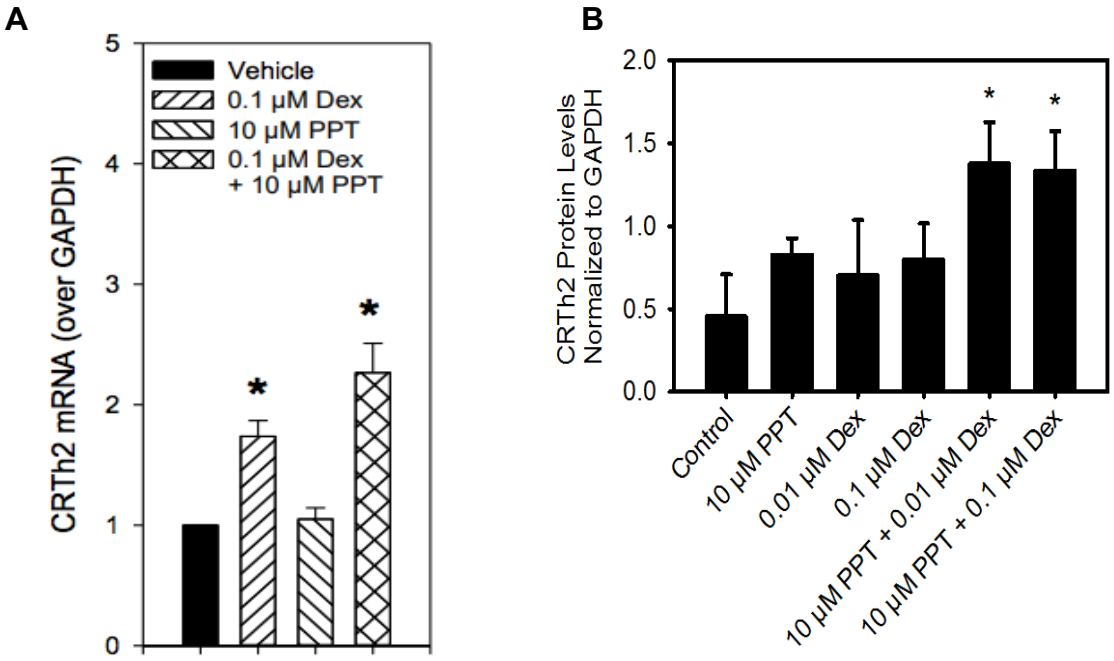
Appendix 1.1: Proportion of Th2 Cells in Men and Women with Severe Asthma

CRTh2 surface staining in circulating peripheral whole blood cells of asthmatics at Asthma Clinic at University of Alberta separated by sex and asthma phenotype (mild/moderate or severe). Data represented as mean ± SEM and statistical significance,* indicating $P<0.05$ *vs* control, was determined through one-way repeated measures ANOVA. Data was assessed by flow cytometry and generated by Dr. Nami Shrestha Palikhe and Dr. Lisa Cameron.



