University of Alberta

Molecular regulation and endogenous expression of CRTh2 in *in vitro* differentiated CRTh2^{+} Th2 cells

by

Emily Iris MacLean

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

in

Experimental Medicine

Department of Medicine

Fall 2011

Edmonton, Alberta

Abstract

Allergic inflammation is mediated by T helper 2 (Th2) cells. Chemoattractant receptor homologous molecule expressed on Th2 cells (CRTh2) is expressed by Th2 cells and mediates chemotaxis and production of the Th2 cytokines interleukin (IL)-4, IL-5 and IL-13.

To understand the molecular regulation of CRTh2, we studied the promoter and found putative binding sites for GATA3 and nuclear factor in activated T cells (NFAT), transcription factors known to regulate Th2 cytokine expression. We hypothesized these factors also regulate transcription of *CRTh2*. Using a reporter construct with the proximal region of the *CRTh2* promoter, we found transcription of *CRTh2* was increased following stimulation in $CRTh2^+$ Th2 cells. Endogenous CRTh2 was decreased following stimulation. Further, over-expression of NFAT1 decreased a GATA3 dependent increase in CRTh2 promoter activity. The discrepancy between CRTh2 endogenous expression and proximal promoter activity indicates longer regions of the CRTh2 5' regulatory region may be important for regulation.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APC	Antigen presenting cell
BAD	Bcl-xL/Bcl-2-associated death promoter
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CRTh2	Chemoattractant homologous receptor expressed on Th2 cells
DC	Dendritic cell
DK-PGD_2	13,14-dihydro-15-keto-PGD_2
cDNA	Complimentary DNA
DNA	Deoxyribonucleic acid
DP1	D prostanoid receptor 1
EMSA	Electromobility shift assay
FACS	Fluorescence activated cell sorting
FcεRI	Fcε Receptor I
GIF	Glycosylation-inhibiting factor
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3β
ICD	Intracellular domain
IFNγ	Interferon γ
Ig	Immunoglobulin
IP-10	Interferon-γ-inducible protein 10
IL	Interleukin
InsP3	Inositol-1,4,5-triphosphate
MHC	Major histocompatibility complex
MIF 1β	Macrophage inflammatory protein 1β

ND	Not determined
NFAT	Nuclear factor in activated T cells
OVA	Ovalbumin
OX40L	OX40 ligand
PMA	Phorbol 12-myristate 13-acetate
P/I	PMA and Ionomycin
PG	Prostaglandin
PGDS	Prostaglandin D synthase
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PLCγ	Phospholipase C-γ
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rh	Recombinant human
RLA	Relative luciferase activity
TARC	Thymus and activation regulated chemokine
Tc	Cytotoxic T cell
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
Th	T helper cell
Th0	Naïve T cell
Th2	T helper 2 cell
Treg	T regulatory cell
TnT	Transcription and translation
TSLP	Thymic stromal lymphopoietin

CHAPTER I: Introduction

1.1 Allergic Inflammatory Diseases

Allergic inflammatory diseases including rhinitis, asthma and atopic dermatitis are rising in prevalance worldwide (1). Allergic asthma is a disease of chronic airway inflammation and is characterized by infiltration of eosinophils and T helper 2 (Th2) cells, structural remodeling, mucous hypersecretion, airway hyper-responsiveness and reversible airway obstruction (2). Atopic dermatitis is a chronic allergic disease associated with eosinophilia and elevated immunoglobulin (Ig) E both systemically and locally at the site of inflammation in the skin (3). Similarly, allergic rhinitis is characterized as IgE mediated recruitment of eosinophils, basophils and Th2 cells to nasal mucosa (4).

1.1.1 Mast cell sensitization and response to allergen re-exposure

Early responses to allergen exposure are mediated by mast cells sensitized to specific allergen(s). Sensitization is the first step in an allergic response and results from mounting an immune response to a normally innocuous protein, there after known as an allergen. The initial immune response produces allergen specific IgE antibodies which coat mast cells via their Fc receptors (FcεRI), leaving the allergen specific region of the antibody free to capture encountered allergen (**Figure 1,** reviewed in (5)). During secondary exposure, allergen cross-links the bound IgE on the sensitized mast cells and results in the immediate release of factors such as histamine and a slower release of cytokines (interleukin (IL)-4, IL-13 and IL-5) and lipid mediators including prostaglandin (PG) D_2 and leukotrienes (Reviewed in (6)). Cytokines contribute to activation of other immune cells and PGD_2 and leukotrienes result in contraction of smooth muscles,

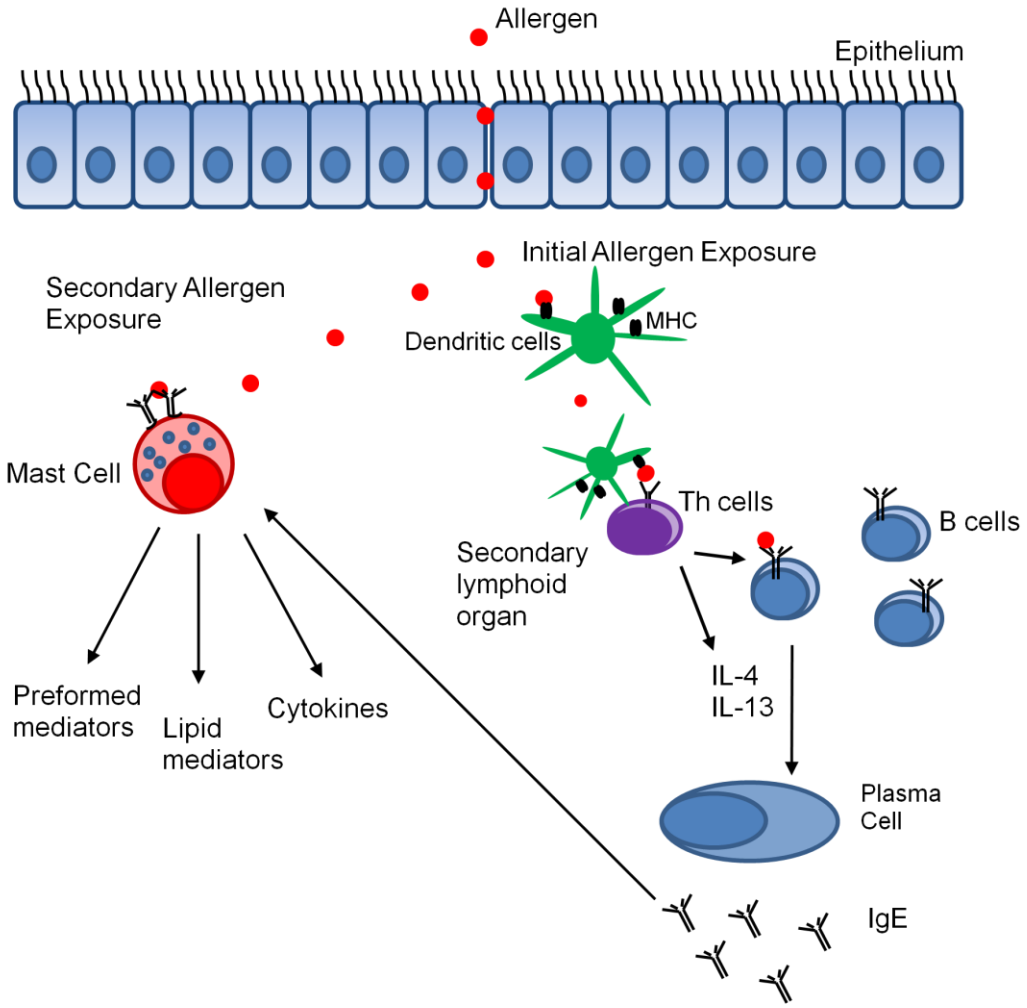

Figure 1: Allergic sensitization and secondary allergen exposure

Allergic sensitization begins with initial allergen encounter when allergen is presented by dendritic cells on major histocompatibility complex (MHC). Dendritic cells migrate to the secondary lymphoid organs. Through cell contact and cytokine help (IL-4 and IL-13) from differentiated Th2 cells, B cells recognize the allergen via surface IgM and produce IgE. IgE then migrates to coat mast cells in the tissue. Upon subsequent allergen exposure, allergen will crosslink the IgE on mast cells and result in release of preformed mediators, including histamine followed by lipid mediators and cytokines which perpetuate the allergic phenotype.

activation of immune cells including Th2 cells and vasodilation which may allow increased transendothelial migration of immune cells (6-11).

1.2 T and B lymphocytes

1.2.1 T cell differentiation – Th1 and Th2

T cells are lymphocytes that can be further characterized as $CD4^+$ helper T cells (Th) cells and $CD8^+$ cytotoxic T cells (T_c) cells. Th cells can be further divided into Th1, Th2, Th17 and T regulatory (T_{reg}) cells, along with other less described lineages that are currently being characterized (12). The first Th cell subsets were discovered in mice and described as having distinct cytokine profiles with different effects on B cell function, especially immunoglobulin production (13-14). These Th cell subsets were named T helper 1 (Th1) and T helper 2 (Th2). Cytotoxic T cells have also been shown to differentiate into similar type 1 and type 2 cells based on cytokine profiles (15).

Both Th1 and Th2 cells differentiate from naïve $CD4^+$ T cells (Th0) in the secondary lymphoid organs depending on signals, such as cytokines, which they receive from antigen presenting cells, including but not limited to dendritic cells (DCs). In the allergic response, DCs are activated at the periphery and then migrate back to the secondary lymphoid organs. Specifically, DCs have been shown to be activated by a cytokine, thymic stromal lymphopoietin (TSLP), to prime naïve Th (Th0) cells to differentiate to Th2 cells (16). TSLP is highly expressed by epithelial cells, especially those associated with atopic dermatitis

lesions (16). TSLP expression has also been shown in the asthmatic lung (17-18), the gut (17) and is up-regulated in mast cells following IgE cross-linking (18). TSLP activated DCs migrate back to the secondary lymphoid organs and present a specific allergen to the T cell along with co-stimulation and Th2 polarizing cytokines. IL-4 will differentiate naïve $CD4^+$ T cells into Th2 cells and up-regulate expression of Th2 cytokines such as IL-5, IL-13 and more IL-4 (19). Conversely, naïve $CD4^+$ T cells will differentiate to Th1 cells when exposed to IL-12 (20) which results in up-regulation of the major Th1 effector cytokine, IFNγ (21). Interestingly, IL-4 and IFNγ are not only the effector cytokines of the Th2 and Th1 subsets, respectively, but also inhibit production of the opposing subset (reviewed in (22)). Highly polarized Th2 cells will also up-regulate their expression of **CRTh2** (chemoattractant homologous receptor expressed on Th2 cells), a marker of Th2 cells and a receptor for PGD_2 (23-25).

1.2.2 B cells and isotype switching play a role in allergic inflammation

The allergic sensitization cascade would not be possible without allergen specific IgE, which is produced by B cells **(Figure 1)**. Cytokines produced by Th2 cells direct immunoglobulin (Ig) isotype switching in B cells (26-27). During B cell development, the germ-line DNA encoding the immunoglobulin genes undergoes dynamic rearrangements to produce antigen specificity and a mature B cell (28). B cells then express antigen specific IgM on their surface which recognizes and internalizes antigen to be expressed on major histocompatibility complex II for interaction with Th cells. Following B cell interaction with Th cells, cytokine receptors on the B cell surface are up-regulated (29). Cytokine signaling regulates

the rearrangement of the immunoglobulin constant region gene and therefore determines the isotype of the antibodies produced; a process known as class switching (reviewed in (30)). In human B cells, IL-4 has been shown to cause sequential switching from IgM to IgG_4 and then IgE (31-32). IL-13 also mediates IgG_4 and IgE class switching (33). In addition to the secondary lymph nodes, class switching was also shown to occur at peripheral sites, such as the nasal mucosa in patients with rhinitis (34).

1.2.3 Memory T cell subsets – Central and effector memory cells are maintained to quickly mount an immune response to allergen re-exposure

Allergic sensitization results in a pool of allergen specific Th2 cells that remain in our body as primed memory cells. Maintenance of memory T cells allows the immune system to quickly respond to re-encountered allergens with a lower threshold of initial co-stimulatory molecules such as CD28 and less proliferation (35).

Human $CD4^+$ memory T cells are composed of two distinct populations known as effector memory (T_{EM}) or central memory (T_{CM}) cells (36). Both populations are defined as highly expressing the memory cell markers (CD45RO and CD44) without the naïve cell marker (CD45RA). However central memory cells are $CCR7^+CD62L^+$ and effector memory cells are $CCR7^-CD62L^-$ (35). CCR7 is a chemokine receptor and CD62L is an adhesion molecule also expressed by naïve T cells and mediate homing to the secondary lymphoid organs for activation (35). Similarly, central memory cells migrate periodically back to the secondary lymphoid organs via CCR7 and CD62L molecules. If exposed to their allergen,

T_{CM} cells undergo clonal expansion and generate a population of effector cells (36). Conversely, effector memory cells do not display homing ligands because when they re-encounter allergen they are immediately capable of effector functions such as cytokine production (35). Studies show that circulating $CRTh2^+$ $CD4^+$ Th2 cells display a central memory phenotype, however, they have also been observed in the tissue in atopic dermatitis lesions and nasal mucosa (37-38). Although it has not been investigated, those $CRTh2^+CD4^+$ Th2 cells are considered to represent an effector memory phenotype and maintain the Th2 milieu within the tissues through cytokine production (37, 39).

1.2.4 TSLP activated dendritic cells maintain the Th2 phenotype through OX40 and OX40 ligand (OX40L) co-stimulation

$CRTh2^+CD4^+$ cells are not only *polarized* but also *maintained* by TSLP activated dendritic cells (37). TSLP activated dendritic cells induce a robust expansion of $CRTh2^+CD4^+$ T cells in an autologous TSLP-DC co-culture system (37). This effect is dependent on the OX40/OX40L co-stimulatory pathway. OX40/OX40L interactions appear to prolong $CRTh2^+CD4^+$ T cell survival (37). In this model system, the TSLP-induced Th2 polarization relies on the OX40/OX40L interactions (37, 40).

1.3 PGD_2 is a metabolite of arachidonic acid and binds two receptors, D prostanoid receptor 1 (DP_1) and CRTh2

One of the major mast cell mediators released following IgE cross-linking is PGD_2. PGD_2 is metabolized from arachidonic acid in the cellular membrane. Phospholipase A_2 releases arachidonic acid from the membrane and cyclooxygenase -1 (COX-1) and COX-2 metabolize free arachidonic acid to

prostaglandin H_2 (PGH_2) which is then metabolized to PGD_2 via PGD synthase (reviewed in (41-42)). PGD_2 has two known receptors, D prostanoid receptor 1 (DP_1) and chemoattractant homologous receptor expressed on Th2 cells (CRTh2) (also known as; DP2, GPR44 and CD294); both are G-protein coupled receptors (GPCRs) (43). DP_1 is a $G_{\alpha s}$ coupled GPCR and when activated, increases intracellular cyclic adenosine monophosphate (cAMP). CRTh2 is a $G_{\alpha i}$ coupled GPCR and when activated, increases intracellular calcium (Ca^{2+}) and decreases intracellular cAMP (25, 43-45). PGD_2 can be metabolized further to other active molecules that act with high affinity at the CRTh2 receptor such as 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2) (44), Δ^{12}-PGD_2 (46), Δ^{12}-PGJ_2 (47), 15-deoxy-$\Delta^{12,14}$-PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (48) **(Figure 2)**.

1.4 CRTh2 and DP_1 are receptors for PGD_2

PGD_2 has two known receptors, D prostanoid receptor 1 (DP_1) and chemoattractant homologous receptor expressed on Th2 cells (CRTh2) (also known as; DP2, GPR44 and CD294); both are G-protein coupled receptors (GPCRs) (43). DP_1 is a $G_{\alpha s}$ coupled GPCR and when activated, increases intracellular cyclic adenosine monophosphate (cAMP). CRTh2 is a $G_{\alpha i}$ coupled GPCR and when activated, increases intracellular calcium (Ca^{2+}) and decreases intracellular cAMP (25, 43-45). The DP_1 receptor is widely expressed on both hematopoietic and non-hematopoietic cells (49) in contrast to the CRTh2 receptor which has a more limited expression among hematopoietic cells (25, 43). CRTh2 expression is well documented on Th2, but not Th1 cells, basophils, eosinophils

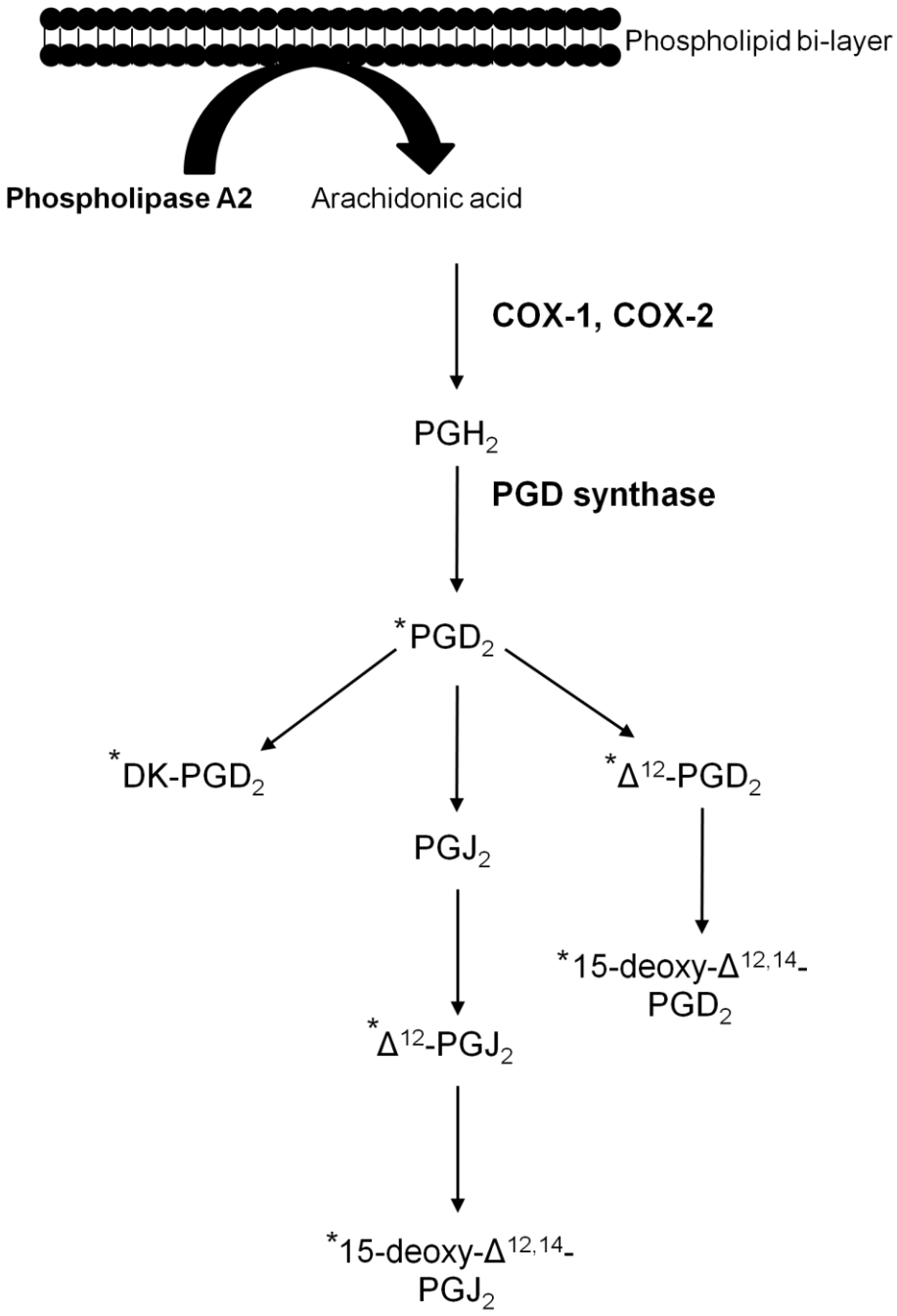

Figure 2. Metabolic pathway of PGD_2 synthesis

PGD_2 is metabolized from membrane bound arachidonic acid. Arachidonic acid is cleaved from the membrane by phospholipase A2 and further metabolized to biologically active molecules, including PGD_2 by cyclooxygenase-1 (COX-1) and COX-2 and prostaglandin (PG) D synthase. * indicates activity reported at CRTh2 receptor.

and low expression on monocytes (25, 43). Further, CRTh2 is expressed by murine mast cells (50).

The differential regulation of CRTh2 is poorly understood, with most of the literature focusing on receptor function. *CRTh2* is located on chromosome 11; location: 11q12-q13.3. This region has been associated with susceptibility genes for asthma in African Americans by genome wide scanning and mapping analysis (51). Further, genetic variations in potential regulatory regions of CRTh2 have been associated with allergic diseases in various populations (52-53).

1.4.1 PGD_2 binding to DP_1 decreases eosinophil migration and IFNγ

Prior to the discovery of CRTh2 as another D prostanoid receptor, PGD_2 was predicted to be a mediator of allergic asthma using DP_1 knock-out C57BL/6 mice. These mice displayed lower lymphocyte and eosinophil infiltration to the lungs, lower Th2 cytokine release and less airway hyper-responsiveness in a mouse model of ovalbumin (OVA)-induced asthma (54). However, in a study with chimeric mice, where irradiated wild type mice were reconstituted with bone marrow from $DP_1^{-/-}$ mice, loss of DP_1 resulted in increased lung eosinophilia in an OVA induced asthma model (55). The difference is considered to be due to DP_1 expression on non-hematopoietic cells including epithelial cells, fibroblast, smooth muscle cells and importantly endothelial cells which may have a pro-inflammatory effect including vasodilation and increased transendothelial migration of immune cells (11, 55). There may also be a pro-inflammatory effect of PGD_2-CRTh2 signaling that may be heightened when DP1 is missing. Using PGD_2 and DP_1 specific agonist BW245C signaling through DP_1 reduces IFNγ and

IL-2 expression from human $CD4^+$ and $CD8^+$ cells. (56). These data imply that the DP_1 receptor, expressed on both hematopoietic and non-hematopoietic cells, has a complex effect, but that overall is considered to be anti-inflammatory.

1.4.2 CRTh2 is expressed on Th2 cells and other leukocytes

CRTh2 is expressed on Th2 but not Th1 cells in humans (25, 43) and is currently the most reliable marker for circulating Th2 (and Tc2) cells in blood (24, 57-58). Although there is a low frequency of circulating $CRTh2^+$ T cells, they are functionally significant and contain allergen specific cells, since when $CRTh2^+$ cells are removed from peripheral blood mononuclear cells (PBMCs) and there is a lack of proliferation in response to a common allergen (house dust mite) (43). Further, when freshly isolated $CRTh2^+CD4^+$ cells from donors with atopic dermatitis are stimulated *in vitro*, 88% express IL-4, IL-5 and IL-13 but no IFNγ; indicating that CRTh2 is *frequently* and *selectively* expressed by Th2 cells (59). In addition, PGD_2-CRTh2 ligation also results in chemotaxis of Th2 cells, eosinophils and basophils (44) which may account for the increased presence of these cells at the sites of allergic inflammation. Further, CRTh2 is expressed by murine mast cells and mediates calcium dependent migration in response to a CRTh2-specific metabolite of PGD_2, DK-PGD_2 (50).

1.5 PGD_2 ligation of CRTh2 results in Th2 cytokine production

Functionally, ligation of CRTh2 on Th cells has been shown to induce Th2 cytokine release, independent of T cell receptor (TCR) activation (44, 56, 60-61). Although the majority of PGD_2 is considered to come from mast cells, there is

also evidence for paracrine activation of CRTh2 because PGD_2 is also produced by Th2 cells (62).

Since CRTh2 is a G-protein coupled receptor bound to $G_{\alpha i}$, ligation results in increased intracellular Ca^{2+} (25, 43). Th2 cytokine production is downstream of calcium flux, through calcineurin mediated dephosphorylation of cytoplasmic nuclear factor of activated T cells (NFAT) proteins. PGD_2-CRTh2 signaling has been shown to result in nuclear translocation of NFAT2 and IL-4 production (63).

Interestingly, PGD_2-CRTh2 ligation has also been suggested to maintain IL-4 expression by keeping NFAT2 in the nucleus. PGD_2-CRTh2 activates the phosphatidylinositol 3-kinase (PI3K) pathway that inhibits glycogen synthase kinase 3β (GSK3β), a kinase that regulates nuclear exports of NFAT proteins, thus turning off cytokine expression (63) (**Figure 3)**.