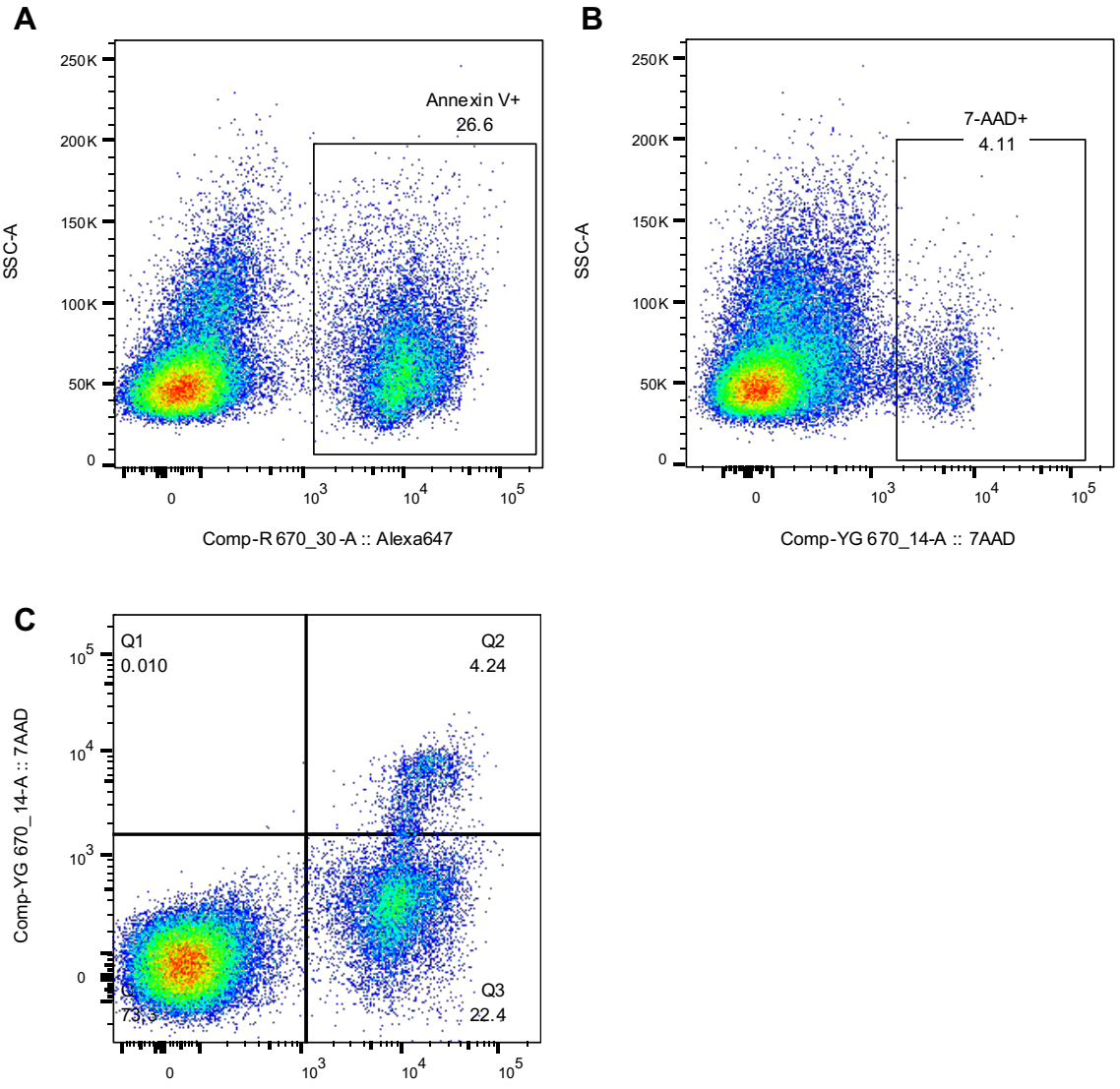
Appendix 1.2: Correlation Between ICS and Th2 Cells Separated by Sex

Relationship between daily dosage of inhaled corticosteroid (μg) and proportion of Th2 cells in circulating peripheral whole blood cells as measured by flow cytometry in males (A) or females (B). Data was analyzed through Pearson's correlation coefficient and generated by Dr. Nami Shrestha Palikhe and Dr. Lisa Cameron.



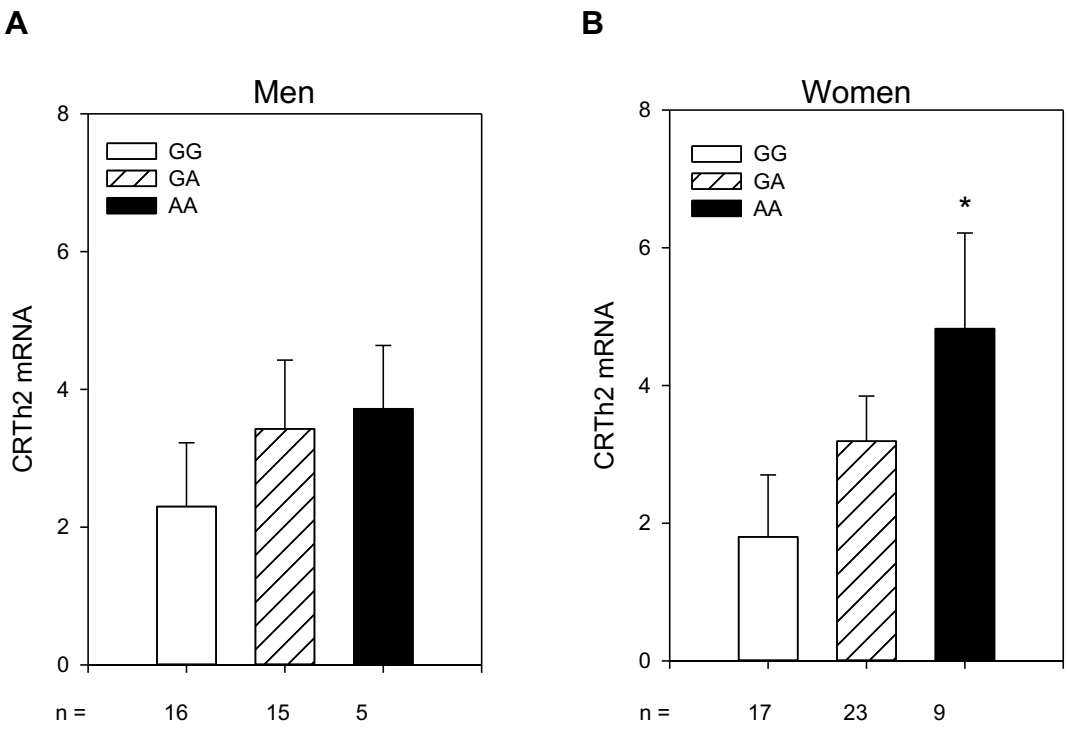
Appendix 1.3: CRTh2 RNA and Protein Levels in Response to GC and ERα Agonist Co-treatment in Primary Th2 Cells