1.5.1 PGD_2 ligation of CRTh2 inhibits apoptosis of Th cells

PGD_2 ligation of CRTh2 has also been shown to inhibit apoptosis in T cells deprived of IL-2 (64). One mode of apoptosis is intrinsic apoptosis which begins by release of cytochrome *c* from the mitochondria. PGD_2-CRTh2 signaling inhibits apoptosis by activation of PI3K-mediated phosphorylation of a serine/threonine protein kinase, protein kinase B (PKB) (64). The activated PKB phosphoryates a pro-apoptotic molecule, Bcl-xL/Bcl-2-associated death promoter (BAD) which normally causes release of cytochrome *c* from the mitochondria (64)

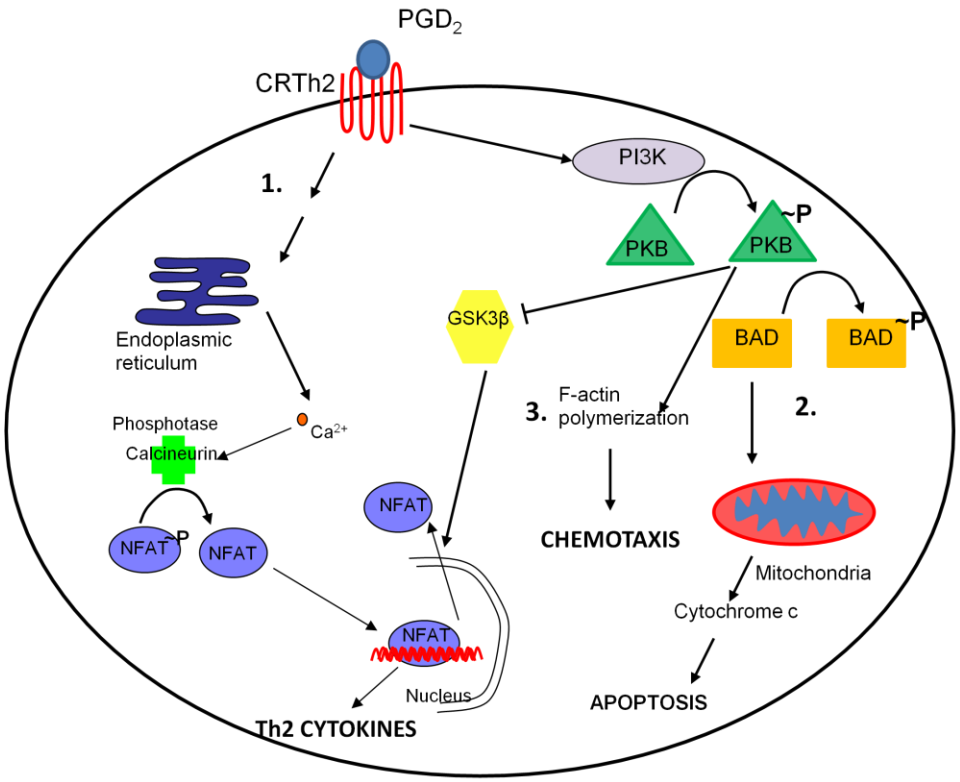

Figure 3. Signaling pathways downstream of PGD_2-CRTh2 ligation

PGD_2 signaling via CRTh2 activates 1) calcium signaling to increase NFAT dependent cytokine expression and 2) PI3K to inhibit apoptosis and 3) polymerization of F-actin for chemotaxis. NFAT: nuclear factor of activated T cells, PI3K: phosphatidylinositol 3-kinase, GSK3β: glycogen synthase kinase 3β, BAD: Bcl-xL/Bcl-2-associated death promoter, PKB: protein kinase B, CRTh2: chemoattractant homologous molecule expressed on Th2 cells, PGD_2: prostaglandin D_2.

(Figure 3). This could lead to prolonged survival of Th2 cells at the site of allergic inflammation.

1.5.2 PGD_2 ligation of CRTh2 induces chemotaxis

Another function of PGD_2-CRTh2 signaling is chemotaxis of Th2 cells, eosinophils and basophils via the CRTh2 $G_{\alpha i}$ mediated activation of PI3K (63). PI3K mediated activation of PKB allows F-actin to polymerize resulting in chemotaxis, independent of the CRTh2 mediated Ca^{2+} signaling (63) **(Figure 3)**.

1.5.3 Evidence for PGD2–CRTh2 mediated inflammatory cell infiltration and local expansion

The relative contribution of infiltration versus local expansion of $CRTh2^+$ cells at sites of allergic inflammation is still not clear. Indeed, higher numbers of CRTh2 expressing leukocytes have been observed in the nasal mucosa of patients with allergic rhinitis and skin of atopic dermatitis patients (38, 65). Since $CRTh2^+$ leukocytes have been shown to undergo chemotaxis in response to PGD_2, the increased numbers of $CRTh2^+$ leukocytes may be due to infiltration. Although $CRTh2^+$ cells undergo chemotaxis in response to PGD_2, they also respond to the Th2 chemoattractant, thymus and activation regulated chemokine (TARC); a CCR4 agonist but not Th1 chemoattractants, macrophage inflammatory protein 1β (MIF 1β); a CCR5 agonist and interferon-γ-inducible protein 10 (IP 10); a CXCR3 agonist (59). This would indicate that recruitment may be important to establish $CRTh2^+$ cells at the site of allergic inflammation. However, there is evidence with a nasal mucosal explant system that IgE/anti-IgE stimulations increase PGD_2 and CRTh2 mRNA (66) indicating that local expansion of $CRTh2^+$ cells may occur.

Regardless of the local expansion or infiltration, in humans, PGD_2 is found in the lungs of asthmatics following allergen challenge (67) and severe asthma has been associated with an increased number of PGD_2 producing mast cells in the lungs (68). Further, the proportion of $CRTh2^+CD4^+$ cells correlates with disease severity in atopic dermatitis patients (59) and circulating $CD4^+$ T cells from atopic dermatitis patients have more CRTh2 mRNA (69). These indicate that $CRTh2^+$ cells have a significant role in these allergic diseases.

1.6 Animal models used to study PGD_2–CRTh2 interaction in allergic inflammation

The effect and mechanisms of PGD_2 in the allergic response has been extensively studied in animal models. One model commonly used is an antigen induced asthma model to mimic immune cell infiltration to the lungs as seen in human asthmatics. In these models, animals are sensitized intraperitoneally to antigen and later challenged (by aerosol or intratracheal) with the same antigen so they develop allergic asthma. In studies of CRTh2, two antigens used are OVA (70) and soluble cockroach antigen (71). Other mouse models were used to assess the role of CRTh2 in allergic skin inflammation, especially to study atopic dermatitis (AD). These models involve exposing the skin of the animals repeatedly to small hapten molecules (72), tape stripping an area of skin to cause irritation and then exposing it to an allergen (73) or by epicutaneous sensitization to cedar pollen. A model of allergic rhinitis in mice is also used, where animals develop allergic symptoms following repeated intranasal sensitization with a common conifer pollen (74).

1.6.1 Over-expression of PGD synthase increases allergic phenotypes

Over-expression of human lipocalin-like PGDS in mice (FVB strain) resulted in increased eosinophils and lymphocyte infiltration in an OVA induced asthma model (75). Saline washes (bronchoalveolar lavage) from lungs of PGDS transgenic mice showed increased IL-4, IL-13 and an eosinophil recruitment chemokine (eotaxin) as well as decreased IFNγ compared to wild type mice. This study indicates a role for the downstream effector of PGDS, PGD_2, in Th2 responses (75).

1.6.2 Activation of CRTh2 using specific agonists results in increased Th2 response

CRTh2 activation with specific agonist DK-PGD_2 increases the number of eosinophils in bronchoalveolar lavage and tissue in mouse models of asthma and atopic dermatitis, respectively (70). Eosinophilia in rat lungs can also be seen following tracheal administration of CRTh2 selective agonists, DK-PGD_2 and 15R-methyl-PGD_2 (76-77).

1.6.3 Antagonism of CRTh2 decreases allergic responses

There are also animal studies showing that blocking CRTh2 inhibits various aspects of the allergic response, including airway hyperresponsiveness and leukocyte infiltration into airways (71). To block CRTh2 in BALB/c mice, a CRTh2 antagonist was used in a soluble cockroach antigen asthma model. The CRTh2 antagonist reduced antigen specific IgE, IgG_1, and IgG_{2a} as well as decreased mucous accumulation and leukocyte infiltration in the airways (71). Further, BALB/c mice treated with the CRTh2 antagonist also showed decreased

inflammatory infiltrate and allergen specific IgE in an atopic dermatitis model (78).

1.6.4 *Crth2* deficient mice show conflicting changes in allergic phenotypes

Mice deficient in *Crth2* (*Crth2*$^{-/-}$) have also been studied. Several groups have used a similar *Crth2*$^{-/-}$ mouse that was created on the BALB/c background. In models of atopic dermatitis, *Crth2*$^{-/-}$ mice show a decrease in cellular infiltrate (eosinophils and lymphocytes) at the site of allergic inflammation, compared to wild type mice (72-73, 79). Similarly, in a model of allergic rhinitis, *Crth2*$^{-/-}$ mice have decreased allergen-specific IgE, infiltrating eosinophils and IL-4 in the regional lymph node compared to wild-type mice (74). Interestingly, another group has created *Crth2*$^{-/-}$ mice on the C57Bl/6 background; although they observed a decrease in IL-4 from splenocytes, there was increased eosinophilia in an asthma model due to increased IL-5 compared to wild-type mice (80).

The differences in these knockout mice could be due to: 1) the mouse strain, i.e. BALB/c versus C57Bl/6; 2) disease models (skin versus lung model) and/or 3) differences or defects in animal response due to incomplete backcrossing of the *Crth2*$^{-/-}$ in the C57Bl/6 mice. Studies have shown that BALB/c mice may be more susceptible to developing Th2 responses (81-82). One mechanism may be due to differences in mast cell production of PGD_2, since one study shows BALB/c mice produce more PGD_2 than C57Bl/6 mice (81). Mice have also been shown to express CRTh2 on both Th1 and Th2 cells (80, 83) which may lead to unexpected dysregulation of IL-5 as seen in the C57Bl/6 *Crth2*$^{-/-}$ mouse. Even with the contradicting results from *Crth2*$^{-/-}$ mice, the bulk of the literature suggest that

CRTh2 could be central in maintaining an allergic Th2 cell phenotype at the site of the allergen exposure.

1.7 CRTh2 antagonists – proof of concept and clinical trials

Due to the reduction in allergic responses by blocking CRTh2, there are currently multiple CRTh2 antagonists which have been tested in animal models and a few are in clinical trials. The only CRTh2 antagonist on the market (BAYu3505) is used in Japan for treatment of allergic rhinitis and is commonly known as Ramatroban. This antagonist was originally developed as a thromboxane A_2 inhibitor and was found to decrease bronchial hyper-responsiveness to methacholine challenge in humans (84). Later, Ramatroban was also found to inhibit CRTh2 activity (85-86). Further, TM30089 is a highly selective antagonist for CRTh2 that has no activity at the thromboxane A_2 receptor and also decreases peribronchial eosinophilia and mucus cell hyperplasia in a mouse model of asthma (87).

Developing antagonists for CRTh2 as a treatment of allergic disease is currently underway. Molecules have been developed by Merck (MK-7246) (88-89), Amira Pharmaceuticals (AM432) (90) and AstraZeneca (AZ11665362) (91). In animal models of allergic disease, these molecules have inhibited eosinophil activation (89-91), recruitment of eosinophils and basophils (91) and airway hyperresponsiveness (89). These molecules are a proof of concept, and indicate the likelihood that this approach may be effective as a treatment in human allergic disease.

Currently the pharmaceutical company Actelion and an Oxford biotech company Oxagen both have CRTh2 antagonists in Phase II clinical trials for dose range efficacy in patients with allergic rhinitis and asthma (www1.actelion.com/en/scientists/development-pipeline/phase-2/crth2-antagonist.page) and (http://www.oxagen.co.uk/pdfs/CRTH2summary.pdf), (reviewed in (92)).

1.8 Molecular regulation of the Th2 phenotype

1.8.1 DNA and chromatin structure

Gene expression can be regulated by DNA accessibility; this is accomplished through epigenetic modification of chromatin. Eukaryotic genomes exist in a 3-dimensional structure called chromatin, where DNA is strategically wrapped around protein complexes called histones. The histone-DNA complexes are known as nucleosomes. When DNA is in a closed chromatin conformation (heterochromatin), the genes are not accessible for transcription. To allow for transcription, cells open the chromatin conformation (euchromatin) in a region of the genome when local histone acetylation occurs (reviewed in (93)). Histone modifications that regulate accessibility of DNA are used to regulate genes that are not needed in a specific cell type or stage of cell development/differentiation.

1.8.2 Epigenetic changes to DNA modify gene accessibility

In addition to histone modifications, epigenetic modifications involving changes to DNA, but not to the DNA sequence, also control gene accessibility and transcription. One of the major modifications is methylation of the five carbon on cytosine bases in the dinucleotide sequence CpG (94). Regions with high CpG

content and hyper-methylation are associated with gene silencing whereas hypomethylated CpG motifs have been associated with an open DNA conformation (94). Methylated DNA has been associated with recruitment of histone deacetylases which results in a closed chromatin structure (reviewed in, (95)). Demethylation of CpG motifs has been shown as a regulatory mechanism for several cytokine genes including IL-2 and IL-4 during differentiation (96-97). Importantly, DNA methylation patterns are maintained between cell generations by maintenance DNA methyltransferases (98-99), even in the absence of the original stimulus (reviewed in (100)).

Chromatin remodeling, specifically histone acetylation, is important in opening the Th2 cytokine locus in differentiating Th2 cells (101). Importantly, this occurs downstream of TCR activation and IL-4 dependent STAT6 signaling (102-103). Specifically, this chromatin remodeling is GATA3 dependent (104-105). Without chromatin remodeling, transcription factors, such as NFAT and GATA3 could not activate transcription of Th2 cytokine genes (106).

1.9 GATA3 and NFAT, transcription factors involved in regulating the Th2 phenotype

1.9.1 GATA3 is the transcription factor largely responsible for Th2 differentiation

Several mouse models have shown that GATA3 is a major transcription factor responsible for Th2 differentiation *in vivo* (107-109). Most convincingly, when GATA3 is inhibited, expression of Th2 cytokines is significantly decreased, thus GATA3 is necessary for Th2 cytokine production. Further, when GATA3 is

expressed ectopically in Th1 clones, Th2 cytokine mRNA increases substantially indicating that GATA3 is sufficient for Th2 cytokine expression (110).

GATA3 expression can be induced by IL-4. IL-4 signaling through its receptor on T cells results in dimerization and activation of STAT6. STAT6 was shown to be necessary and sufficient for IL-4 dependent Th2 differentiation (111). More specifically, STAT6 activation is upstream of GATA3 expression (112) and interacts at the GATA3 promoter (113). In resting Th2 cells, GATA3 found in the cytoplasm and upon T cell receptor activation p38MAP kinase phosphorylates GATA3 for nuclear translocation via importin-α (114).

1.9.2 High expression of GATA3 is strongly associated with the CRTh2$^+$ T cell fraction in humans

A study in a human population missing one GATA3 allele showed decreased serum levels of Th2 associated immunoglobulins (IgG_4 and IgE) and an increase in Th1 associated IgG1 (115). In humans, GATA3 expression *per se* is not a marker of Th2 cells, since low level GATA3 is also expressed in Th1 cells (23, 116). Importantly, the level of GATA3 expression is significantly higher in CRTh2$^+$ versus CRTh2$^-$ cells (23).

However, like in mice, ectopic expression of GATA3 in committed human Th1 cells is sufficient to induce Th2 cytokine expression and in naïve T cells results in up-regulation of CRTh2 (117). This plasticity was shown to be less present as cells matured to effector memory cells (117).

1.9.3 NFAT1 and NFAT2 are expressed in activated T cells

Nuclear factor of activated T cells (NFAT) is another family of transcription factors involved in Th2 differentiation. NFAT1 (NFATp, NFATc2) was the first NFAT to be cloned and is constitutively present in the cell cytoplasm (118). NFAT2 (NFATc, NFATc1) is present in low amounts in the cytoplasm of resting cells, but is greatly induced by T cell receptor activation and NFAT1 binding to its promoter (118-120).

1.9.4 NFAT activation is calcium dependent

In mice and humans, NFAT1 and low amounts of NFAT2 are found in the cytoplasm of resting T cells (119). Upon TCR activation, the signaling molecule phospholipase C-γ (PLCγ) is activated and hydrolyzes phosphatidylinositol-4-5-biphosphate to inositol-1, 4, 5-triphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ binds its receptor on the endoplasmic reticulum and induces the release of intracellular calcium stores. Calcium activates calmodulin which in turn activates the phosphatase calcineurin to dephosphorylate cytoplasmic NFAT and allow nuclear translocation where NFAT can function as a transcription factor (reviewed in (121)) **(Figure 4)**. NFAT nuclear translocation can be inhibited by Cyclosporin A, which interferes with the phosphatase activity of calcineurin.

1.9.5 NFAT1 and NFAT2 regulate Th2 differentiation

NFAT proteins mediate Th2 cytokine production (IL-4 and IL-13) (122). NFAT1 significantly increased early IL-4 in human primed T cells (123) and mouse *in vitro* differentiated Th2 cells (106, 124). At the *Il4* enhancer, NFAT1 and GATA3 synergize to increase IL-4 (106). Interestingly, NFAT1 was also shown to bind

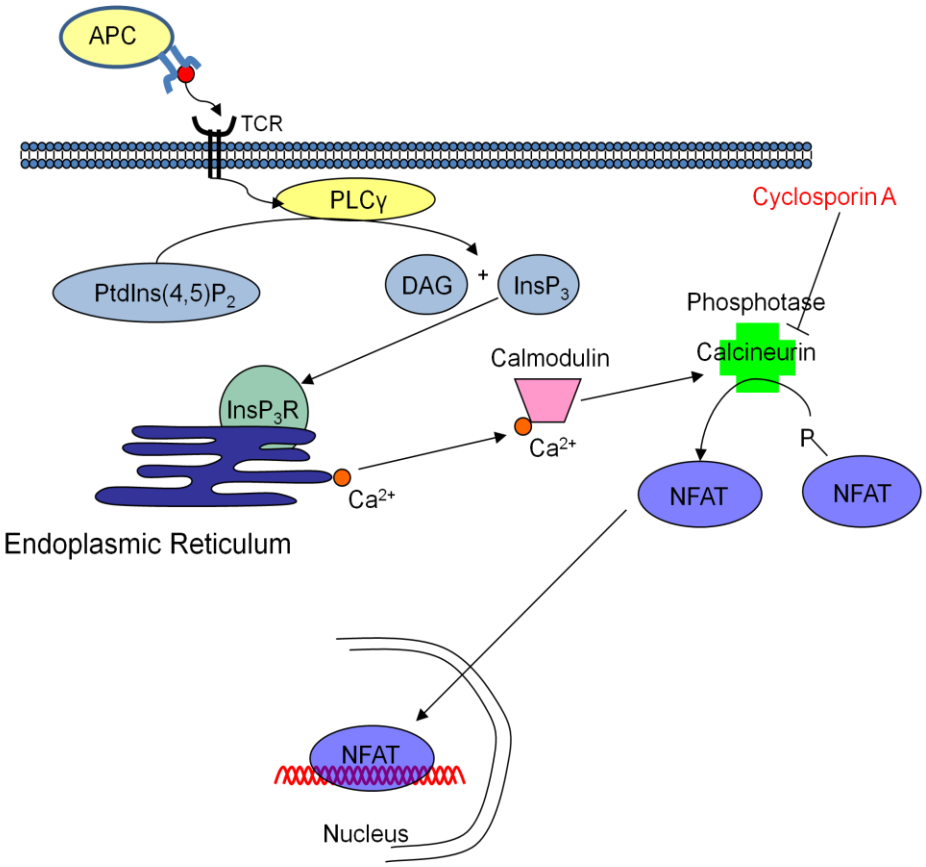

Figure 4. NFAT activation cascade following TCR activation

Signaling downstream of TCR activation results in increased intracellular calcium (Ca^{2+}) which activates a phosphatase (calcineurin) to dephosphorylate NFAT and allow nuclear translocation. APC: antigen presenting cell, TCR: T cell receptor, PLCγ; phospholipase C γ, PtdIns (4, 5) P_2: Phosphatidylinositol 4, 5-bisphosphate, DAG: diacylglycerol, $InsP_3$: Inositol triphosphate, $InsP_3R$: inositol triphosphate receptor.

the *IFNγ* promoter in Th1 cells and increase IFNγ production, indicating that NFAT1 is not cell type specific and that chromatin regulation appears to also direct the cytokine genes upon which NFAT1 acts (106, 124). Similarly, NFAT2 in mice was shown to be necessary for IL-4 production following stimulation (125-126). Mice deficient in NFAT2 show decreased lymphocyte proliferation, IL-4 and decreased IL-4 driven isotype switching (to IgE) in B cells (125-126).

Although NFAT1 has been associated with early IL-4 production (reviewed in (127-128), NFAT1 knockout mice show increased and prolonged late phase IL-4 response (129-130). NFAT1 knockout mice also show an enhanced Th2 response in a parasite model (*Leishmania major***)** (131). Also, when both NFAT1 and NFAT4 (NFATc3) are knocked out in mice, naïve Th0 cells intrinsically differentiate into Th2 cells (126, 132-133). This is further substantiated in a glycosylation-inhibiting factor (GIF) deficient mouse which showed that normally GIF maintained NFAT1 in the nucleus resulting in decreased IL-4 expression (134). These studies indicate overall NFAT1 may limit Th2 responses, possibly in a negative feedback mechanism.

Although NFAT1 is important in initial up-regulation of IL-4 and IL-13 (122), NFAT1 knockout mice show that the major role may be to down-regulate IL-4 in a negative feedback mechanism (129). There are also complex interactions of NFAT proteins with several co-factors to direct gene expression (reviewed in (128)). These complicated actions of NFAT1 indicate that in different cellular,

genetic environments and stages of differentiation, NFAT1 may act in variable ways.

1.10 Rationale for this study

The regulation of *CRTh2* is poorly understood but since CRTh2 mRNA levels have been shown to correlate with protein expression (43-44), we hypothesized that CRTh2 expression is regulated at the level of transcription. Transcription can be regulated by chromatin conformation and DNA methylation status. If the gene is constitutively unmethylated or open, transcription could be primarily regulated by transcription factor binding. Ectopic expression of GATA3 in naïve T cells has been shown to up-regulate surface expression of CRTh2 (117) and CRTh2 expression is induced by IL-4, an upstream effector of GATA3 (23). Our lab previously showed that GATA3 over-expression in Jurkat T cells increases *CRTh2* promoter activity, using a reporter gene assay (135). The human *CRTh2* gene contains putative binding sites for common Th2 cytokine transcription factors, such as GATA and NFAT (135). To further study the molecular regulation of *CRTh2*, we developed a $CRTh2^+$ Th2 cell line and examined promoter activation and endogenous gene expression. My overall hypothesis is that NFAT and GATA3 contribute to molecular regulation of CRTh2 and expression in $CRTh2^+$ Th2 cells.

1.11 Study objectives

To study the molecular regulation of CRTh2 expression

The objective of this thesis was to study the regulation of the proximal region of the *CRTh2* promoter. Chromatin structure was studied by assessing methylation in

CRTh2^{high} and CRTh2^{low} cells. Transcriptional regulation was also studied using a luciferase promoter reporter construct containing a fragment of the *CRTh2* promoter. Transcription factor binding was determined by electromobility shift assay (EMSA) and transcription factors were over-expressed in CRTh2^{low} cells to assess their role in CRTh2 transcription.

To study the regulation of endogenous CRTh2 expression

To determine whether the stimulus induced promoter activity observed with reporter constructs reflects endogenous expression, CRTh2 expression was studied by surface staining and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

To assess whether OX40L co-stimulation regulates CRTh2 expression

Since OX40L has been associated with maintenance of CRTh2^{+} cells in a DC-T cells co-culture system, we asked whether exogenous OX40L could alter *CRTh2* gene responses. Exogenous OX40L was added to cultures at various time points following stimulation and CRTh2 expression was examined by qRT-PCR.

.

CHAPTER II: Materials and Methods

2.1 Cell culture

2.1.1 Jurkat cells

Jurkat cells (clone E6-1) were purchased from American Type Culture Collection (VA, USA) and were cultured in RPMI 1640 media (Sigma Aldrich, ON, Canada) supplemented with Fetal Bovine Serum (10%; Hyclone Scientific, Fisher Scientific, Ontario, Canada) and Penicillin, Streptomycin and glutamine (1X; Gibco, ON, Canada).

2.1.2 *In vitro* differentiated CRTh2$^+$ cells

Peripheral blood mononuclear cells (PBMCs) were isolated from self reported non-allergic (n=1) and allergic donors (n=8) by density centrifugation over Ficoll histopaque PLUS (GE Healthcare, Sweden) and CD4$^+$ cells were isolated by negative selection with CD4$^+$ cell Isolation Kit II (Miltenyi Biotech, CA, USA). On one occasion, as indicated, following CD4$^+$ isolation, naïve CD4$^+$ CD45RA$^+$ cells were further isolated using a CD45RO depletion kit (CD45RO microbeads, Miltenyi Biotech, CA, USA). Cells were activated on plate bound anti-CD3 (Clone UCHT1, 1 µg/mL) and anti-CD28 (Clone 37407, 1 µg/mL) in Th2 differentiating conditions; recombinant human (rh) IL-2 (5 ng/mL), rhIL-4 (10 or 20 ng/mL), and blocking antibody against IFNγ (polyclonal, 1µg/mL) and IL-12 (Clone C8.6, 1µg/mL) for 3 days. Cells were then proliferated with cytokines and blocking antibodies but without activating CD3 and CD28 for 4 days. After 7 days of differentiation, CRTh2$^+$ cells were isolated by positive selection (CRTh2$^+$ cell selection kit, Miltenyi Biotech, CA, USA) or sorted for CRTh2 using anti-

CRTh2 PE (Miltenyi Biotech, CA, USA) with the fluorescence activated cell sorting (FACS) Aria cell sorting flow cytometer (Becton Dickson, ON, Canada) to 98% purity. $CRTh2^+$ cells were maintained for up to 60 days on cycles of **activation** (3 days αCD3/CD28 + IL-2) followed by **proliferation** (4 days IL-2 alone) **(Figure 5)**. Experiments were performed between days 10 and 60, when CRTh2 expression was >50%. Recombinant human cytokines and monoclonal antibodies were all from R&D Biosystems Inc. (MN, USA) except anti-IL-12 was from eBiosciences Inc. (CA, USA). All primary cells were cultured in X-Vivo15 media (Lonza, MD, USA**)**, supplemented with Fetal Bovine Serum (10%; Hyclone, Fisher Scientific, Canada) and Penicillin, Streptomycin and glutamine (1X; Gibco, ON, Canada). All stimulations were done with cells at 2 x 10^6 cells/mL (unless otherwise indicated) in X-Vivo15. As indicated, phorbol 12-myristate 13-acetate (PMA) (20 ng/mL, Sigma Aldrich, ON, Canada), ionomycin (1μM, Sigma Aldrich, ON, Canada), N-terminal histidine tagged OX40L (50 – 200 ng/mL, R&D Biosciences, MN, USA, 1054-OX) and/or α-polyhistidine (9.2 μg/mL, R&D biosciences, MN, USA, MAB050) were added.

2.1.3 Extracellular flow cytometry

Isolated $CD4^+$ T cells and *in vitro* differentiated $CRTh2^+$ Th2 cells were stained for surface markers following both the activation and proliferation phases during culture. All antibodies were purchased from BD Pharmingen (ON, Canada), unless otherwise indicated. Cells were incubated with antibody or matched antibody control at 4°C (30 min) before fixation with 2% paraformaldehyde + 0.54% sucrose. Staining was assessed by flow cytometry (FACSCalibur, Becton

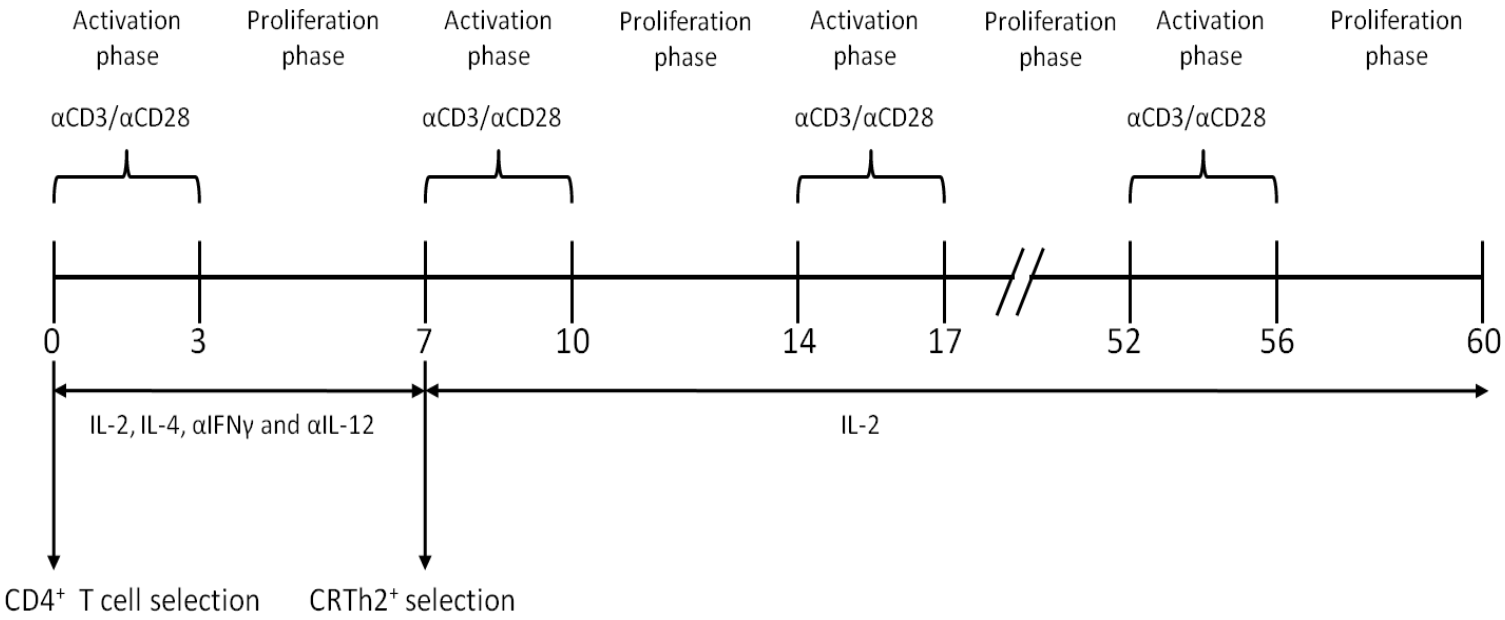

Figure 5. ***In vitro*** **differentiation of CRTh2$^+$ Th2 cells**

Schematic timeline for *in vitro* differentiation of CD4$^+$ T cells, isolation of CRTh2$^+$ cells and carrying the differentiated CRTh2$^+$ Th2 cells.

Dickson, ON, Canada). Cells were stained for CD4 (Clone RPA-T4, Isotype mouse IgG1 FITC, AbD serotech), CD8 (Clone RPA-T8, isotype mouse IgG1κ PE), OX40 (Clone ACT35, isotype mouse IgG1κ FITC), CD62L (Clone DREG-56, isotype mouse IgG1κ PE), CD45RA (Clone L48, mouse IgG1κ FITC) and/or CD45RO (Clone UCHL-1, isotype mouse IgG2κ PE-Cy7). Only cells stained for OX40 were blocked with normal mouse IgG (Invitrogen, CA, USA) at room temperature (30 min) prior to incubation with antibody.

CRTh2 expression was assessed throughout the culture by surface staining after the proliferation phase or after the activation phase. Cells were blocked with normal rat IgG **(**Invitrogen, CA, USA**)** at room temperature (30 min) followed by staining with primary biotin conjugated CRTh2 antibody (Clone BM16, Miltenyi biotech, CA, USA) or rat IgG2a isotype (ABD Serotech, NC, USA) at 4°C (30 min) followed by Streptavidin-APC (eBioscience, CA, USA), at 4°C (30 min). Samples were fixed and assessed by flow cytometry as above.

2.1.4 Intracellular flow cytometry

Intracellular staining for IL-4, IL-13 and IFNγ was performed following the proliferation phase. *In vitro* differentiated $CD4^+$ $CRTh2^+$ cells (1.3 x 10^6 cells/mL) were stimulated (4h) with PMA (20 ng/mL) and ionomycin (1 µM) in the presence of Brefeldin A (10 µg/mL, Sigma Aldrich, ON, Canada). Cells were fixed on ice (10 min) with paraformaldehyde (4%; Sigma Aldrich, ON, Canada) and permeabilized on ice (10 min) with saponin (0.4%, Sigma Aldrich, ON, Canada). IL-13-PE (Clone JES10-5A2, isotype rat IgG1κ PE), IFNγ-Alexa 647 (Clone B27, isotype mouse IgG1κ Alexa 647) and IL-4-Alexa 488 (Clone 8D4-8,

isotype mouse IgG1κ Alexa 488) antibodies (BD Pharmingen, ON, Canada) were added and samples were incubated at 4°C (30 min) and after washing cells, fluorescence was assessed by flow cytometry (FACS Calibur, Becton Dickson, ON, Canada).

2.1.5 Supernatant analysis for cytokines

Supernatant from differentiated CRTh2$^+$ Th2 cells were collected following the activation phase and frozen at -80°C until use. Supernatants were analyzed for cytokines by 42-plex discovery assay, using Multiplex LASER Bead Technology (EVE technologies, Calgary, Alberta).

2.2 *CRTh2* sequence analysis and assessment of promoter region methylation

2.2.1 Sequence analysis

CRTh2 sequence was obtained from Ensembl (http://www.ensembl.org) and species conservation was determined using VistaPlot (http://genome.lbl.gov/vista/index.shtml). *In silico* analysis of transcription factor binding sites was performed using MatInspector (www.genomatix.de) and matrix search parameters were set at a core similarity of 0.75 and an optimized matrix similarity.

2.2.2 Bisulphite treatment of DNA and sequencing

Genomic DNA was extracted from freshly isolated CD4$^+$ T cells (Day 0) or from *in vitro* differentiated CRTh2$^+$ Th2 cells (Day 45) using the Wizard genomic DNA extraction kit (Promega, WI, USA) and eluted in nuclease free water. Genomic DNA was treated with sodium bisulphite according to manufacturer's instructions (EZ DNA methylation Kit; ZymoResearch, CA, USA) and resuspended in nuclease free water. Sodium bisulphite treatment results in

deamination of unmethylated cytosines **(Figure 6a)**. A fragment of the 5' region of *CRTh2* (-646 to +66) was amplified by polymerase chain reaction (PCR) from bisulphite treated DNA with primers designed using MethPrimer (www.urogene.org/methprimer/index1.html) to recognize bisulphite treated DNA: TTTTAGAGGAGAAGGAAGTTG – forward and ACAATCAACTCCTAACAACAAC – reverse. Untreated DNA was also amplified by PCR as a negative control using the following primers: CCTCAGAGGAGAAGGAAGCTG - forward and GCAGTCAGCTCCTGACAGCAGC – reverse. Amplified DNA was gel extracted (Gel extraction kit, Qiagen) and 4 µl of PCR product was cloned (pCR2.1TOPO vector, TOPO-TA cloning kit, Invitrogen, CA, USA). From each cloned PCR amplification, 20 clones were sequenced and analyzed for methylation status. With this procedure, methylated cytosines are protected from deamination **(Figure 6b)** and are amplified as cytosine **(Figure 6c)**. Unmethylated cytosines are deaminated and undergo a conformational change to uracil, which is then amplified as thymine **(Figure 6c)**. Results are presented as percent of clones methylated at each CpG site [(number protected (methylated) cytosines/ (total number of cytosines)]. Sequencing was performed by Molecular Cloning Laboratories (McLab, San Francisco, California, USA).

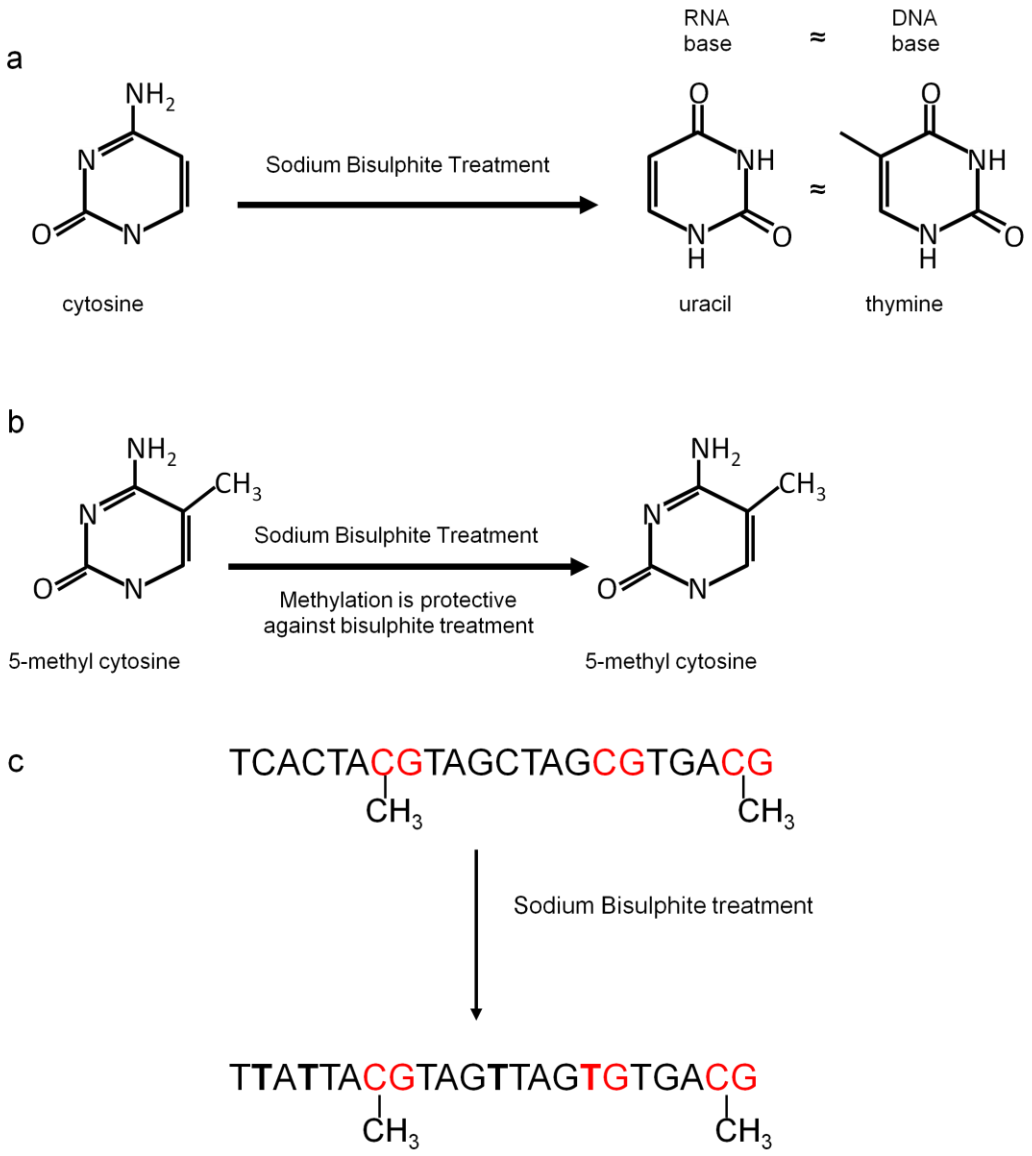

Figure 6. Sodium bisulphite deamination of unmethylated cytokines

Treatment of DNA with sodium bisulphite results in deamination of cytosine to uracil **(a)**. Methylated cytosines are protected from deamination **(b)**. All unmethylated cytosines will be deaminated to uracil **(c)**.

2.3 Transient transfection and nucleofection

2.3.1 Plasmids

Luciferase reporter plasmids were constructed by cloning the region 450 base pairs, upstream of the transcription start site (TSS, **Figure 7a**) into the pGL3 basic (Promega, WI, USA, **Figure 7b**) construct (*CRTh2*-450/Luc).

The complimentary DNA (cDNA) for GATA3 and NFAT1 (OriGene, United States) were sub-cloned into pcDNA3.1^{+} (Invitrogen, ON, Canada).

For over-expression experiment, expression vectors were added at molar equivalents relative to GATA3; GATA3 (5 µg), pcDNA3.1^{+} (3.95 µg) and/or NFAT1 (6.1 µg). Furthermore, in order to add equal amounts of plasmid DNA during the over-expression assay, pcDNA3.1^{+} was added to NFAT1 and GATA3 conditions to equal the molar equivalent of the total DNA in the NFAT1 and GATA3 co-transfection (10 µg molar equivalents to GATA3).

2.3.2 Dual Luciferase Assay

The luciferase reporter construct will produce Firefly luciferase under the control of the promoter region cloned 5' of the luciferase gene (ie, proximal *CRTh2* promoter region, *CRTh2*-450/Luc). Luciferase production was measured with the Dual Luciferase Assay system, used according to manufacturer's instructions (Promega, WI, USA) and light output was quantified using a luminometer (20/20n, Turner Biosystems, WI, USA). The amount of light that is emitted from the Firefly luciferase reports the activity of the *CRTh2* promoter region. A control plasmid (pRL-TK) is co-transfected with the Firefly luciferase reporter plasmid.

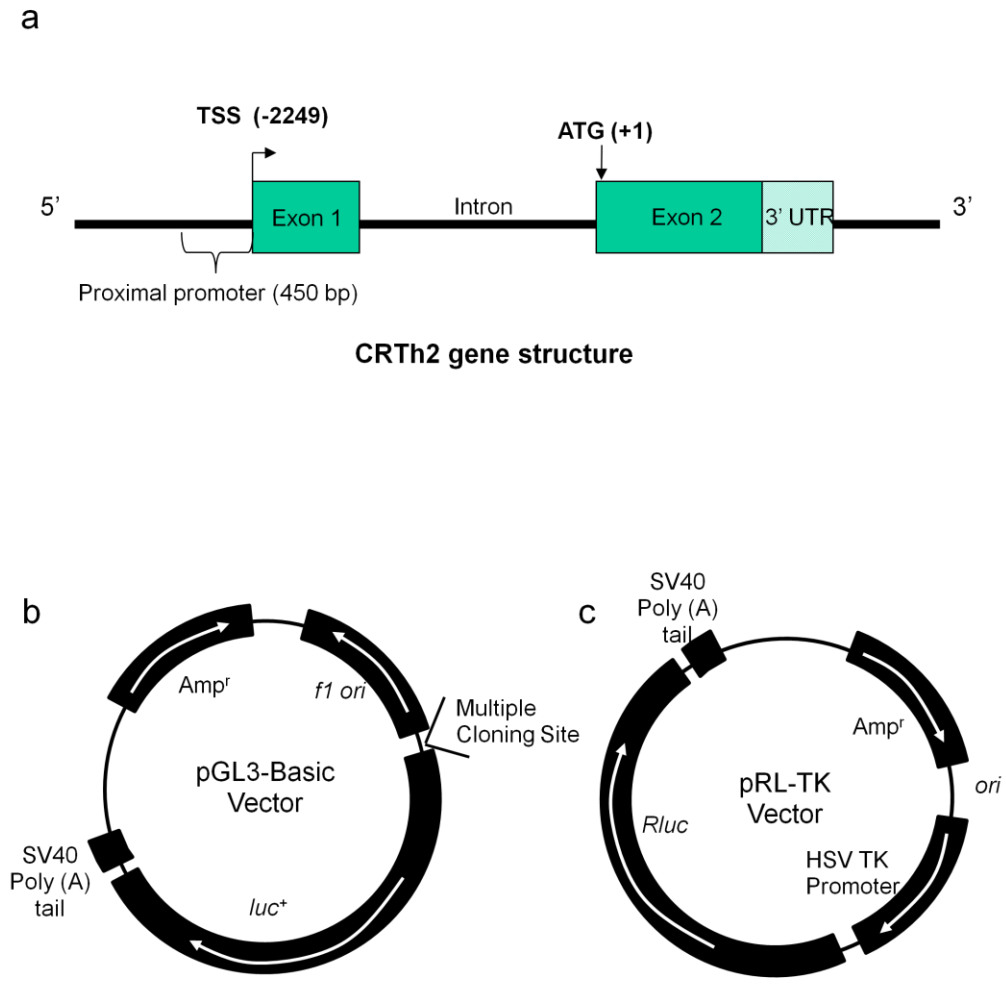

Figure 7. The *CRTh2* gene structure and luciferase reporter constructs

The *CRTh2* gene contains a large 5' region, up to -7000 base pairs upstream of the coding region (ATG), an untranslated exon (exon 1), one intron, a translated exon (exon 2) and a 3' untranslated region (3' UTR) **(a)**. Luciferase constructs in pGL3-basic (Promega) were generated by cloning a 450 base pair region 5' of the TSS. pGL3-basic vector contains no eukaryotic promoter and the *firefly luciferase* cDNA **(b)** pRL-TK vector (Promega) contains the constitutively active herpes simplex thymidine kinase promoter and is used as a control in dual luciferase transfections **(c)**.

The pRL-TK plasmid contains a Sea Pansy luciferase (Renilla luciferase) under the control of the constitutive Herpes Simplex thymidine kinase promoter **(Figure 7c).** The Renilla luciferase plasmid acts as a transfection efficiency control. The expression of the Renilla luciferase indicates that the transfection or nucleofection was successful and we can assume that the experimental plasmid containing the Firefly luciferase was also successfully transfected or nucleofected. The Firefly and Renilla luciferase enzymes react with different substrates to emit light. The Firefly luciferase reacts with luciferin and the Renilla luciferase with colenterazine. To measure the two light outputs independently, luciferin is added first to the transfected cell lysates and the light is quantified. Immediately following, the luciferin/Firefly luciferase reaction is quenched and the colenterazine is added and the light output from the Renilla luciferase reaction is quantified.

2.3.3 Transient Transfection

Jurkat T cells were transiently transfected with *CRTh2*-450/Luc (10 µg) and pRL-TK (5 ng) using electroporation. Jurkat T cells (5×10^6) were transfected in log phase growth. The control plasmid (pRL-TK) was added to all cells as a master mix, after which they were incubated on ice (10 min). *CRTh2*-450/Luc ± expression plasmids or control DNA was added and rested on ice (10 min). Cells were electroporated with 1 pulse (240 mV) using a square wave electroporator (ECM 830, Harvard Apparatus, MA, USA). Cells were rested on ice (15 min) before incubating in media alone (16h) or with stimulus (16h, PMA; 20 ng/mL and ionomycin; 1 µM).

2.3.4 Transient Nucleofection

In vitro differentiated $CRTh2^+$ Th2 cells (1.25 x 10^6 cells) were transiently nucleofected with *CRTh2*-450/Luc after the activation phase. Nucleofection was performed using a nucleofection kit (Human T Cell Nucleofector® Kit; Lonza, MD, USA). Cells, reporter plasmid (1µg) and control pRL-TK (10ng) were combined in Amaxa nucleofection solution and immediately electroporated. To control for background levels of Firefly luciferase, control nucleofections were performed without a Firefly construct, only the pRL-TK construct. Cells were electroporated using a Nucleofector™ Device (Amaxa, MD, USA, program T-23). Cells were left to recover in X-vivo15 media at 37°C (15 min) and incubated with or without PMA (20 ng/mL) and ionomycin (1 µM) stimulation (16h). When indicated Cyclosporin A (Sigma Aldrich, ON, Canada) was added to 16h stimulations.

Nucleofection efficiencies >25% (~ 30%) were achieved, as determined by flow cytometry analysis following nucleofection with pMAXGFP plasmid (1 µg/1.25 x 10^6 cells) (Lonza, MD, USA). Following stimulation (16h, PMA and Ionomycin, P/I), GFP transfected cells were stained with propidium iodide (Invitrogen, CA, USA) to exclude dead cells and GFP transfection was assessed by flow cytometry (FACSCalibur, Becton Dickson, ON, Canada).

2.3.5 Quantifying the Dual Luciferase Assay

For nucleofections and transfections, cells were harvested and lysed in 100 µl and 250 µl respectively, of passive lysis buffer (1X PLB, Promega, WI, USA) and frozen and thawed to increase lysis. For Jurkat transfections, 10 µl of lysate was

added to 50 µl of Firefly luciferase substrate (luciferin) followed by addition of 50 µl of Renilla luciferase substrate (colenterazine). For primary CRTh2$^+$ Th2 cell nucleofections, 50 µl of lysate was added to 100 µl of firefly luciferase substrate followed by addition of 100 µl of Renilla luciferase substrate. Total protein in each lysate was quantified using a BCA assay (Thermo scientific, IL, USA) and 20 µl of lysate. Transfection and nucleofection results were normalized for transfection efficiency (as determined by *Renilla* luciferase activity) and protein concentrations and expressed as relative luciferase activity (RLA). (Firefly luciferase count * (mean baseline Renilla / Renilla count) * (mean protein / protein count)).

2.4 Nuclear extracts and electromobility shift assays

2.4.1 Nuclear extracts

Nuclear extracts were made from Jurkat T cells in log phase growth (12 x 10^6 cells), *in vitro* differentiated CRTh2$^+$ Th2 cells following the proliferation phase (12 x 10^6 cells), or activation phase (12 x 10^6 cells). Most cells were stimulated with PMA (20ng/mL) and ionomycin (1 µM) (3h), others were left unstimulated in media alone (3h). When indicated, Cyclosporin A (1.2 µg/mL) was added to 3h stimulations (P/I). All subsequent steps were performed on ice. Cells were harvested and resuspended in lysis solution (Buffer A: 10 mM HEPES, 3 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 5% glycerol, 0.20% NP-40, 1 mM PMSF, 5 mM β-glycerophosphate, 1 mM benzamidine, 1 mM orthosodium vanadate, 1 mM sodium fluoride, 10 µg/mL antipain, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin: 120 µL). Lysis was assessed with trypan blue and once swollen;

cell nuclei were pelleted (4°C) in a microcentrifuge at 13,200 RPM. Nuclear pellets were resuspended in an appropriate volume of Buffer C (420 mM NaCl, 10 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM DTT, 1 mM PMSF, 5 mM β-glycerophosphate, 1 mM benzamidine, 1 mM orthosodium vanadate, 1 mM sodium fluoride, 10 µg/mL antipain, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin)**.** PMSF and DTT were added to Buffer A and C immediately before use. Nuclear extracts were flash frozen with liquid nitrogen and stored at -80°C until use.

2.4.2 Recombinant proteins

Recombinant human GATA3 and NFAT1 proteins were synthesized using expression vectors described above and *in vitro* transcription and translation (TnT), with the TnT coupled rabbit reticulocyte lysate system and T7 polymerase (according to manufacturers' instructions, Promega, WI, USA). Plasmid (1µg) was incubated with 25 µl rabbit reticulocyte lysate, 2 µl reaction buffer, 1 µl T7 polymerase, 0.5µl each of leucine and methionine in a final reaction volume of 50µl in water. Reactions were incubated in a 30°C water bath (90 min) and lysates were stored at -80°C.