Primary Th2 cells were treated for 24 hours with DEX and/or PPT. Total CRTh2 mRNA was measured through qRT-PCR, using $2^{-\Delta\Delta Ct}$, normalized to GAPDH (A). Through western blot analysis, CRTh2 total protein abundance was measured through densitometry and normalized to GAPDH (n=3) (B). Statistical differences represented through asterisks (*) indicating significance *vs* control ($P<0.05$). Data generated by Dr. Lauren Solomon and Meerah Vijeyakumaran.



Appendix 3.1: Gating Strategy of Apoptotic Th2 Cells

Primary Th2 cells were treated for 48 hours with 0.1 µM DEX and 1.0 µM PPT. Through flow cytometry, percept of Alexa Fluor™ 647 Annexin V conjugate (Annexin V^+; A) and 7-AAD^+ (B) stained cells were measured. Further analysis through FlowJo computer software allowed for the determination of percent of cells in an apoptotic state, Annexin V^+/7-AAD^- (C) through quadrant gating. Annexin V^+/7-AAD^+ cells are in quadrant 1, Annexin V^+/7-AAD^+ cells in quadrant 2, Annexin V^+/7-AAD^- cells in quadrant 3, and Annexin V^-/7-AAD^- cells are in quadrant 4. This is a representative figure from one experiment that is used to explain the gating analysis used throughout all experiments.



Appendix 4.1: Variation in CRTh2 mRNA amongst *CRTh2*-6388G>A within Males and Females

CRTh2 mRNA measured through qRT-PCR of whole blood of severe asthmatic patients. Data normalized to GAPDH and presented as fold change. Patients were sorted by genotype and sex. Significance (*) represents differences between condition and control (P<0.05). Data was generated by Drs. Nami Shrestha Palikhe and Lisa Cameron.

Curriculum Vitae

Name:	Jenna Fortunato
Post-secondary Education and Degrees:	Laurentian University Sudbury, Ontario, Canada 2014-2018, B.Sc. Biomedical Biology
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Honours and Awards:	Western Graduate Scholarship 2018-2020
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Regional Presentations:

Fortunato J., Vijeyakumaran, M., Kanagalingam T., Solomon L., Palikhe N., Vligaoftis H., and Cameron L. "The Influence of Estrogen on Th2 Cell Sensitivity to Glucocorticoid" London Health Research Day, Lawson Health Research Institute, London, Ontario (*April 30, 2019*).

Fortunato J., Vijeyakumaran, M., Kanagalingam T., Solomon L., Palikhe N., Vligaoftis H., and Cameron L. "The Influence of Estrogen on Th2 Cell Sensitivity to Glucocorticoid" Pathology and Laboratory Medicine Research Day, Western University, London, Ontario (*March 28, 2019*).

Manuscripts:

Vijeyakumaran, M., Solomon, L., Palikhe, N.S., **Fortunato, J**., Vliagoftis, H., and Cameron, L. "Asthma severity in women is associated with type 2 inflammation" (Manuscript in preparation).

Palikhe, N., **Fortunato, J**., Solomon, L., Vliagoftis, H., and Cameron, L. "The CRTh2 SNP rs533116 associates with asthma severity and increased CRTh2 mRNA in women" (Manuscript in preparation).