2.4.3 Electromobility shift assay (EMSA)

Gel shift assays were performed with an oligonucleotide probe (5' – cggcattgatggaaattgatgatatttgaac) representing position -125/-94 of the *CRTh2* promoter region (relative to the transcription start site). Annealed oligonucleotides (100 ng) were 5' end labeled with [γ-^{32}P] – ATP using T4 polynucleotide kinase (New England Biolabs, ON, Canada) and excess radioactivity was removed using

a Mini Quick Spin DNA column (Roche, Sweden). Labeling efficiency was measured using a scintillation counter (counts per minute per µl). Probes were stored at -20°C and used within one week. The EMSA binding reaction included nuclear extract (5 µg) or recombinant protein (1 µL of TnT reaction), 50 mM NaCl, 10 % glycerol, poly dI:dC (50 ng/µL, Sigma Aldrich, ON, Canada), BSA (0.2 µg/µL) and 1X protease inhibitor (Roche, IN, USA). Nuclear extracts or recombinant proteins were incubated on ice (30 min) in the described binding conditions; either alone, with super-shifting antibodies (4 µg) or with molar excess (100X) of unlabeled *CRTh2* proximal promoter oligonucleotide, consensus probes for transcription factors or mutants of the *CRTh2* proximal promoter oligonucleotide **(Table 1)**. To mutate transcription factor binding sites, transversion mutations (purine to pyrimidine or vice versa) were done to core binding nucleotides. Most super-shifting antibodies were purchased from Santa Cruz Biotechnology (CA, USA); GATA3 (HG3-31 and HG3-35), NFAT1 (4G6-G5), isotype matched control antibody (ATF2; Clone F2BR-1). The super-shifting antibody for NFAT2 (MA3-024) was purchased from Affinity Bioreagents (CO, USA). Probes were then incubated on ice (30 min) with the nuclear extract binding reactions as described above. Binding reactions were loaded onto a 5% non-denaturing polyacrylamide gel and run at 19 mA for 5-6 hours with 0.5X Tris-buffered EDTA at 4°C. Gels were dried at 80°C (1h) (Hoefer Slab Gel Dryer, GD2000) and exposed to film for up to 48 hours at -80 °C.

Table 1: EMSA oligonucleotide sequences

	OLIGONUCLEOTIDE MUTATION		SEQUENCE		LENGTH
CRTh2 proximal		5'	CGGCATTGATGGAAATTGATGATATTTGAAC	3'	31 bp
NFAT concensus		5'	CGGAGGAAAAACTGTTTCATACAGAAGGCGTG	3'	32 bp
GATA3 concensus		5'	CCTCTATCTGATTGTTAGCC	3'	25 bp
CRTh2 proximal	NFAT1-m2	5'	CGGCATTGATGG**t**[1]**t**ATTGATGATATTTGAAC	3'	31 bp
CRTh2 proximal	NFAT1-m4	5'	CGGCATTGAT**cctt**ATTGATGATATTTGAAC	3'	31 bp
CRTh2 proximal	GATA3-m1	5'	CGGCATT**c**ATGGAAATTGATGATATTTGAAC	3'	31 bp
CRTh2 proximal	GATA3-m2	5'	CGGCATTGATGGAAATT**c**ATGATATTTGAAC	3'	31 bp
CRTh2 proximal	GATA3-m3	5'	CGGCATTGATGGAAATTGATGA**a**ATTTGAAC	3'	31 bp
CRTh2 proximal	GATA3-m1 + m3	5'	CGGCATT**c**ATGGAAATTGATGA**a**ATTTGAAC	3'	31 bp

1. Lower case letters indicate transversion mutations

2.5 Polymerase Chain Reaction

2.5.1 Quantitative reverse transcription (qRT)-PCR

RNA was extracted from frozen cell pellets (2 - $10x10^6$ cells) using the RNeasy extraction kit (Qiagen, ON, Canada). RNA was eluted with 30 µl RNase/DNase free water (Qiagen, ON, Canada) and 2 - 10µl were diluted in Tris-EDTA buffer to determine the concentration by spectrophotometry (BioRad SmartSpec 3000). Complimentary DNA (cDNA) was synthesized with 1 µg RNA using dNTPs, oligoDT and SuperScriptII. RNA was incubated with oligodT, dNTPs and water at 65°C (5 min). 1X First Stand Buffer, DTT and RNase out were added at 42°C (2 min). Finally Superscript II was added for 50 minutes at 42°C followed by 15 minutes at 70°C. cDNA was stored at -20°C until needed. All reagents for cDNA synthesis were purchased from Invitrogen (CA, USA). Quantitative reverse transcription PCR (qRT-PCR) TaqMan gene expression assays for CRTh2 (Hs00173717_m1) and IL-4 (Hs00174122_m1) were purchased from Applied Biosystems (Applied Biosystems, CA, USA) and used as per manufacturer's instructions. All TaqMan probe based real time qRT-PCRs were standardized to GAPDH using primers: Forward 5' CTGAGAACGGGAAGCTTGTCA 3' and Reverse 5' GCAAATGAGCCCCAGGGTT 3'. A specific probe, recognizing the GAPDH PCR product, was designed and custom ordered from ABI, 5' 6FAMAAA TCCCATCACCATCTTCCAGGAGCGA TAMRA 3'. qRT-PCRs were performed using TaqMan gene expression master mix (Applied Biosystems, CA, USA), 1 µl cDNA (final reaction volume: 20µl) and an Eppendorf RealPlex 4 machine with the following program; 2 min 50°C, 10 min 95°C and 40 cycles of

15 sec 95°C and 1 min 60°C. CRTh2 qRT-PCRs were run with a CRTh2 and GAPDH standard curve to obtain copy number.

To make standards, quantitative real time PCR products were cloned into pCR2.1TOPO vector (Invitrogen, CA, USA) and CRTh2 and GAPDH products were linearized using BamHI and KpnI, respectively. The DNA concentration following purification of the linearized vector was determined by spectrophotometry (BioRad SmartSpec 3000) and dilutions of 10^1 to 10^9 copies/µl were made and stored at -80°C. CRTh2 qRT-PCRs were run with 10^1, 10^3 and 10^5 standards and GAPDH qRT-PCRs were run with 10^5, 10^6 and 10^7 standards.

2.6 Statistical Analysis

Statistical significance for transfection and nucleofection experiments was determined using Mann-Whitney (Rank Sum) test for non-parametric data using SigmaStat software. This method was chosen due to non-parametric data. For over-expression transfection assays and flow cytometry, statistical significance was determined by ANOVA with post hoc analysis using Holm-Sidak method for parametric data. Data is considered significant if $p<0.05$ (*).

CHAPTER III: Results - Molecular regulation of *CRTh2*

3.1 Background and rational

Molecular regulation of *CRTh2* is poorly understood, with most research focusing on receptor function. To date, it is known that surface expression of CRTh2 is increased by IL-4 and over-expression of GATA3 (117, 136). Our lab has shown that over-expression of GATA3 in Jurkat T cells can directly increase *CRTh2* promoter activity using a luciferase reporter system (135). To further study the molecular regulation of *CRTh2,* we developed a $CRTh2^+$ Th2 cell line and examined promoter activation and endogenous gene expression.

3.2 *In vitro* differentiated $CRTh2^+$ Th2 cells

Endogenous expression of CRTh2 is low on both the immortalized Jurkat T cell line (2.8% ± 1.1, n=4, **Figure 8a**) as well as human circulating $CD4^+$ T cells (3.0% ± 1.1, n=3, **Figure 8b**). Therefore to study the molecular regulation of *CRTh2* we developed a $CRTh2^+$ Th2 cell line. To do this, $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs), using a magnetic column and negative selection and cultured in Th2 differentiating conditions. $CD4^+$ T cell purity was 95% ± 0.63 (93% – 98%, n=9, **Figure 9a**). On day 7 of differentiation, 22% ± 3.1 (11-42%, n=9, **Figure 9b)** of $CD4^+$ cells were $CRTh2^+$. Blood was obtained primarily from allergic donors but on one occasion differentiation was performed on cells from a non-allergic donor. No differences were observed in the propensity of $CRTh2^+$ differentiation between the non-allergic (25%, n=1) and allergic (21%, n=8) donors. Isolated $CRTh2^+$ cells were

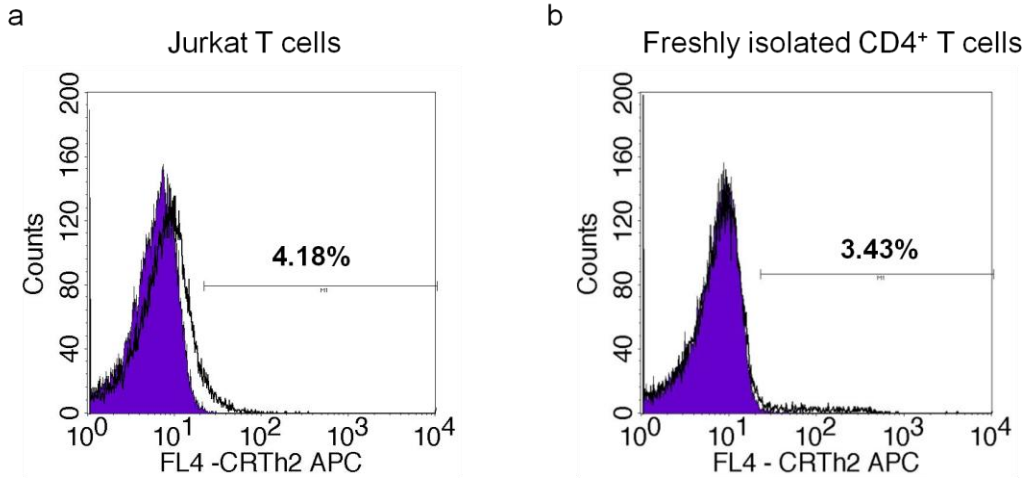

Figure 8. CRTh2 expression on Jurkat T cells and freshly isolated $CD4^+$ cells

Jurkat T cells **(a)** and freshly isolated $CD4^+$ cells **(b)** express low levels of CRTh2 by surface staining. Empty histogram indicates CRTh2 staining and filled histogram indicates corresponding isotype control. Figure is representative of n= 2-4 experiments.

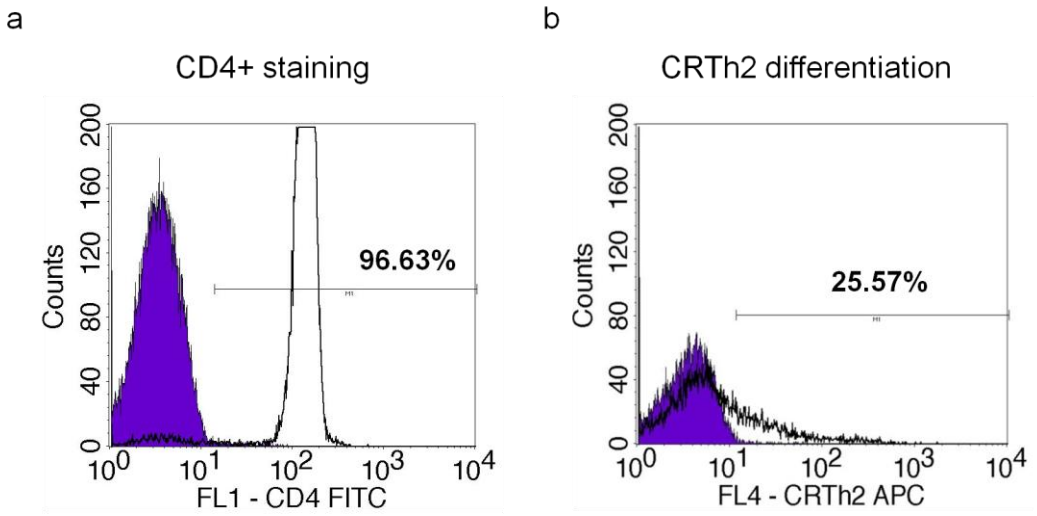

Figure 9. Staining following CD4 isolation and CRTh2 differentiation

Following $CD4^+$ isolation from PBMCs, 96.6% of cells are $CD4^+$ **(a)**. After 7 days in Th2 differentiation conditions, 25.6% of cells are $CRTh2^+$ **(b)**. Empty histogram indicates CD4 **(a)** or CRTh2 **(b)** staining and filled histogram indicates corresponding isotype control. Figure is representative of n=9.

maintained on cycles of an activation phase (3 days αCD3/CD28 + rhIL-2) and a proliferation phase (4 days rhIL-2 alone) **(Figure 5)**.

Maintenance of CRTh2 expression and the Th2 cell phenotype was assessed by surface expression of CRTh2 and intracellular staining for IL-4, IL-13 and interferon γ (IFNγ) following the proliferation phase. Over the course of all experiments CRTh2 surface staining following CRTh2 enrichment on day 7 was 80% ± 2.5 (50-96%, n=24) **(Figure 10a).** CRTh2 expression was highest on cells immediately following $CRTh2^+$ isolation and decreased as cells were kept in culture **(Figure 10c).** *In vitro* differentiation resulted in $CRTh2^+$ Th2 cells expressing IL-4 (17% ± 3.7, n=23), IL-13 (59% ± 4, n=23), IFNγ (19.7% ± 4, n=23) and double positive for IL-13 and IFNγ (15.3% ± 3.9, n=22) over the course of 9 independent cell lines **(Figure 10b)**. To better understand the magnitude of the IFNγ positive cells, we also analyzed supernatants from three independent cell lines by Multiplex bead array following the activation phase **(Table 2)**. Even though the percent of cells expressing IFNγ was higher than expected, there was consistently more IL-4 (8777 - >11 000 pg/mL) and IL-13 (> 10 000 pg/mL) produced than IFNγ (20 – 4568 pg/mL). Further, the amount of IFNγ from our $CRTh2^+$ Th2 differentiated cell lines was considerably lower than reported from Th1 differentiated cells lines (>80 000pg/mL) (117).

$CRTh2^+$ Th2 cell lines were also assessed for markers of the central memory phenotype. $CRTh2^+$ Th2 cells expressed CD45RO (69% ± 11.8, 40 - 93%, n=4)

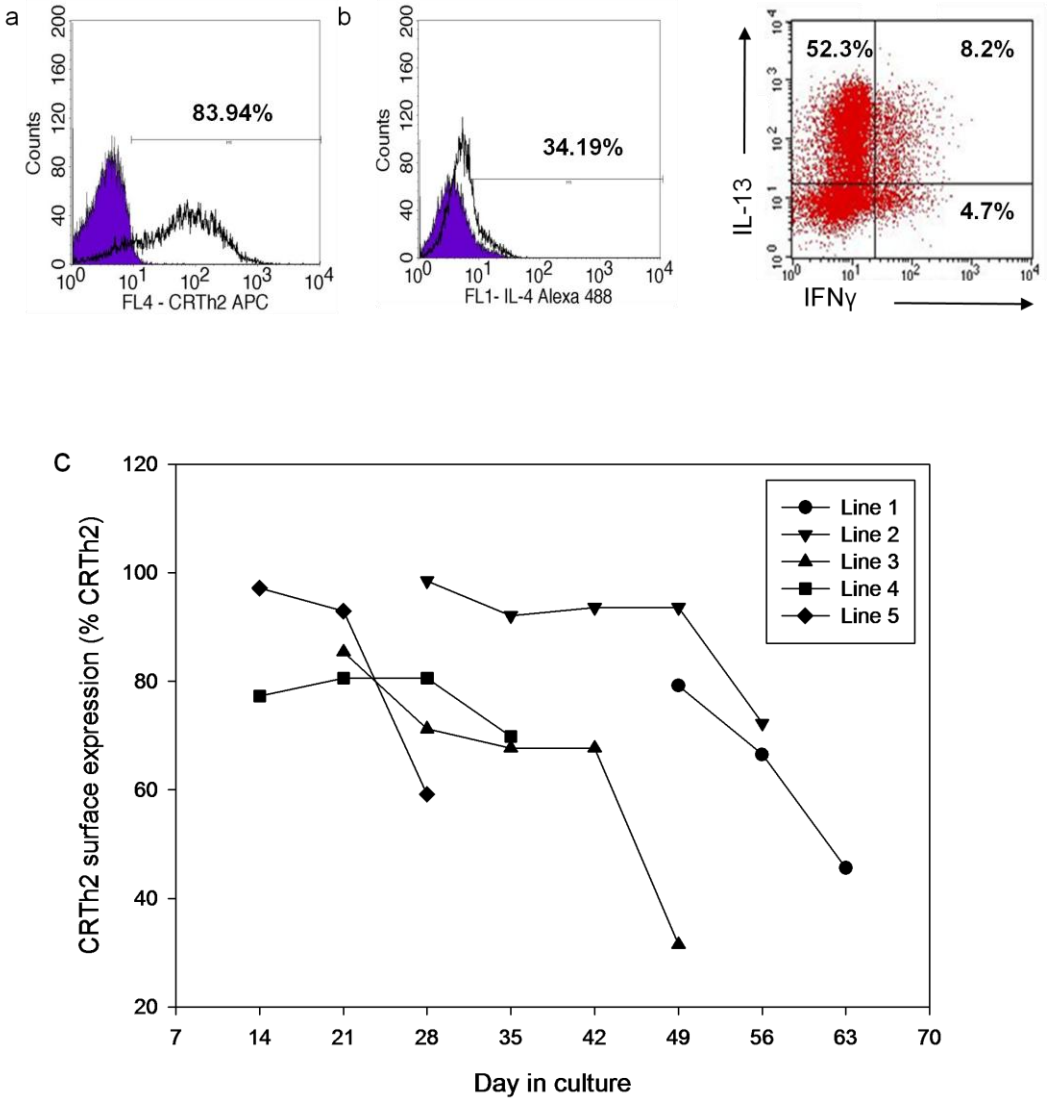

Figure 10. CRTh2 and cytokine expression from *in vitro* differentiated CRTh2$^+$ Th2 cells

In vitro differentiated CRTh2$^+$ Th2 cells express CRTh2 (83.94%) following proliferation phase **(a)**, and IL-4 (34.19%), IL-13 (52.3%), IFNγ (4.74%) and are double positive for IL-13 and IFNγ (8.2%) by intracellular staining **(b)** following mitogenic stimulation. Empty histogram indicates CRTh2 **(a)** or IL-4 **(b)** staining and filled histogram indicates corresponding isotype control. Figure is representative of n= 13-24 over 9 independent cell lines. CRTh2 surface expression on isolated CRTh2$^+$ T cells decreases with days in culture **(c)**. Graph shows 5 representative cells lines at various time points following CRTh2 isolation.

Table 2: Cytokine expression by differentiated CRTh2$^+$ Th2 cells

	IFNγ (pg/mL)	IL-4 (pg/mL)	IL-13 (pg/mL)
Line A Day 38	4568	>11000	>10000
Line B Day 17	20	>11000	>10000
Line C Day 17	3131	8777	>10000

(Figure 11a) and CD62L (66% ± 5.9, 29% - 96%, n=15) following the proliferation phase **(Figure 11b)** and the activation phase **(Figure 11c)**. It is interesting to note that as cells were kept in culture CD62L expression decreased. These data indicate that our *in vitro* differentiation protocol generates $CRTh2^+$ Th2 cells exhibiting a central memory phenotype as has been shown *in vivo* (37).

3.3 Conservation of the *CRTh2* promoter region

To begin assessing the molecular regulation of *CRTh2*, we looked at the region immediately upstream of the transcription start site (TSS), which is also the start of the untranslated exon 1 **(Figure 6a)**. This region has been shown to contain putative initiator sites and Sp1 sites although there is no TATA box within the 5 kb upstream of exon 1 (135). We also examined the sequence conservation of this region since conserved non-coding sequences are likely important for gene regulation. Interestingly, the 450 base pair region immediately upstream of the TSS is highly conserved in Chimpanzee and Rhesus monkey and moderately conserved in Marmoset and Horse **(Figure 12a)**. *In silico* analysis also shows this region contains putative binding sites for transcription factors known to be involved in regulating other Th2 genes, such as GATA3, NFAT and STAT6 (135) **(Figure 12b)**.

3.4 The proximal *CRTh2* promoter is not differentially methylated in freshly isolated $CD4^+$ T cells and $CRTh2^+$ Th2 cells

Since the transcriptional regulation of *CRTh2* may be regulated by chromatin structure and accessibility of the promoter, we first examined the profile of DNA methylation at the *CRTh2* promoter. The region from +66 to -646 (relative to the

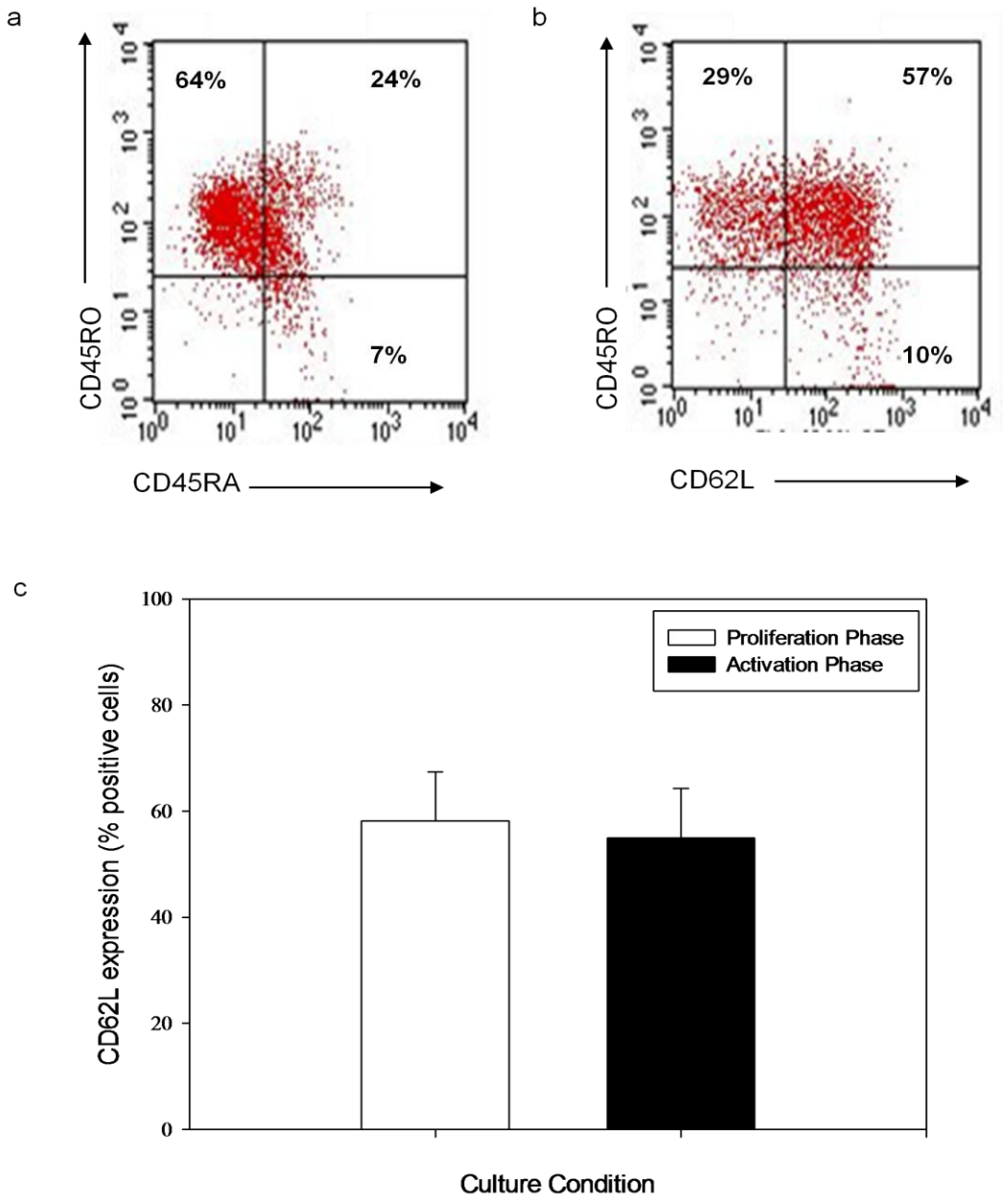

Figure 11. CD45RO and CD62L expression by *in vitro* differentiated CRTh2$^+$ Th2 cells

In vitro differentiated CRTh2$^+$ Th2 cells highly express CD45RO **(a)** and CD62L **(b)** following the proliferation phase. CD62L is also expressed following the activation phase **(c)**, n= 6 following the proliferation phase and n=8 following the activation phase, from 3 independent cell lines.

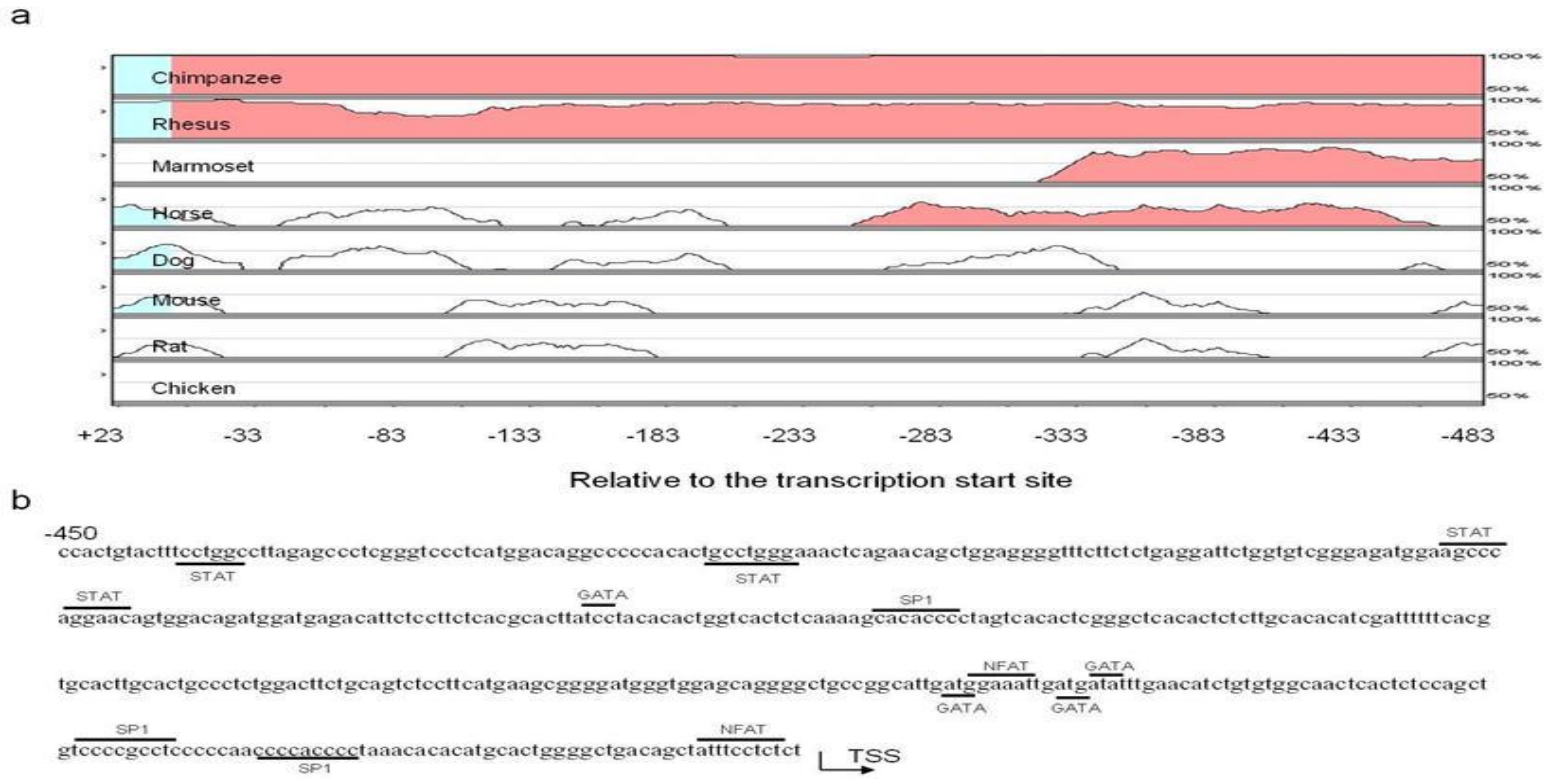

Figure 12: Conservation and putative transcription factor binding sites at the proximal region of the *CRTh2* promoter

Sequence conservation of the *CRTh2* proximal promoter region using VistaPlot **(a)** Marmoset sequence missing up to -315. Teal coloring indicates exon 1 and pink coloring indicates 5' conserved region (>70% conserved). Predicted transcription factor binding sites, filtering for transcription factors (GATA3, NFAT and STAT) shown to be found in lymphocytes, using MatInspector, Genomatix software **(b)**.

TSS) in the proximal region of the *CRTh2* promoter was examined due to the high density of CpG sites (14 CpG sites) **(Figure 13a)**. DNA methylation at dinucleotide CpG sites has been associated with gene silencing (94). Therefore we hypothesized that the *CRTh2* proximal promoter was hypomethylated in cell populations highly positive for CRTh2 and hypermethylated in populations with low positivity for CRTh2.

DNA methylation profiles were examined using freshly isolated $CD4^+$ T cells ($CRTh2^{low}$) and *in vitro* differentiated $CRTh2^+$ Th2 cells ($CRTh2^{high}$). Both $CRTh2^+$ Th2 cells ($CRTh2^{high}$, 88% $CRTh2^+$) and freshly isolated $CD4^+$ T cells ($CRTh2^{low}$, <5% $CRTh2^+$) displayed similar methylation patterns **(Figure 13b, n=2)**. This indicated that differential methylation was not a method of regulation at this region of the proximal promoter. However, hypomethylation was only observed at the first 7 CpG sites (+28 to – 232) and interestingly, in $CRTh2^{low}$ and $CRTh2^{high}$ cells, the region from -284 to -646 appears to be consistently hypermethylated regardless of CRTh2 surface expression. This indicated that this region is most likely in a closed chromatin conformation.

3.5 $CRTh2^+$ Th2 cells support transcription of *CRTh2*

Our results from the methylation analysis indicated that the region immediately upstream of the TSS is not differentially methylated in $CRTh2^{high}$ compared to $CRTh2^{low}$ cells **(Figure 13b)**. Therefore, molecular regulation of *CRTh2* does not appear to be at the level of chromatin accessibility, at least at this proximal region of the *CRTh2* promoter. As such, we next tested whether transcriptional regulation of this proximal region of the *CRTh2* promoter was dependent on nuclear

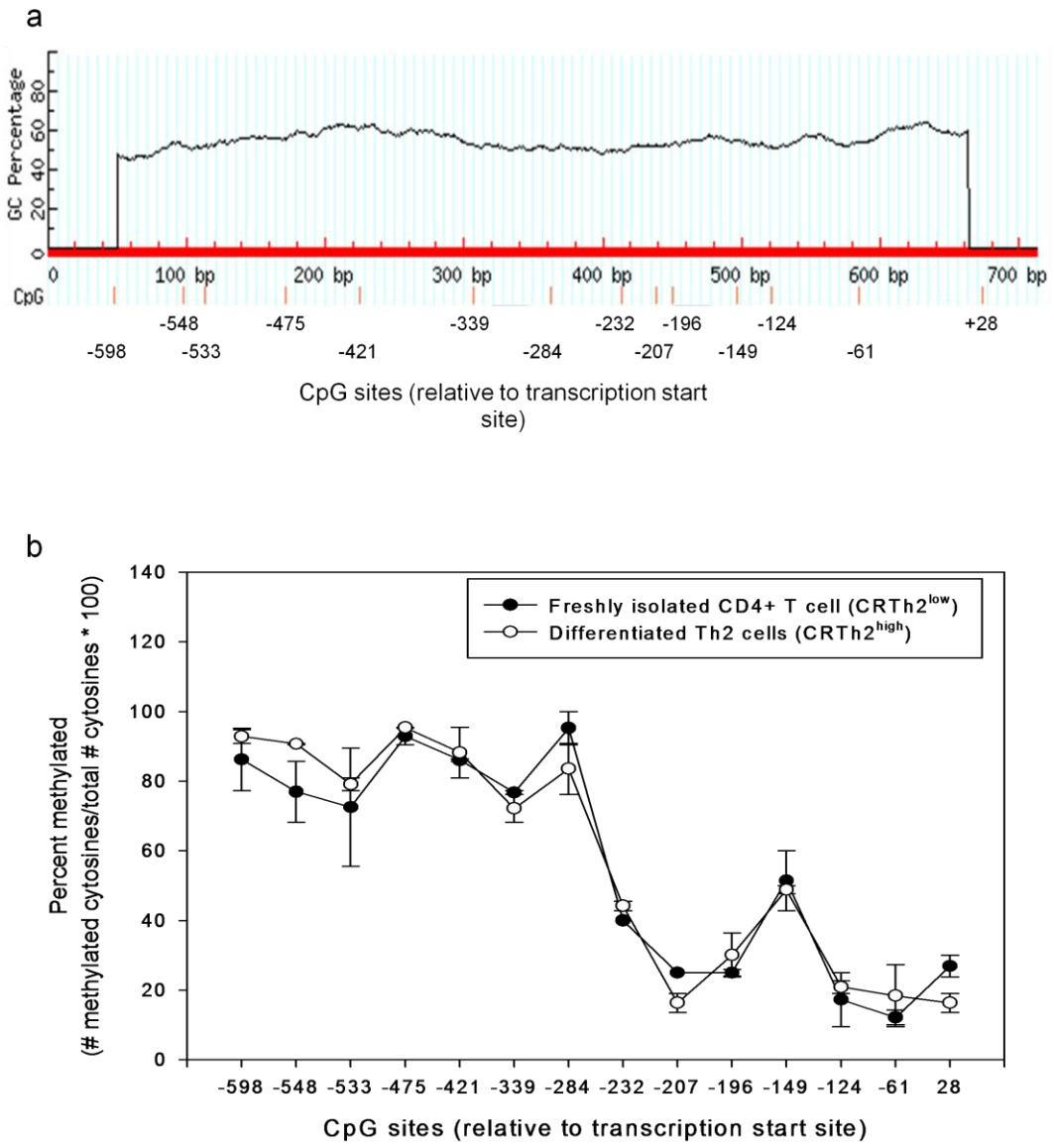

Figure 13. CpG sites and methylation at the proximal region of the *CRTh2* promoter

There are 14 CpG sites in the proximal region of the *CRTh2* promoter (+66 to -646) **(a)**. DNA methylation profiles from *in vitro* differentiated $CRTh2^+$ Th2 cells ($CRTh2^+$ 76% ±12.5) and freshly isolated $CD4^+$ T cells show that the *CRTh2* proximal promoter is hypomethylated from the transcription start site to -232 **(b)**. The x-axes show CpG sites with numbering relative to the transcription start site. Results are represented as percent methylated, the percent of clones amplified as a cytosine. n=2, 20 clones from two independent cell lines.

environment. To do this, we transfected or nucleofected a luciferase reporter construct containing the 450 base pairs immediately upstream of the TSS (*CRTh2*-450/Luc) into Jurkat or CRTh2$^+$ Th2 cells. This region of the *CRTh2* promoter was assessed because it is mostly hypomethylated in primary CD4$^+$ cells **(Figure 13b)** and is relatively conserved in higher species **(Figure 12a)**.

CRTh2-450/Luc was transiently transfected into Jurkat cells and demonstrated a modest 1.3-fold (1.3 ± 0.194; n=15) induction of relative luciferase activity (RLA) in response to PMA and ionomycin (P/I) stimulation (16h). Whereas nucleofection of *CRTh2*-450/Luc into CRTh2$^+$ Th2 cells showed an 8-fold (8.2 ± 1.8, n=19) induction of relative luciferase activity with stimulation (16h, P/I). The fold induction was significantly higher in CRTh2$^+$ Th2 cells compared to Jurkat cells **(Figure 14a, $p<0.05$)**. These data suggest higher transcriptional activity of the proximal region of the *CRTh2* promoter in stimulated Th2 cells could be mediated by variation in their respective nuclear environments.

It is also important to note that in CRTh2$^+$ Th2 cell nucleofections, *CRTh2*-450/Luc was not active without stimulation, since relative luciferase activity was similar to background (i.e., no Firefly luciferase construct) **(Figure 14b)**. This indicates that, in isolation, this promoter region requires T cell activation for activity.

3.6 Characterizing transcription factor binding to the *CRTh2* proximal promoter

To examine transcription factor binding to the proximal *CRTh2* promoter, electromobility shift assays (EMSAs) were performed using nuclear extracts from

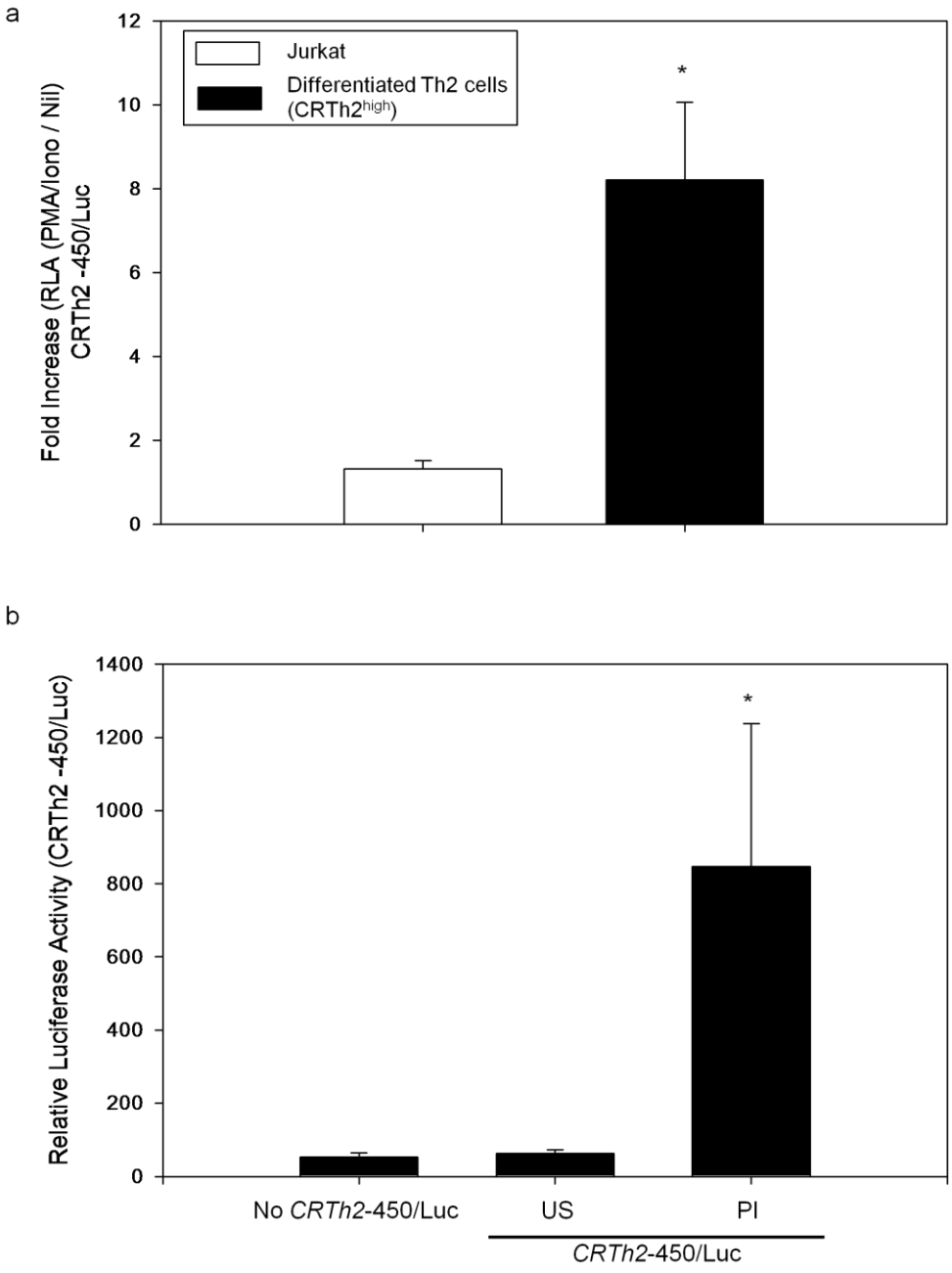

Figure 14. Transcriptional activity of the proximal *CRTh2* promoter in Jurkat and CRTh2$^+$ Th2 cells in response to stimulation

In vitro differentiated CRTh2$^+$ Th2 cells show 6.2 fold higher induction of the proximal *CRTh2* promoter (*CRTh2*-450/Luc) than non-polarized Jurkat cells **(a)**. No change in relative luciferase activity of *CRTh2*-450/Luc was observed without stimulation (16h, P/I) using *in vitro* differentiated CRTh2$^+$ Th2 cells **(b)**. (Jurkat n=15 and Th2 n=19, No *CRTh2*-450/Luc control n=4) $p<0.05$ Mann-Whitney Rank sum test.

stimulated Jurkat T cells and CRTh2$^+$ Th2 cells (3h, P/I), after the proliferation phase (4 days rhIL-2) along with a probe representing a proximal region of the *CRTh2* promoter (-94/-125) **(Figure 15a)**. This region was chosen for the presence of putative NFAT and GATA3 binding sites and was unmethylated indicating that it is accessible to transcription factors *in vivo*. Figure 15b shows that the binding profile from stimulated Jurkat cells (3h, P/I) is very different from that of CRTh2$^+$ Th2 cells, indicating the presence or relative abundance of transcription factors differs between these two cell types **(Compare lane 1 with lane 4)**. Indeed neither of the complexes from CRTh2$^+$ Th2 cells were present in the binding profile from Jurkat T cells. However, the transcription factors from Jurkat T cells did bind to the probe specifically since we competed by molar excess of unlabelled (cold) *CRTh2* promoter oligonucleotide (self 100X) **(lane 2)**. Importantly, there is no GATA3 binding with Jurkat T cells **(lane 3)** as we see no supershift with a GATA3 antibody (αGATA3).

Analysis of the bands from CRTh2$^+$ Th2 cells show they are specific because all binding was competed with cold self competitor (100X) **(Figure 15b, lane 5, 13).** Furthermore, we identified the lower complex as GATA3, since it was competed with molar excess of a cold GATA3 consensus oligonucleotide **(lane 6)** and super-shifted (or missing) with two independent α-GATA3 antibodies **(lane 9, 10)** but not an isotype matched control antibody **(lane 8)**. The upper complex was shown to be NFAT1, since it was competed with molar excess of cold NFAT consensus oligonucleotide **(lane 7, 14)** and super-shifted by an NFAT1 antibody

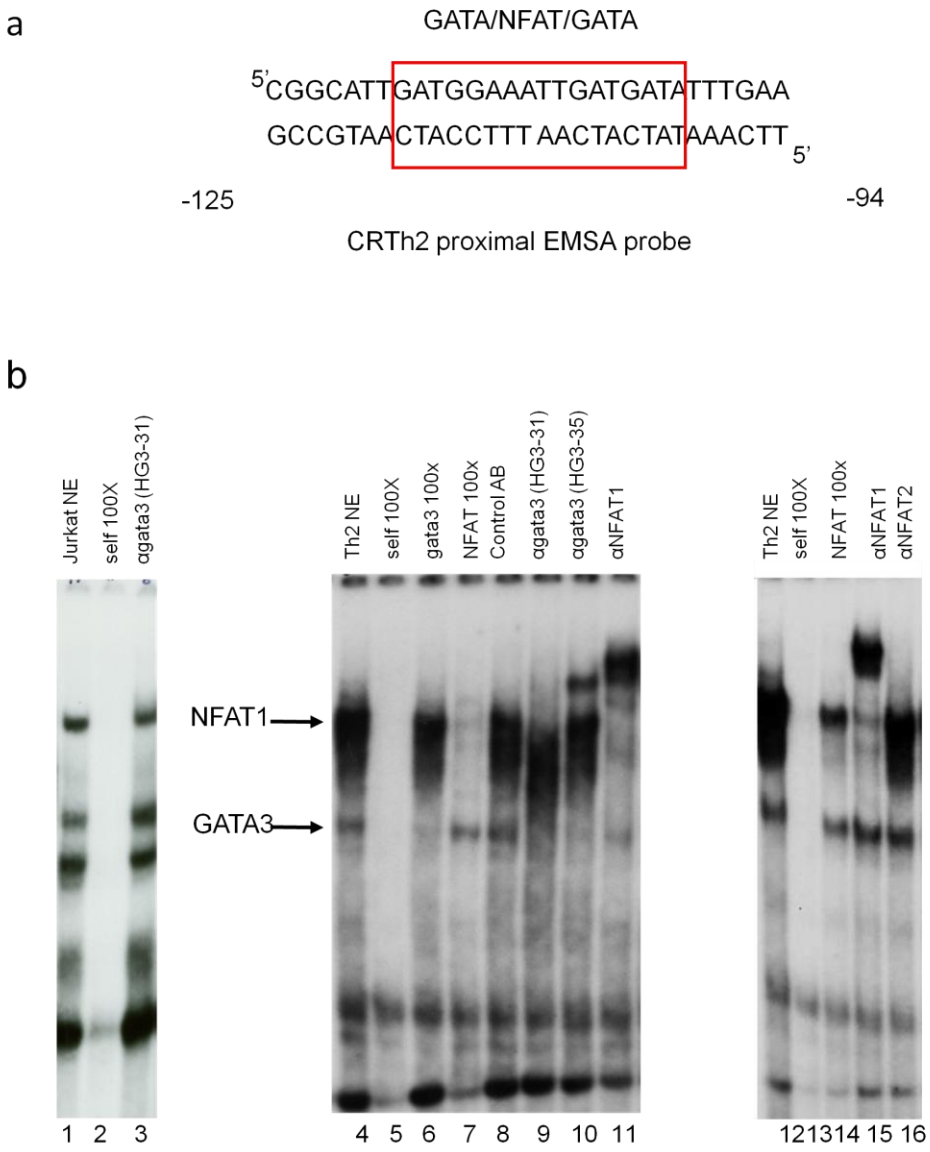

Figure 15. The *CRTh2* EMSA probe and transcription factor binding from Jurkat and CRTh2$^+$ Th2 cells

A proximal region of the *CRTh2* promoter used as an EMSA probe has putative binding sites for GATA and NFAT, as indicated **(a)**. GATA3 does not bind to the *CRTh2* promoter from stimulated Jurkat cells (Jurkat NE) **(b, lanes 1-3)**. NFAT1 and GATA3 bind to the *CRTh2* promoter from stimulated *in vitro* differentiated CRTh2$^+$ Th2 cells (Th2 NE) **(b, lanes 4-11)**. NFAT1, not NFAT2 from *in vitro* differentiated CRTh2$^+$ Th2 cells binds the *CRTh2* promoter **(b, lanes 12-16)**. The competitors and super-shifting antibodies used are noted above the corresponding lanes. Antibodies denoted as α, bracketed numbers distinguish different antibody clones. Gels are representative of n=8 with 6 independent cells lines.

(lane 11, 15). Since NFAT family members bind similar sites, we also investigated NFAT2 and confirmed the upper complex is not NFAT2 **(lane 16)**.

3.6.1 Identifying GATA3 binding sites

Since there are three potential GATA3 binding sites in the *CRTh2* promoter probe **(Figure 15a)**, we next determined which sites bind GATA3. *CRTh2* promoter oligonucleotides containing mutations of the predicted GATA3 sites were created **(Table 1)** and used as cold competitors (100X) to determine the ability of these sites to bind GATA3 **(Figure 16)**. To do this, recombinant GATA3 was generated by *in vitro* transcription and translation (TnT) with expression vectors and rabbit reticulocyte lysates and incubated with the *CRTh2* promoter probe **(lane 1)**. Binding was shown to be specific by cold self competitor (100X) **(lane 2)**. GATA3 binding was verified by a super-shift with a GATA3 antibody **(lane 7).** To test putative GATA3 binding sites, rhGATA3 was incubated with cold competitors (100X) of the mutated *CRTh2* proximal oligonucleotides **(Table 1)**; GATA3- m1 **(lane 3)**, m2 **(lane 4)**, m3 **(lane 5)**, or a double GATA mutant GATA3-m1 + m3 **(lane 6)**. To ensure no protein from that reaction interacted with the *CRTh2* promoter probe, a TnT reaction using empty pcDNA3.1^{+} was used as a control **(lane 8)**. If an excess of mutated *CRTh2* promoter oligonucleotide was unable to compete the binding of rhGATA3 to the labeled probe, this would indicate the original GATA3 site was necessary for binding. Since GATA3-m1, GATA3-m3, and the two mutations combined (GATA3-m1 + m3) were unable to compete the rhGATA3, these sites appear necessary for GATA3 binding. Further, since mutation of one site cannot be compensated by

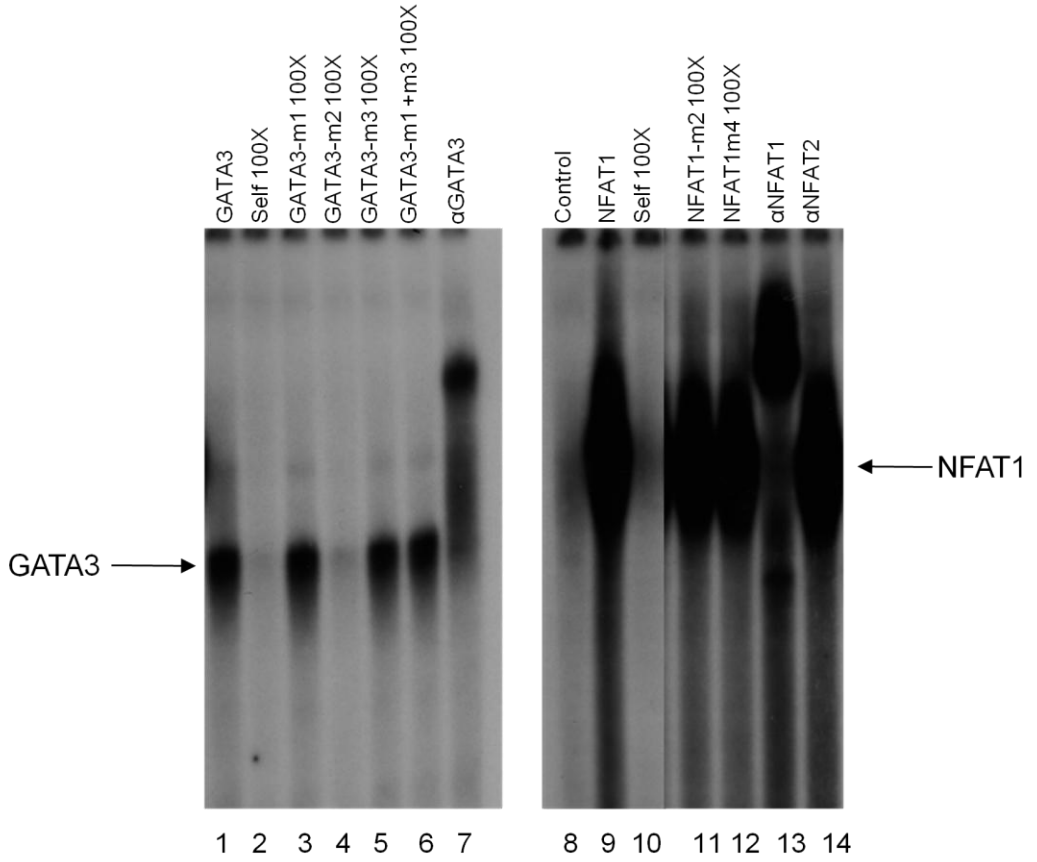

Figure 16. Recombinant GATA3 and NFAT1 binding at the *CRTh2* proximal EMSA probe

GATA3 (**Lane 1-7)** and NFAT1 **(Lanes 8-14)** recombinant protein bind to the *CRTh2* proximal promoter probe. Specific point mutations in the *CRTh2* EMSA oligonucleotides inhibit the ability to compete either GATA3 or NFAT1 recombinant protein binding. Vertical labelling indicates recombinant protein, competitors and/or antibodies used. GATA3 antibody used was clone HG3-31. Figure is representative of n=2.

the other non-mutated site **(lanes 3, 5 and 6)**, both sites appear necessary for rhGATA3 binding.

3.6.2 Confirming the NFAT binding site

Although, there was only one predicted NFAT site, we also mutated this site. Since we saw NFAT1 but not NFAT2 binding the *CRTh2* promoter with nuclear extracts from $CRTh2^{+}$ Th2 cells **(Figure 15)**, recombinant human NFAT1 was used. NFAT1 was incubated with labeled *CRTh2* promoter probe **(Figure 16, lane 9)**. Again binding specificity was verified using cold self competitor (100X) **(lane 10)** and by incubating the control TnT reaction with the labeled probe **(lane 8)**. To ensure the band was NFAT1 we used a NFAT1 antibody and saw a super-shift **(lane 13)** but not with α-NFAT2 **(lane 14)**.

To verify we could inhibit NFAT1 binding by mutating the binding site, recombinant human NFAT1 was incubated with cold competitors (100X) of *CRTh2* promoter mutation oligonucleotides. The mutated oligonucleotide contained either two or four nucleotide changes to the predicted NFAT site **(Table 1)**; NFAT1-m1 **(lane 11)** or NFAT1-m2 **(lane 12)**. Neither oligonucleotide was able to compete NFAT1 binding, indicating both mutations disrupted the NFAT1 binding.

3.7 Endogenous CRTh2 expression decreases with stimulation

Since both GATA3 and NFAT1 nuclear translocation have been shown to increase following TCR activation (114, 119, 121) and both can bind to the proximal region of the *CRTh2* promoter following stimulation (3h, P/I), we next examined endogenous CRTh2 expression following activation or acute

stimulation. After the activation phase (3 days αCD3/CD28 + rhIL-2) or the proliferation phase (4 days rhIL-2) + acute stimulation (16h P/I), surface expression of CRTh2 is significantly decreased **(Figure 17)**. These data were surprising, in light of our *CRTh2*-450/Luc nucleofections showing increased transcription following acute stimulation (16h, P/I) **(Figure 14a)**.

To assess whether this decrease in CRTh2 surface expression was due to receptor internalization or decreased transcription of *CRTh2*, we next examined CRTh2 mRNA levels. CRTh2 mRNA was decreased 5.1-fold following the activation phase compared to the proliferation phase. Furthermore, CRTh2 mRNA was log units lower at both time points (proliferation or activation phase) after acute stimulation (16h P/I) **(Figure 18, n=1-2)**. The decrease in mRNA following the activation phase and acute stimulation (16h P/I) is surprising since these are the conditions of the nucleofection experiments that showed an increase in transcriptional activity from the *CRTh2* promoter region **(Figure 14a)**.

We next considered whether the discrepancy in mRNA expression versus promoter activity following acute stimulation (16h P/I) could be due to mRNA stability. CRTh2 mRNA stability may be regulated by the 3' untranslated region (3' UTR) and although mRNA is decreased at 16h post-stimulation, there may have been an initial increase. Since the luciferase reporter vector does not have a 3' UTR, the luciferase transcript (which reports *CRTh2* promoter activity) is not subject to degradation. Therefore we also assessed CRTh2 mRNA at earlier time

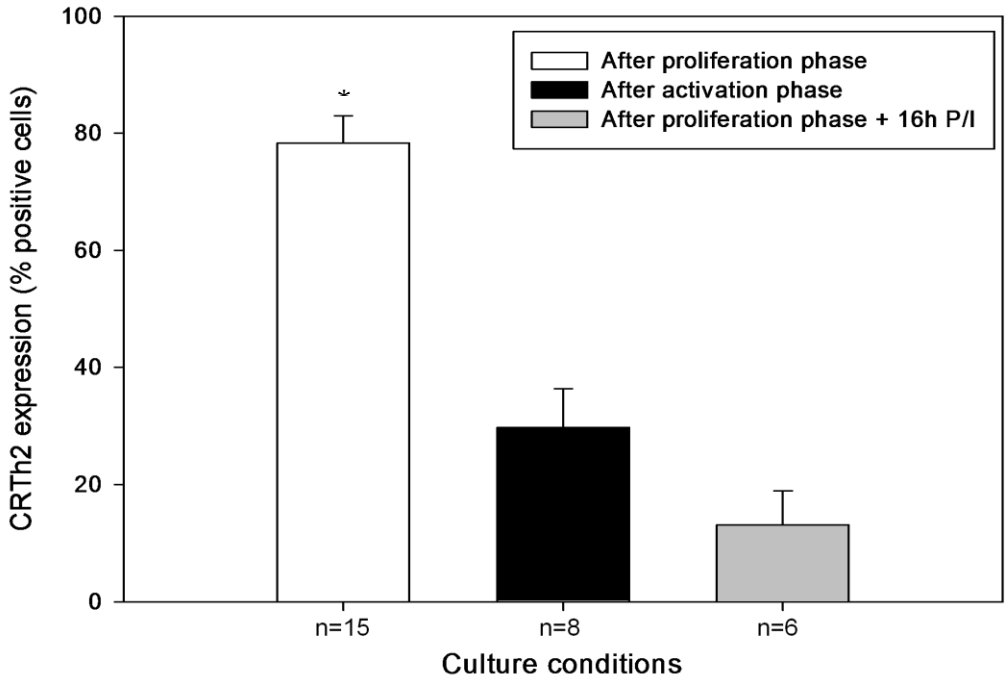

Figure 17. CRTh2 surface expression following proliferation and activation phase

CRTh2 surface expression on *in vitro* differentiated CRTh2^{+} Th2 cells decreases following T cell stimulation. CRTh2 surface expression was determined by flow cytometry following the proliferation phase + mitogenic stimulation (16h, PMA and Ionomycin) or activation phase. n= 6-15, from 3 independent cell lines, * indicates p> 0.05.

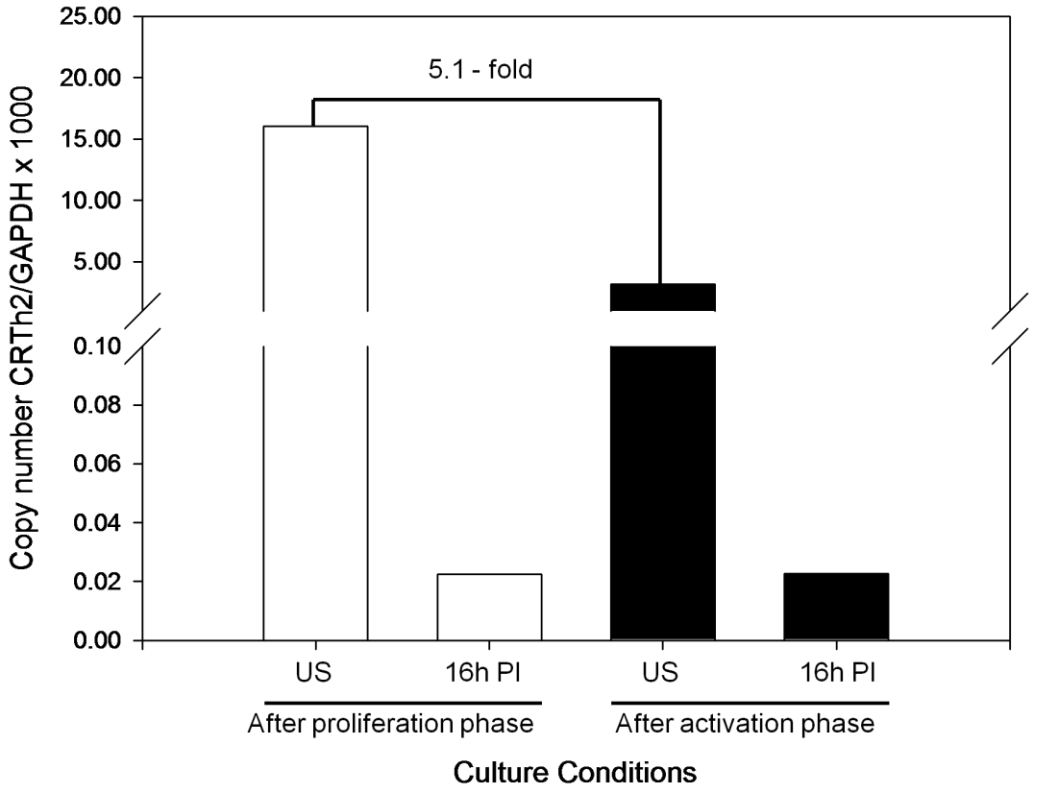

Figure 18. CRTh2 mRNA following proliferation and activation phase

CRTh2 mRNA decreases in *in vitro* differentiated CRTh2^{+} Th2 cells following T cell stimulation. Cells were stimulated with PMA and ionomycin (16h) following either the proliferation (n=1) or activation phase (n=2) and mRNA was assessed by qRT-PCR. Representative of n=1-2.

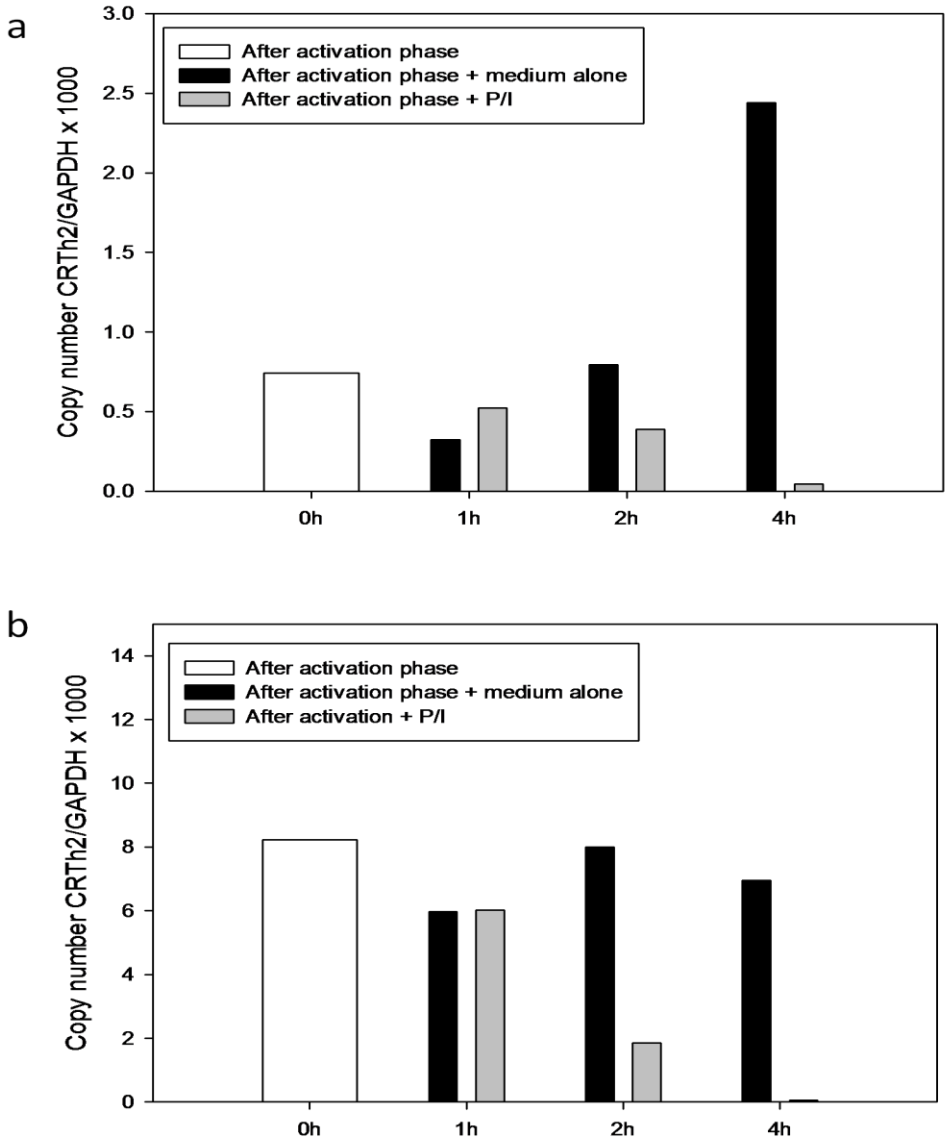

Figure 19. CRTh2 mRNA decreases rapidly following stimulation

CRTh2 mRNA from *in vitro* differentiated CRTh2^{+} Th2 cells is low following the activation phase but falls even further following stimulation (PMA + Ionomycin) **(a)**. The trend is similar in a different cell line but the copy number of CRTh2 varies **(b)**. Data are representative of triplicates in one experiment and data is presented as copy number of CRTh2 over copy number of GAPDH, n=1.

points following stimulation. Figure 19a and 19b show kinetic experiments in two independent CRTh2$^+$ Th2 cell lines. Figure 19 shows there may be an early increase in CRTh2 mRNA after 1 hour of stimulation (P/I), but that the CRTh2 transcript is clearly reduced after 2 and 4 hour stimulations (P/I). It is interesting to note that although the later trend (2h and 4h) is similar in two independent cell lines, the initial (1h) increase in CRTh2 mRNA and the baseline CRTh2 mRNA after the activation phase varies greatly between lines **(Figure 19)**. Together, these data show that following mitogenic stimulation (2-16h P/I) or long term physiological (3 days αCD3/CD28) activation, CRTh2 expression is decreased, both at the mRNA and protein levels.

3.8 NFAT1 binding coincides with decreased CRTh2 expression

To examine the profile of transcription factor binding in relation to endogenous CRTh2 expression, EMSA was performed with nuclear extracts from unstimulated (3h, media alone) CRTh2$^+$ Th2 cells following the proliferation phase (4 days rhIL-2, **Figure 20)**. Binding reactions with unstimulated CRTh2$^+$ Th2 cells after the proliferation phase (when CRTh2 expression is highest) are shown in **lane 1**. All bands were specific **(lane 2,** 100X**)**. There was very little NFAT1 binding, as seen with the small super-shift using αNFAT1 **(lane 3)** and not αNFAT2 **(lane 4)**. GATA3 also binds, as seen by the missing lower band when αGATA3 is added **(lane 5)**.

On the other hand, with nuclear extracts from proliferating CRTh2$^+$ Th2 cells following stimulation (3h P/I; when CRTh2 is the lowest) we saw a strong NFAT1 binding to the *CRTh2* promoter probe **(Figure 20, lane 6).** This binding

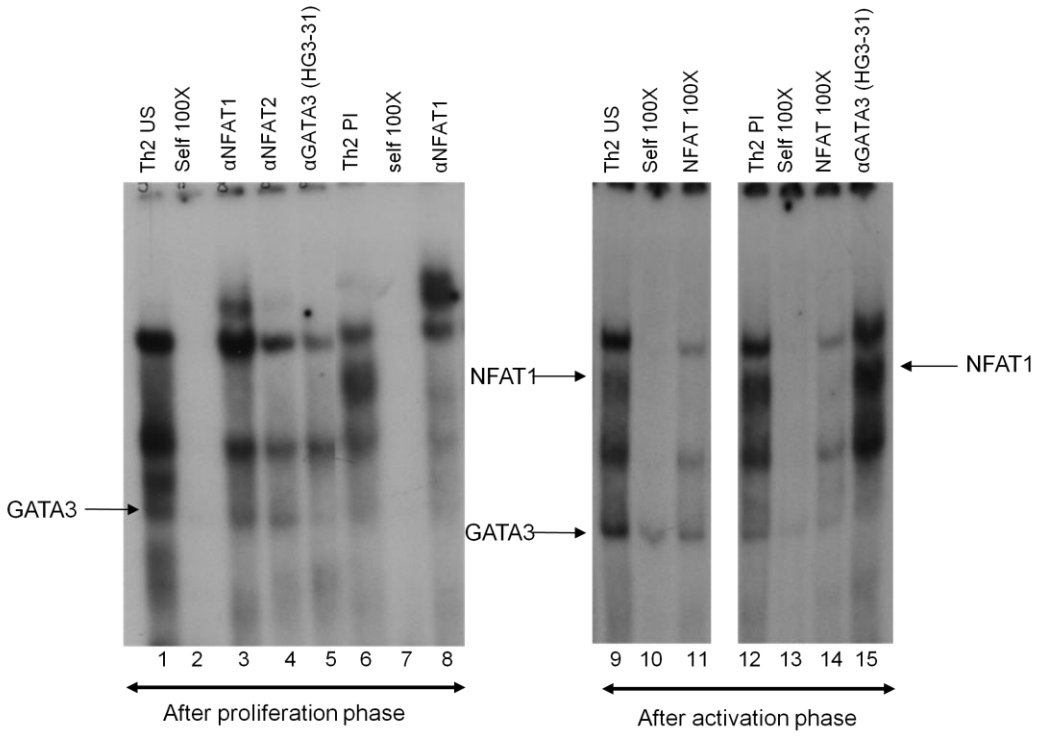

Figure 20. NFAT1 binds to the *CRTh2* proximal probe following the proliferation and activation phase

NFAT1 only binds to the *CRTh2* proximal promoter probe following stimulation of *in vitro* differentiated CRTh2^{+} Th2 cells; proliferation phase + mitogenic stimulation (3h P/I) or activation phase ± mitogenic stimulation (3h P/I). GATA3 also binds following proliferation phase and stimulation. CRTh2^{+} Th2 cell nuclear extracts used: After proliferation phase + 3h unstimulated (Lanes 1-3), after proliferation phase + 3h P/I (Lanes 4-8), after activation phase + 3h unstimulated (Lanes 9-11), after activation phase + 3h P/I (Lanes 12-15). Vertical labels about figure indicate nuclear extracts used, excess cold competitors used or supershifting antibodies used, including clone number.

was confirmed by cold competition **(lane 7)** and super-shifting with αNFAT1 **(lane 8)**.

Since we had only looked at transcription factor binding after the proliferation phase, we also examined cells following the activation phase (3 days αCD3/CD28 + rhIL-2, when CRTh2 is decreased). Nuclear extracts from $CRTh2^+$ Th2 cells after the activation phase show that NFAT1 binding is seen with both the unstimulated (medium alone, 3h) and stimulated (P/I, 3h) conditions **(Figure 20, lanes 9 and 12)**. The binding was specific using excess cold self oligonucleotide **(lane 10 and 13)** and excess cold NFAT consensus **(lane 11 and 14)**. The lower complex also shows GATA3 binding following stimulation by the lack of a lower band with the GATA3 antibody **(lane 16)**. Collectively, these data indicate that while GATA3 binding is relatively constant, NFAT1 binding is most prominent following either the activation phase or acute stimulation (when CRTh2 is lowest, **Figure 17).**

3.9 Over-expression of NFAT1 reduces transcriptional activity of the *CRTh2* proximal promoter

Based on our EMSA and mRNA/protein data showing that NFAT1 binding corresponds to the conditions of lowest expression of CRTh2, we hypothesized that NFAT1 could inhibit *CRTh2* promoter activity. To test this, NFAT1 was over-expressed in Jurkat cells by co-transfection of an NFAT1 expression vector with the *CRTh2* promoter construct (*CRTh2*-450/Luc). If NFAT1 is a negative regulator of *CRTh2* transcription, we would expect decreased transcriptional

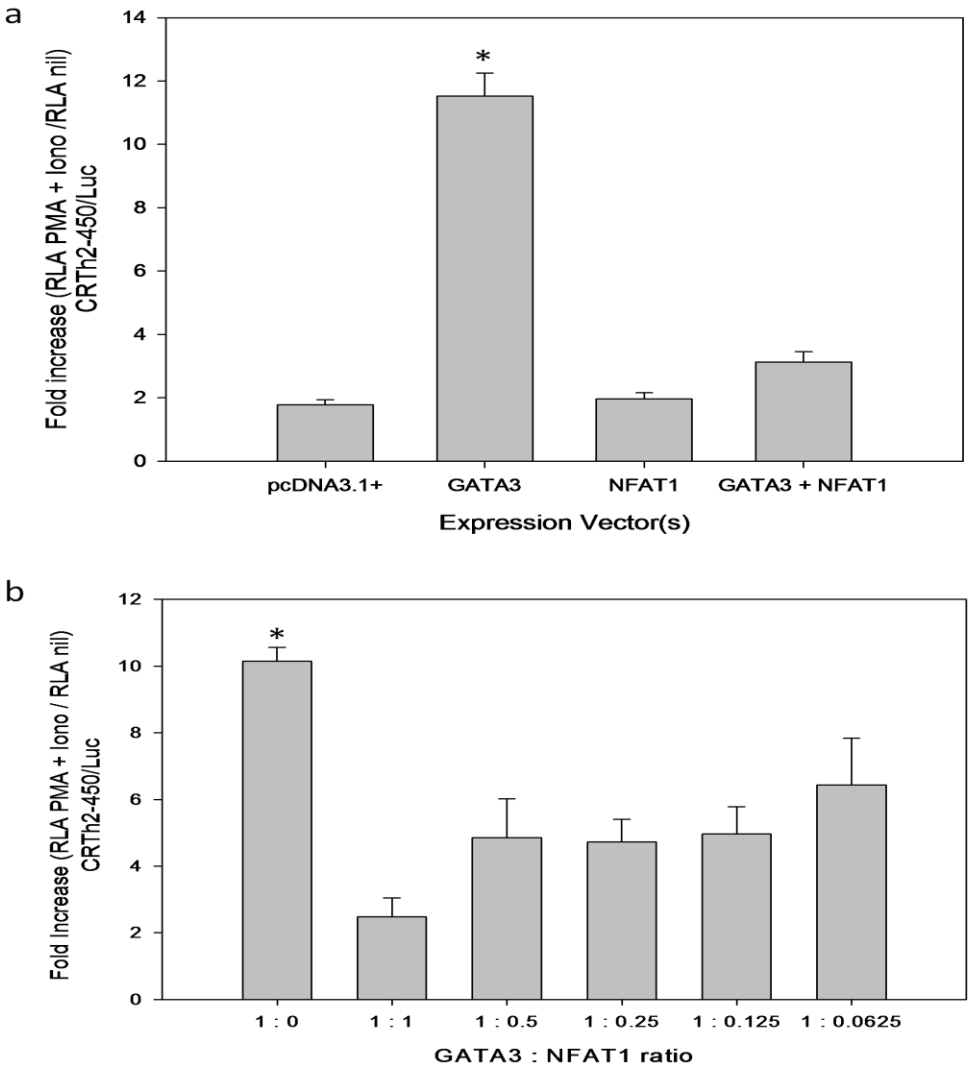

Figure 21. Over-expression of GATA3 and NFAT1 with the *CRTh2* proximal promoter construct in Jurkat T cells

GATA3, but not NFAT1, over-expression in Jurkat T cells increases (6.5 fold) transcriptional activity of CRTh2-450/Luc. GATA3 + NFAT1 co-expression inhibits GATA3-mediated activity **(a)**. Titrating the NFAT1-to-GATA3 ratio down (1/16) still results inhibits *CRTh2* promoter activity **(b)**. Transcriptional activity of the *CRTh2* proximal promoter (CRTh2-450/Luc) is measured by fold increase of relative luciferase activity (RLA) in stimulated (16h PI) over unstimulated samples. n=3-9, * = $p < 0.05$, determined by ANOVA with post hoc analysis using Holm-Sidak method.

activity. However, Figure 21a shows there was no significant decrease in promoter activity when NFAT1 was over-expressed. Since our group has previously shown that GATA3 over-expression in Jurkat cells increases *CRTh2*-450/Luc activity (135), we examined whether NFAT1 could influence this. Figure 21a shows that GATA3 increases promoter activity 6.5–fold over the control, pcDNA3.1^{+} **(n=9, p<0.05)** and when NFAT1 was co-transfected with GATA3 at molar equivalents, promoter activity was reduced down to similar fold induction as the control *CRTh2*-450/Luc + pcDNA3.1^{+}. Even as NFAT1 was titrated down, as low as 1/16 of GATA3, there was only a slight increase in transcriptional activity from the *CRTh2* proximal promoter (*CRTh2*-450/Luc), which never achieved the level of GATA3 alone **(p>0.05, Figure 21b)**. Therefore, these experiments indicate that NFAT1 inhibits the GATA3-induced transcription of *CRTh2*, at least from this region of the promoter.

3.10 GATA3 and NFAT1 bind to the *CRTh2* proximal probe independently

To further investigate how NFAT1 decreases GATA3 induced *CRTh2* promoter activity, we considered whether GATA3 and NFAT1 binding was cooperative or independent. To address this, we determined if disrupting a GATA3 site would affect NFAT1 binding and vice versa; if disrupting the NFAT1 site would affect GATA3 binding. Figure 22a shows that rhNFAT1 binds to the *CRTh2* promoter probe **(lane 2)**. This binding is specific because it is competed by cold self oligonucleotide (100X) **(lane 3)** and there is no binding when we incubated the control TnT reaction (pcDNA3.1^{+}) with the labeled probe **(lane 1)**. NFAT1 binding was verified by α-NFAT1 super-shift **(lane 8)** and no super-shift with α-

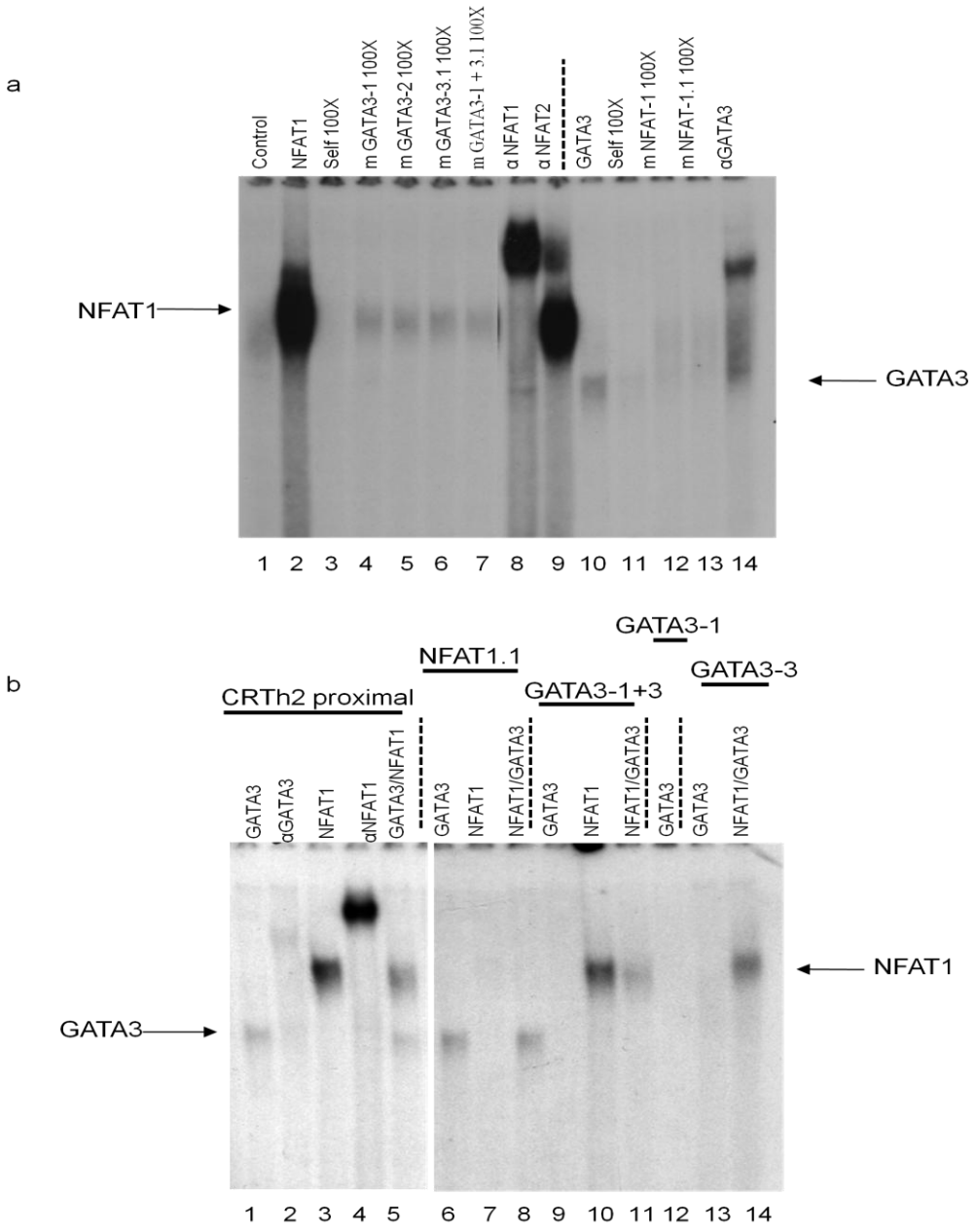

Figure 22. Mutating the *CRTh2* promoter oligonucleotide to determine GATA3 and NFAT1 binding dependency

Specific point mutations in the *CRTh2* EMSA oligonucleotide do not affect the binding of recombinant human NFAT1 or GATA3 **(a)**. Point mutations in the GATA3 sites do not affect NFAT1 binding and vice versa **(b)**. Vertical labelling indicates recombinant protein, 12 hours after an initial stimulation but within 2 hours following re-stimulation.

NFAT2 **(lane 9)**. Mutating the individual GATA3 sites does not affect the oligonucleotides' ability to compete rhNFAT1 binding **(lanes 4-7)**. Similarly, rhGATA3 binds to the *CRTh2* promoter probe **(lane 10)** specifically **(**100X, **lane 11)** and was confirmed by an α-GATA3 super-shift **(lane 14)**. GATA3 binding is not impaired by either mutation of the NFAT1 site **(lane 12, 13)** because both mutated oligonucleotides could still compete rhGATA3 binding when added in excess.

To further support independent binding, GATA3 and NFAT1 do not appear to bind the *CRTh2* proximal probe at the same time **(Figure 22b)**. Figure 22b shows that GATA3 alone **(lane 1)** binds and is verified by a super-shift with α-GATA3 **(lane 2)**. Similarly, NFAT1 alone binds higher on the gel **(lane 3)**, confirmed by α-NFAT1 super-shift **(lane 4)**. If GATA3 and NFAT1 bound the probe simultaneously, we would expect three bands on the gel when both recombinant GATA3 and NFAT1 are added, one band for GATA3 alone, one for NFAT1 alone and finally a higher band representing GATA3 + NFAT1 binding. Figure 23b shows this is not the case **(lane 5)**.

To confirm independent binding, the mutated oligonucleotides were labeled and used as probes **(Figure 22b).** NFAT1-m2 can bind rhGATA3 **(lane 6)** but not rhNFAT1 **(lane 7)**. Even when both rhNFAT1 and GATA3 are added together, only rhGATA3 binds **(lane 8)**, further supporting that GATA3 and NFAT1 are not forming a complex. Similarly, GATA3-m1+m3 can bind rhNFAT1 **(lane 10)** but not rhGATA3 **(lane 9)**. Finally the individual mutant probes GATA3-m1 and

GATA3-m3 do not bind rhGATA3 **(lanes 12 and 13)**. This confirms that GATA3 binding requires both intact GATA3 binding sites. Even when both rhNFAT1 and GATA3 were incubated with the GATA3 mutant probe, only rhNFAT1 could bind **(lanes 11 and 14)**. Together, these data indicate that GATA3 and NFAT1 can independently bind this region of the *CRTh2* promoter.

3.11 Cyclosporin A inhibits transcriptional activity of the *CRTh2* proximal promoter in CRTh2$^+$ Th2 cells

We observed NFAT1 binding to the *CRTh2* promoter probe following the activation phase or mitogenic stimulation, when CRTh2 surface and mRNA expression was decreased. Therefore, we next examined the effect of inhibiting NFAT activity, using Cyclosporin A (CsA), on *CRTh2* promoter activity. CsA inactivates the ability of calcinuerin to dephosphorylate NFAT proteins and therefore inhibits their nuclear translocation **(Figure 4)**. When we examined CRTh2$^+$ Th2 cells stimulated in the presence of CsA, NFAT1 binding to the *CRTh2* proximal promoter was inhibited **(Figure 23a, lane 2)**. Since we found that over-expression of NFAT1 decreased promoter activity, we next hypothesized that inhibiting NFAT1 with CsA would result in an increase in transcriptional activity of the proximal region of the *CRTh2* promoter. However, when *CRTh2*-450/Luc was nucleofected into differentiated CRTh2$^+$ Th2 cells in the presence of CsA, transcriptional activity did not increase, in fact it was significantly decreased, even with doses as low as 312.5 ng/mL of CsA **(Figure 23b)**. These data were unexpected and indicate further experiments examining the effect of CsA on endogenous CRTh2 expression may be more informative.

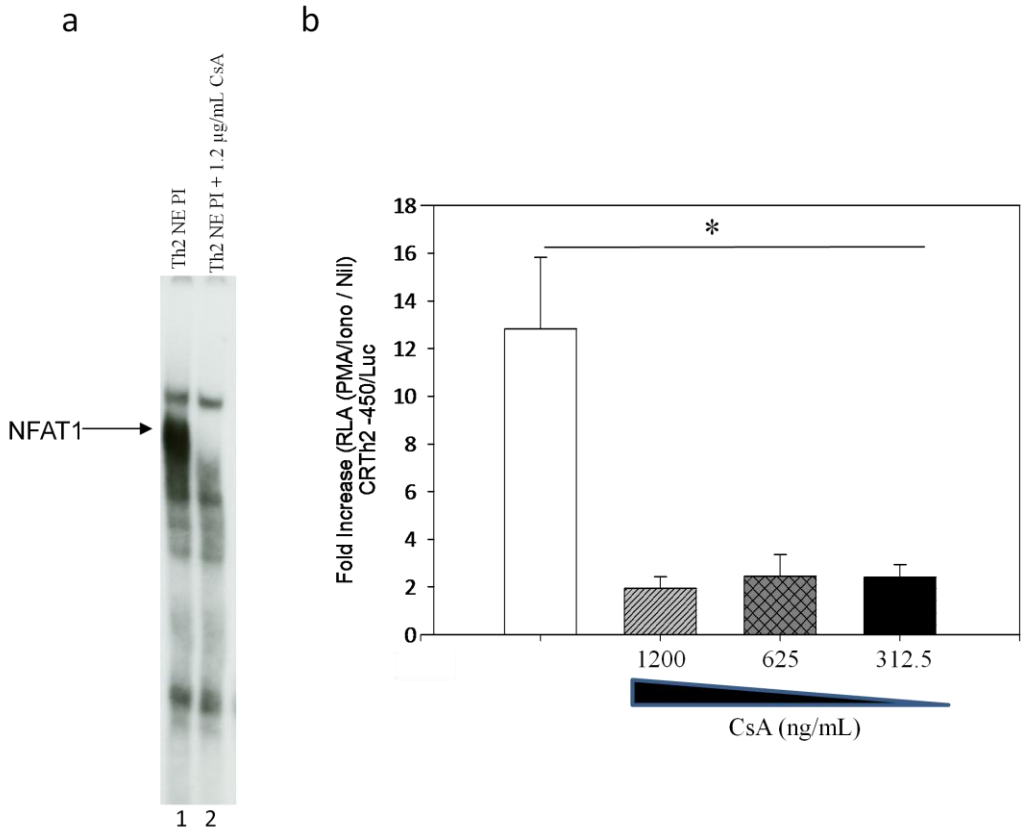

Figure 23. Binding profile and promoter activity of *CRTh2*-450/Luc following Cyclosporin A addition

Cyclosporin A inhibits NFAT1 binding from *in vitro* differentiated CRTh2$^+$ Th2 cell nuclear extracts to the *CRTh2* promoter probe **(a)**. *In vitro* differentiated CRTh2$^+$ Th2 cells show higher transcriptional activity of the proximal (CRTh2-450/Luc) promoter than the CRTh2$^+$ Th2 cells treated with CsA **(b)**. Transcriptional activity of *CRTh2* (*CRTh2*-450/Luc) measured through relative luciferase activity from cells with or without CsA treatment $p<0.01$ Mann-Whitney Rank sum test.

Chapter IV: Results - Stimulation induced down-regulation of CRTh2 may be due lack of OX40/OX40L co-stimulation

4.1 Background and rational – OX40 co-stimulation may be necessary to maintain CRTh2 following activation

TSLP activated dendritic cells (DCs) have been shown to maintain CRTh2 expression and the Th2 phenotype through the OX40/OX40L pathway (37). Sorted peripheral $CRTh2^{+}CD4^{+}$ cells cultured with TSLP-activated DCs maintained higher CRTh2 expression than those cultured with activating CD3 and CD28 (37). Further, the TSLP- DCs were able to maintain the Th2 phenotype (IL-4, IL-5 and IL-13) whereas $CRTh2^{+}CD4^{+}$ cells expanded with activating CD3 and CD28 up-regulated IFNγ (37). This may indicate that for $CRTh2^{+}CD4^{+}$ cells to maintain CRTh2 expression and their Th2 phenotype, they require additional signaling from antigen presenting cells such as the OX40/OX40L pathway.

Since we observed CRTh2 down-regulation following both mitogenic stimulation (16h, P/I) and the activation phase in our $CRTh2^{+}$ Th2 cell cultures, we hypothesized this may be due to the absence of OX40/OX40L co-stimulation from dendritic cells.

4.2 *In vitro* differentiated $CRTh2^{+}$ Th2 cells express OX40

To address this, we first assessed the expression of OX40 by *in vitro* differentiated $CRTh2^{+}$ Th2 cells. OX40 is expressed at low levels following the proliferation phase (4 days rhIL-2, 8.6% ± 3) **(Figure 24, n=10)**, however is highly expressed following the activation phase (3 days αCD3/CD28 + rhIL-2, 57.8% ±8.72) **(Figure 24, n=9)**. OX40 is also up-regulated with mitogenic stimulation (16h P/I; 80.8% ± 5.33) **(Figure 24, n=8)**

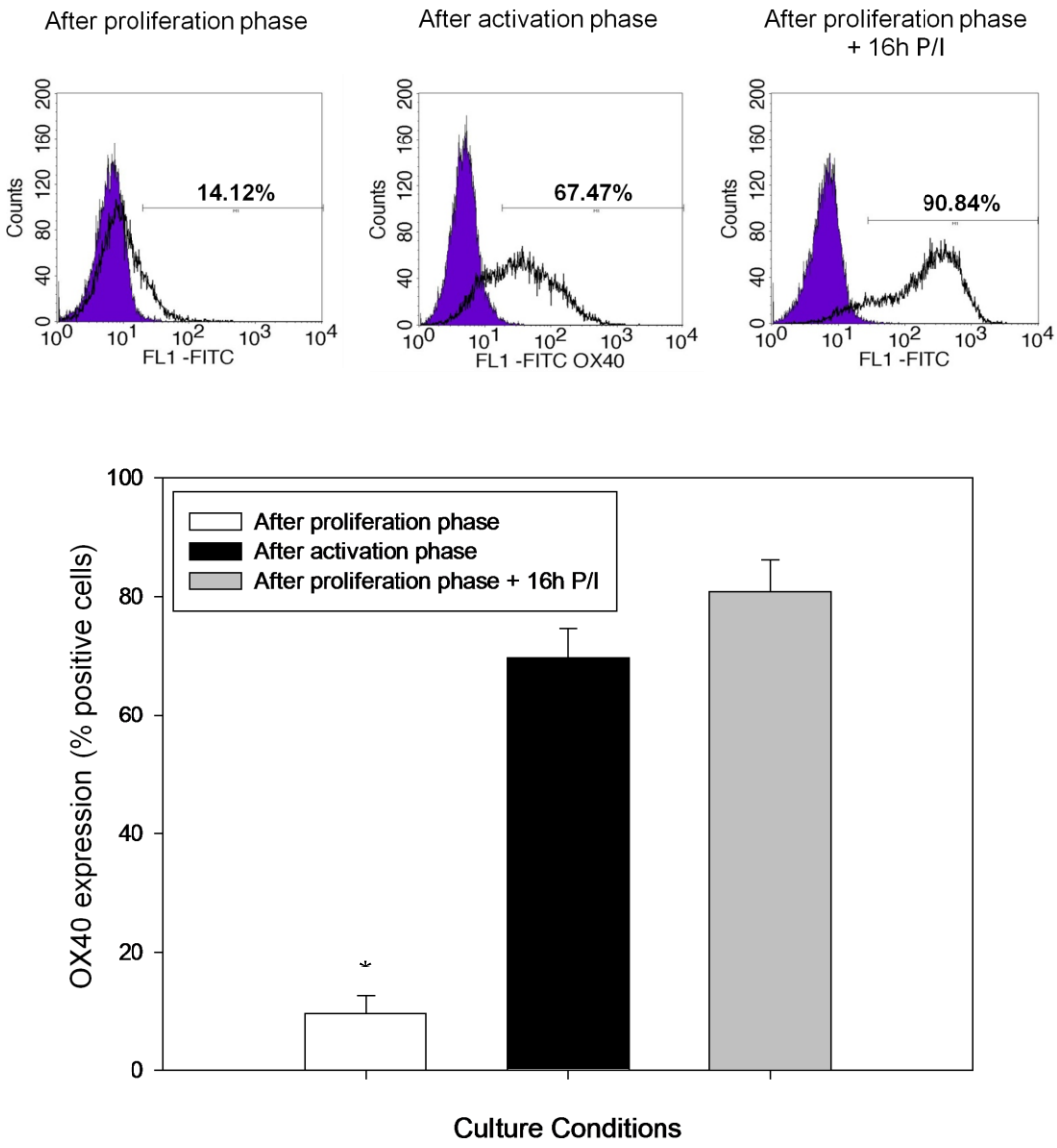

Figure 24. OX40 expression on *in vitro* differentiated CRTh2$^+$ Th2 cells

OX40 is expressed on *in vitro* differentiated CRTh2$^+$ Th2 cells following T cell stimulation. OX40 surface expression was determined by flow cytometry following the proliferation phase ± mitogenic stimulation (16h, P/I) or the activation phase. Empty histograms indicate OX40 staining and filled histogram indicates corresponding isotype control. Histograms are representative and graph is the means of n= 8-10, from 3 independent cell lines, * indicates p> 0.05.

and this is consistent with other studies that have also shown OX40 up-regulation (137).

4.2.1 Addition of exogenous OX40L does not overcome stimulation induced down-regulation of CRTh2 mRNA

To determine if the decrease in endogenous CRTh2 was due to lack of co-stimulation through OX40, recombinant OX40L was added to mitogenic stimulation (4h P/I) following the activation phase (3 days αCD3/CD28 + rhIL-2). Since OX40 expression was high (58% ± 8.7) following the activation phase, we hypothesized that adding OX40L to cultures further stimulated (4h and 16h, P/I) would inhibit the decrease we observed in CRTh2 mRNA **(Figure 19)**. OX40L is localized in the cell membrane as a trimer, to mimic this *in vitro*, α-polyhistidine was added with the recombinant OX40L to allow aggregation. Contrary to our hypothesis, adding OX40L + α-polyhistidine did not inhibit CRTh2 down-regulation following 4 or 16 hours of stimulation (P/I or CD3/CD28) **(Figure 25 a, b)**.

Since surface expression of OX40 was low following the proliferation phase (4 days IL-2, 8% ± 3) but has been shown to be up-regulated within 2 hours following re-stimulation of cells (137) we also tested whether the cells taken after the proliferation phase could respond to OX40L. Adding OX40L + α-polyhistidine to stimulated $CD4^+$ $CRTh2^+$ Th2 cells (4 or 16h, P/I or CD3/CD28) showed similar results and was not able to inhibit the stimulation induced decrease in CRTh2 mRNA **(Figure 26 a, b)**. It is interesting to note that

stimulation with CD3/CD28 does not decrease CRTh2 mRNA as much as stimulation with P/I **(Figure 25 and 26)**.

Previous studies showed that $CD4^+$ $CRTh2^+$ T cells produced more Th2 cytokines in response to TSLP activated DCs with up-regulated OX40L compared to CD3/CD28 stimulation (37). To be sure the lack of an OX40L effect on CRTh2 mRNA was not merely due to assay optimization we also tested whether the OX40L had a biological effect on IL-4 expression. However, this was not the case, since Figure 27 shows that OX40L was able to up-regulate IL-4 mRNA when added to $CRTh2^+$ Th2 cell stimulation (4 or 16h P/I).

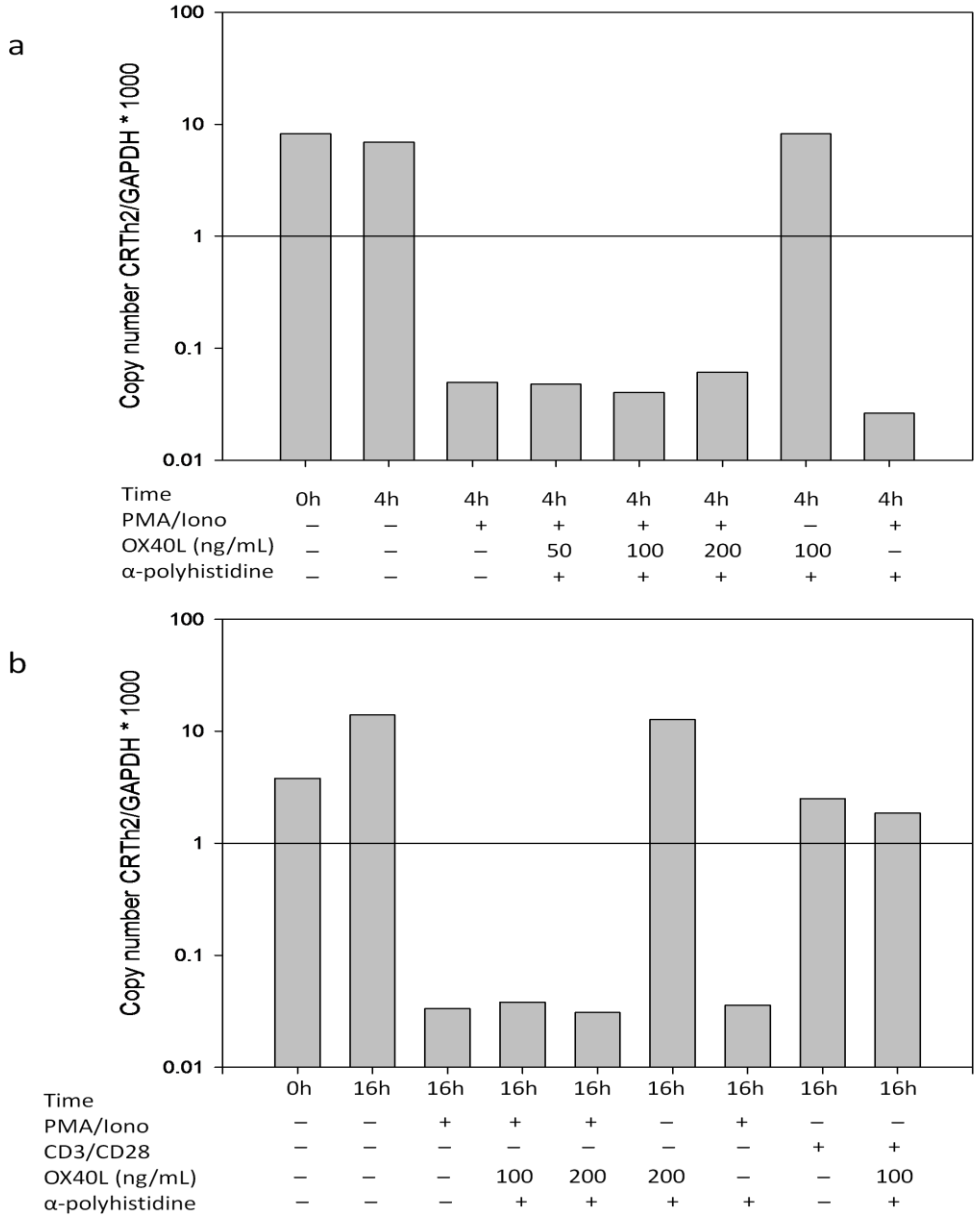

Figure 25. CRTh2 mRNA expression following addition of exogenous OX40L to stimulated CRTh2$^+$ Th2 cells following the activation phase

Exogenous OX40L cannot inhibit CRTh2 down-regulation in *in vitro* differentiated CRTh2$^+$ Th2 cells following 4h **(a)** or 16h **(b)** mitogenic stimulation (P/I) after the activation phase (3d αCD3/CD28). Data is from one experiment performed in triplicate, data is presented as copy number of CRTh2 over copy number of GAPDH x 1000.

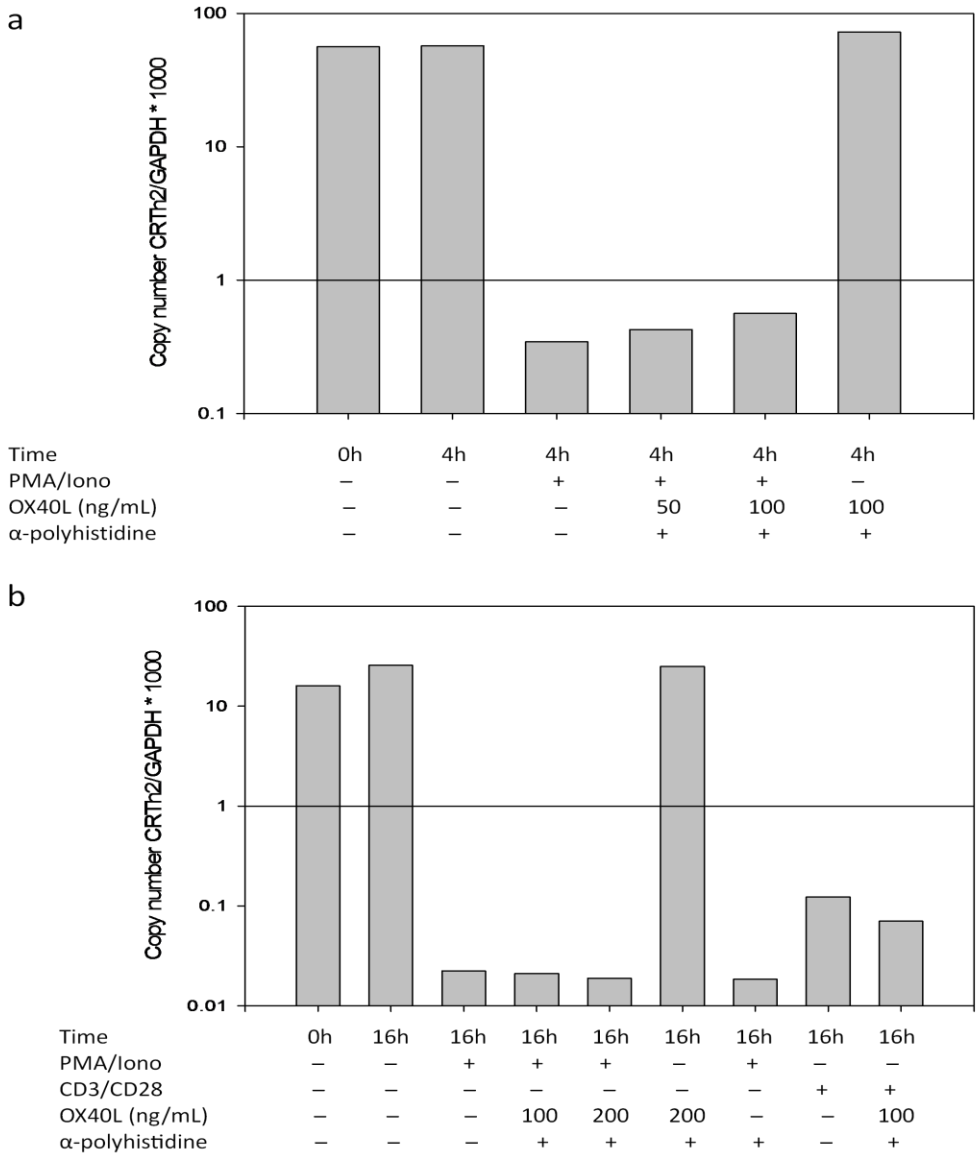

Figure 26. CRTh2 mRNA expression following addition of exogenous OX40L to stimulated CRTh2$^+$ Th2 cells following the proliferation phase

Exogenous OX40L cannot inhibit CRTh2 down-regulation in *in vitro* differentiated CRTh2$^+$ Th2 cells following 4h **(a)** or 16h **(b)** mitogenic stimulation after the proliferation phase (4d IL-2). Data is from one experiment performed in triplicate, data is presented as copy number of CRTh2 over copy number of GAPDH x 1000.

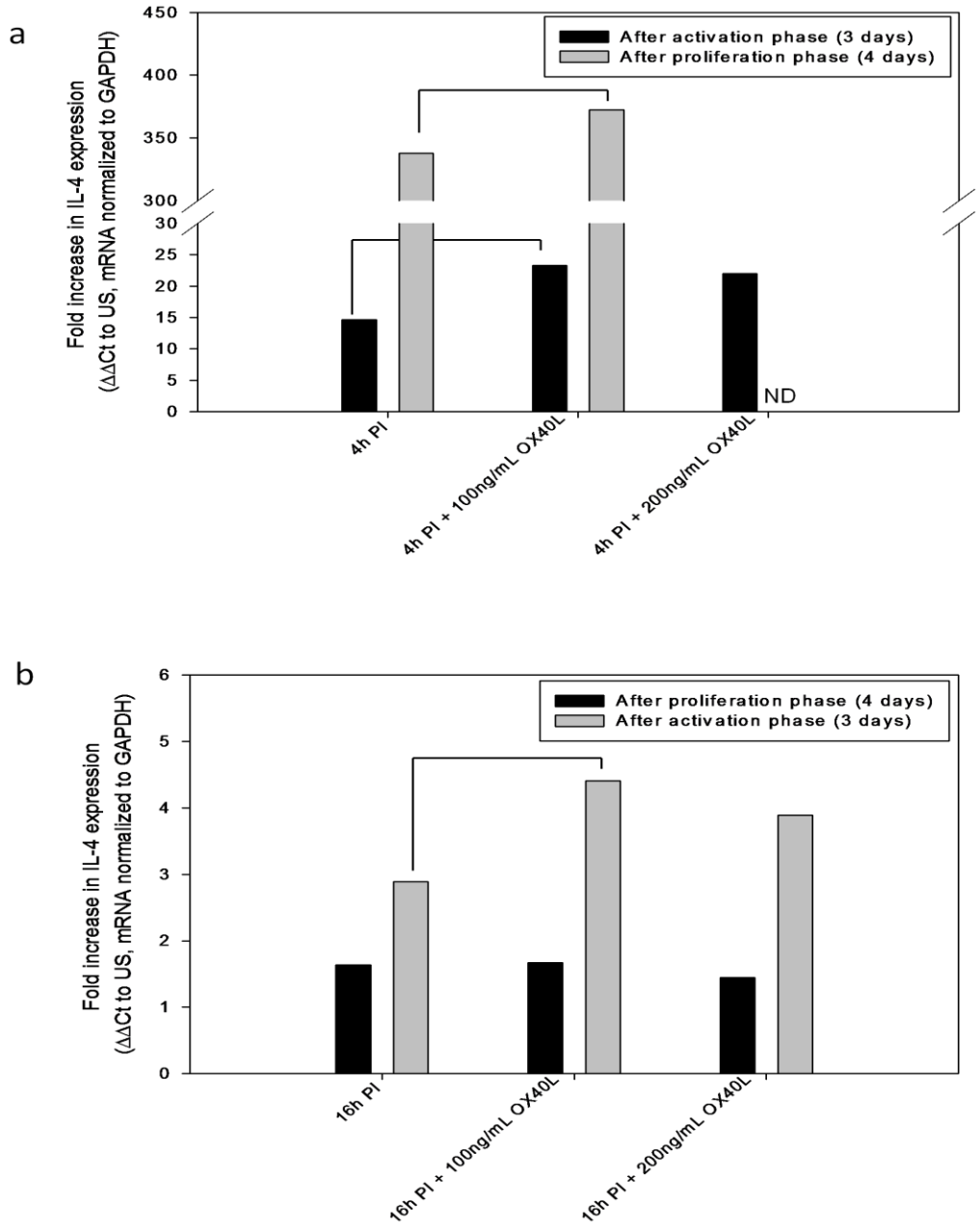

Figure 27. IL-4 mRNA expression following addition of exogenous OX40L to stimulated CRTh2$^+$ Th2 cells

Recombinant OX40L increases IL-4 mRNA from *in vitro* differentiated CRTh2$^+$ Th2 cells **(a,b)** with mitogenic stimulation (4h **(a),** 16h **(b)** P/I) after the proliferation phase (4d IL-2) but not the activation phase (3d αCD3/CD28) **(a, b)**. Lines show increased IL-4 expression with OX40L. Data is from one experiment performed in triplicate, data is presented as fold increase of ΔΔCt (to GAPDH) and normalized to respective unstimulated (US) condition. ND, not determined.

Chapter V: Discussion

Summary

Using *in vitro* differentiated CRTh2^{+} Th2 cells, developed in our lab, we studied the molecular regulation of CRTh2 expression. Since we found no difference in methylation of the *CRTh2* proximal promoter in CRTh2^{high} and CRTh2^{low} cells, we examined transcription factor binding and nuclear environments associated with CRTh2 expression. Using transient nucleofection of a luciferase reporter construct containing the proximal region of the *CRTh2* promoter (*CRTh2*-450/Luc), we found that transcriptional activity was increased following acute (16h) mitogenic stimulation. This may be due to the presence and ability of GATA3 from CRTh2^{+} Th2 cells to bind the promoter. To confirm the results from the promoter construct, we studied CRTh2 surface and mRNA expression in CRTh2^{+} Th2 cells. Surprisingly, we found both to be down-regulated following stimulation. This may be through repression by NFAT1, since its over-expression inhibited the GATA3-induced increase in transcriptional activity. Furthermore, NFAT1 binds the promoter following the activation phase and acute stimulation, conditions shown to decrease CRTh2 surface and mRNA expression **(Table 3)**. In conclusion, our data indicate that endogenous CRTh2 is down-regulated in response to stimulation, but that transcriptional activity from a reporter construct containing the proximal region of the *CRTh2* promoter (-450 bp) was increased. We believe this indicates further regulatory regions are required to adequately recapitulate the transcriptional regulation of endogenous CRTh2 expression.

Table 3: CRTh2 expression decreases with stimulation and this corresponds to NFAT1 binding

		Expression[3]		Binding[4]	
Cell Cycle	Stimulation	mRNA	surface	NFAT1	GATA3
Activation		+[1]	+	+	+
Activation	P/I	-[2]	-	+	+
Proliferation		+++	+++	-	+
Proliferation	P/I	-	-	+	+

1. + refer to more degrees of binding or CRTh2 surface or mRNA expression
2. – refers to low to no binding observed in EMSA or low to no expression by surface or mRNA expression
3. When indicated, CRTh2 expression was determined following 16h P/I stimulation
4. When indicated, NFAT1 and GATA3 binding was determined from $CRTh2^+$ Th2 cells following 3h P/I stimulation

There are still many questions that remain to be answered, however these data will help us direct those future experiments and to understand the limitations of our experimental system.

In vitro differentiated CRTh2$^+$ Th2 cells

We cultured freshly isolated CD4$^+$ T cells in Th2 differentiating conditions and isolated a relatively stable population of CRTh2$^+$ Th2 cells. Although cells produced IL-4 and high IL-13, there was still a considerable percentage of cells that were capable of producing the Th1 cytokine IFNγ, as determined by intracellular flow cytometry. This was unexpected, since T cell differentiation literature in humans and mice shows that *in vivo* differentiated Th2 cells do not produce IFNγ due to a closed chromatin conformation at the *IFNγ* locus which occurs during differentiation (136, 138). However, others have shown that *in vitro* differentiated Th2 cells produce some IFNγ (100-500 pg/mL) following stimulation (117), but at much lower levels than from stimulated *in vitro* differentiated Th1 cell (>80 000pg/mL) (117).

Quantification of CRTh2$^+$ Th2 supernatants, by Multiplex Bead Array, showed that the concentration of IFNγ in our cultures was highly variable (20 -4568 pg/mL) following the activation phase. Importantly, the IL-4 (>11 000 pg/mL) and IL-13 (>10 000 pg/mL) levels were always much higher than IFNγ. For all three cell lines examined, CRTh2 was expressed on 67-79% of cells. Therefore, it is also possible that all the IFNγ$^+$ cells are CRTh2$^-$. We were unable to look at this because the stimulation needed to see IFNγ corresponds with reduction of CRTh2,

as we have shown herein. However, a recent study shows a protocol for CRTh2/IFNγ co-staining so this could be performed in future experiments (57).

This production of IFNγ may be due to the use of only recombinant cytokines and antibodies during *in vitro* differentiation, as opposed to the many signals that a T cell receives *in vivo* from an activated dendritic cell (37). Furthermore, PMA and ionomycin mimic TCR stimulation but they do so in a strong non-physiological manner by directly activating down-stream effectors of TCR and CD28 stimulation. Therefore, stimulation with PMA and ionomycin prior to intracellular staining may over-represent the number of cells producing IFNγ during the normal culture where activation is accomplished by activating antibodies against CD3 and CD28.

Interestingly, one cell line was created for which naïve T cells ($CD4^+$ $CD45RA^+$) were used as the starter population. This was accomplished by depleting the $CD45RO^+$ cells from the initial $CD4^+$ T cell population. These cells were differentiated *in vitro* and $CRTh2^+$ cells were isolated and carried in the same conditions as the other cell lines. This difference in the starting population may be important because this line expressed the lowest IFNγ (20 pg/mL) based on the supernatant analysis. This could be due to removal of all the *in vivo* differentiated effector cells ($CD45RO^+$), including Th1 effector cells which can up-regulate IL-4 and IL-13 in Th2 differentiating conditions but also maintain their expression of IFNγ (136).

Interestingly, there was no increased differentiation of $CD4^+$ T cells isolated from allergic or non-allergic donors. Although the allergic donors may have started with a higher percentage of CRTh2 expressing cells (unpublished data from our lab), the *in vitro* differentiation was robust enough to differentiate the $CD4^+$ T cells from the non-allergic donor (n=1) to the same degree as the cells from allergic donors (n=8) (21% in allergic compared to 25% in the non-allergic). This would indicate that our *in vitro* protocol is capable of differentiating cells, not simply expanding the population of *in vivo* differentiated $CRTh2^+$ Th2 cells.

In an effort to evaluate this further, we took the $CRTh2^-$ fraction following the CRTh2 isolation (Day 7) and further cultured them in differentiating conditions (rhIL-2, rhIL-4, αIL-12, and αIFNγ). These cells up-regulated CRTh2 following the extra 7 days (14 days total) in differentiating conditions (n=2). These data indicate that the cells are capable of differentiating *in vitro* since any *in vivo* differentiated $CRTh2^+$ cells would have been removed on day 7 during the CRTh2 isolation.

Our *in vitro* differentiated $CRTh2^+$ Th2 cells began as $CD62L^{high}$ central memory T cells, but there was a large range of CD62L expression resulting from down-regulation as cells were kept in culture (data not shown). This may be due to loss of the T_{CM} phenotype or changes to effector memory cells as our cultured cells were submitted to several cycles of activation (36). Wang *et al* reported that the $CD4^+$ $CRTh2^+$ population was a subset of T_{CM} cells and this was maintained

through culture with TSLP-DCs. Therefore, the loss of CD62L expression may be due to lack of signals from activated DCs (37).

Molecular regulation of *CRTh2* from the proximal promoter region

In this study we showed that the transcriptional activity from the proximal region of the *CRTh2* promoter (*CRTh2*-450/Luc) was significantly increased in response to stimulation but only in cells that endogenously expressed CRTh2. This was accomplished with a *CRTh2* promoter luciferase reporter construct, a standard approach to studying promoter activity. We studied the 450 base pair region immediately up-stream of the *CRTh2* transcription start site because it is highly conserved in the primate order. The *CRTh2* proximal region of the promoter is most conserved in Chimpanzees (a Hominoid family) followed by Rhesus monkeys (an Old World Monkey family) and finally Marmosets (a New World Monkey family); with Hominoids being evolutionarily closest and New World Monkeys the furthest to humans. Since the degree of conservation of the proximal promoter region of *CRTh2* between human and Marmoset is less conserved than Chimpanzee and Rhesus Monkey, this would indicate that the regulation of *CRTh2* has been evolving.

We only studied the transcriptional activity of CRTh2 from the proximal region of the *CRTh2* promoter (*CRTh2*-450/Luc) although there is another ~4kb of conserved sequence 5' region that may act as promoter, enhancer or repressor elements to modify CRTh2 expression. In addition to the 5' region, there is also an intron separating the untranslated exon 1 from the coding region (exon 2). These regions could separately or collectively affect the expression of CRTh2 not

only in response to stimulation but also in resting cells. Since the decrease in endogenous expression of CRTh2 in response to stimulation differed from the increase seen with the *CRTh2*-450/Luc, our construct may be missing regulatory regions. For this reason, our lab has generated luciferase constructs containing a longer 5' region (4kb), 5' truncations and/or intron regions. These are currently being tested in transient transfection experiments with Jurkat T cells and *in vitro* differentiated CRTh2^{+} Th2 cells to assess if they can replicate the regulation of endogenous gene expression.

Luciferase reporter assays only report transcriptional regulation by transcription factor binding not any chromatin modifications or translational regulation such as mRNA stability. These factors could be affecting endogenous CRTh2 expression following stimulation. We hypothesized that stimulation increases transcription from both endogenous *CRTh2* and our luciferase construct initially, but that endogenous CRTh2 mRNA degrades quickly, unlike the luciferase mRNA. We attempted to exmaine this by looking for early up-regulation of CRTh2 mRNA following stimulation. It is possible that there was an initial increase in CRTh2 mRNA but it was hard to determine due to variation among cell lines. Although we did not see any significant early increase in CRTh2 mRNA to suggest that mRNA stability is regulating CRTh2 expression, further experiments with actinomycin D to examine the mRNA half life are still needed. It is also important to note that the rapid decrease in CRTh2 mRNA (2h) would suggest that CRTh2 mRNA stability is regulated.

Since GATA3 and NFAT1 bind to the *CRTh2* promoter following stimulation, we over-expressed both these factors with the *CRTh2*-450/Luc construct in Jurkat T cells to see if they had an enhancing or repressing effect. We determined not only that over-expression of GATA3 increases proximal *CRTh2* promoter activity in Jurkat T cells but also that this could be inhibited by over-expression of NFAT1. Since we observed binding of both NFAT1 and GATA3 with nuclear extracts of Th2 cells, this competitive interaction is likely occurring *in vivo*

Further, we observed that GATA3 could bind two of the three putative binding sites in the proximal *CRTh2* promoter probe and that binding of both was inhibited by mutating either site. In a similar study of the IL-13 promoter, which contains three GATA3 sites within 35 base pairs, not only does GATA3 bind to all three but optimal binding and expression of IL-13 depends on all three sites being intact (139). Further, GATA3/DNA crystal structures from mice show that GATA3 can either dimerize to bind to adjacent sites (5 bp apart) or a single GATA3 molecule can bind both sites (3 bp apart) with the N and C terminal zinc finger of the same GATA3 molecule (140). Further work is needed to determine if one or two GATA3 molecules bind the GATA3 sites (10 bp apart) in the *CRTh2* proximal promoter probe and alter expression.

We also confirmed that NFAT1 binding could be inhibited by mutating two or four base pairs of the NFAT1 binding site. Although both mutations disrupted the NFAT site, we had made the two base pair mutation to decrease the chance of disrupting the predicted adjacent GATA3 site, which in the end did not bind

GATA3. Since the transcription factor binding sites are in close proximity, we verified that GATA3 and NFAT1 can bind independently. Indeed, when the NFAT1 binding site was mutated it did not affect GATA3 binding. Since NFAT1 and GATA3 do not bind the *CRTh2* proximal probe simultaneously and the binding of each factor does not require the other binding site, the inhibitory effect of NFAT1 may be due to competitive inhibition of GATA3 binding or active repression. This may be similar to the ICOS promoter (141) where both NFAT1 and GATA3 were shown to bind but that activation was GATA3 dependent.

NFAT1 binding to the proximal promoter was observed following stimulation (when CRTh2 expression is lowest) and NFAT1 over-expression could inhibit GATA3 induced transcriptional activity of the *CRTh2* promoter construct (*CRTh2*-450/Luc) in Jurkat cells. Therefore, we hypothesized NFAT1 inhibits CRTh2 expression. However, when NFAT1 binding was inhibited using CsA transcriptional activity of the *CRTh2* promoter construct in $CRTh2^{+}$ Th2 cells was significantly reduced. These data could indicate the NFAT1 induced inhibition was inaccurate, possibly due to having given such excess of NFAT1, compared to normal expression. However, this seems unlikely because we did not see a significant change when NFAT1 was titrated down substantially (1/16 of GATA3). Since CsA inhibits all calcineurin mediated calcium signaling it may have disrupted more than just NFAT1 nuclear translocation which may be affecting transcriptional activity of *CRTh2*. To verify this, a specific NFAT inhibitory peptide (VIVIT), which competes for the calcineurin binding site (142), could be used to assess the effect of NFAT1 inhibition. Ultimately, this line of

investigation needs to be followed up by examining the influence of CsA on endogenous CRTh2 mRNA and surface expression.

By electromobility shift assay, we confirmed GATA3 binding from differentiated $CRTh2^{+}$ Th2 cells, but more importantly, identified NFAT1 binding following T cell activation. EMSA is an excellent tool to determine the potential of a certain sequence to bind a transcription factor, which can be determined by hypothesizing which factors bind and verifying with recombinant proteins. Therefore it is an effective method to understand the potential of transcription factors to bind in certain nuclear environments. Limiting factors of EMSA are that firstly the probe is devoid of 3-dimensional chromatin structure and secondly is given in such excess (compared to genomic DNA) that it is difficult to discern what binding would occur *in vivo*. In the experiments performed herein, the *CRTh2* proximal promoter probe showed only two bands on the gel indicating two different binding scenarios this likely does not occur simultaneously *in vivo*; otherwise there would be an extra band showing both GATA3 and NFAT1 binding the probe together.

To verify binding of GATA3 and NFAT1 *in vivo*, chromatin immunoprecipitation (ChIP) can be used. This technique involves fixing the cells so all DNA binding proteins are cross-linked to the DNA. This is followed by immunoprecipitation of GATA3 and NFAT1 bound fragments of DNA (600 – 1000 bp), using anti-GATA3 or anti-NFAT1 antibodies. Following immunoprecipitation, cross-links are reversed and DNA is amplified by polymerase chain reaction (PCR) with

primers for the *CRTh2* region in question. This will not only help us understand what conditions support GATA3 and NFAT1 binding to the *CRTh2* promoter in $CRTh2^+$ Th2 cells but also primers for other regions could show if the transcription factors bind elsewhere at the promoter. Our lab is currently pursuing this work.

Another important question is whether the transcription factors necessary to initially up-regulate CRTh2 (i.e., during differentiation) are the same as those needed to maintain CRTh2 (i.e., during proliferation with IL-2 alone). To assess this, nuclear extracts can be made from isolated $CD4^+$ T cells during *in vitro* differentiation and again during the proliferation phase following CRTh2 positive selection. Following EMSA analysis, ChIP can be performed in both differentiating and differentiated $CRTh2^+$ Th2 cells to confirm EMSA results.

Since we observed that transcriptional activity of *CRTh2* is up-regulated by over-expression of GATA3 and this is inhibited by over-expression of NFAT1, the next experiment to understand the implications of this binding with the whole *CRTh2* gene would be siRNA. We can 1) use GATA3 siRNA and see if CRTh2 expression is decreased or 2) NFAT1 siRNA to see if CRTh2 expression is increased, stable or decreased in response to stimulation. Although siRNA knock-down is difficult in primary T cells, there are several studies that have shown significant inhibition of GATA3 function (143-144). If siRNA is too difficult or we wish to look further, we could also over-express NFAT1 in differentiated $CRTh2^+$ Th2 cells and observe the effect on endogenous CRTh2 expression.

In an effort to understand the potential role the rest of the 5' region may play in regulation of CRTh2, we examined conservation of the 5' region and intron. We mapped 19 conserved regions (CR) 1-19 relative to horse and the putative Th2 transcription factor binding site(s) within **(Figure 27)**. Along with additional GATA3 sites there are also putative binding sites for STAT, RBPJκ and ETS, all factors associated with Th2 differentiation (138, 145-146). These are the next factors to examine by over-expression with longer promoter constructs, EMSA or ChIP.

Endogenous CRTh2 is decreased with stimulation

There have been reports in the literature that CRTh2 is decreased following stimulation (43, 80). When we observed increased transcriptional activity from the proximal region of the *CRTh2* promoter in response to stimulation we looked at endogenous CRTh2 expression from *in vitro* differentiated CRTh2$^+$ Th2 cells following activation. We saw a decrease in both CRTh2 surface and mRNA expression following stimulation. We did notice that cells stimulated with activating CD3/CD28 down-regulated CRTh2 to a lesser extent than the mitogenic stimulation with PMA and Ionomycin **(Figure 18** and data not shown**)** indicating that the CD3/CD28 stimulation may be less potent.

Interestingly, there is also a hypothesis that lower TCR activation of T cells results in Th2 cell differentiation as opposed to Th1 differentiation (147). The lower potency of TCR activation can come from either low affinity peptides or low doses of antigen. One proposed mechanism is that low affinity peptide stimulation results in increased IL-4 from a higher NFAT2:NFAT1 ratio in the

		Conserved Regions (CR)							
		5' region			Within the intron				
Description	TF Family	1-3	4-9	10-14	16	17	18	19	Total
GATA binding factors	GATA	1	1	3	2		1	1	9
Human and murine ETS1 factor	ETSF		9	2	6	1	2	1	21
Signal transducer and activator of transcription	STAT			3	1				4
Nuclear factor of activated T-cells	NFAT			3		1	1	1	6
RBP-Jkappa	RBPF			1	1			1	3
Ikaros zinc finger family	IKRS								0

Figure 28. Summary of putative transcription factor binding sites 5' of the *CRTh2* coding region

Putative transcription factor binding sites in conserved regions of the 5' and intron regions of *CRTh2*. **TF:** transcription factor.

nucleus which is permissive for IL-4 transcription (148). Since CRTh2 is up-regulated in differentiated Th2 cells, CRTh2 may also be positively regulated by the lower TCR activation. We only tested the transcriptional activity of the *CRTh2* promoter (*CRTh2*-450/Luc) using PMA and ionomycin. Although these mimic TCR stimulation, they do so in a strong non-physiological manner by activating down-stream effectors of TCR and CD28 stimulation. Since CRTh2 is expressed in Th2, not Th1 cells, its expression may also be regulated based on TCR stimulation so it may be insightful to either titrate down PMA and ionomycin and/or stimulating CD3 and CD28. Lowering TCR stimulation may result in higher IL-4, activation of GATA3, and lower NFAT1 in the nucleus (148) which we have shown here may be inhibitory to CRTh2 transcriptional activity.

I*n vitro* differentiated $CRTh2^+$ Th2 cells may differ from *in vivo* differentiated cells because they were cultured without antigen presenting cells. We hypothesized that lack of interaction with antigen presenting cells during activation resulted in decreased CRTh2 expression. In a study by Wang *et al*, TSLP activated dendritic cells appear to be responsible for Th2 polarization and maintenance of CRTh2 expression. Although Th2 cells could be stimulated *in vitro* by CD3 and CD28, they did not maintain their polarization, as they began to produce IFNγ (37). OX40 signaling has also been shown to maintain protein kinase B (PKB) activation which maintains expression of anti-apoptotic molecules (survivin) resulting in cell survival (149-150). Since the OX40/OX40L interactions were required for cell expansion, we hypothesized that the OX40 pathway may maintain the CRTh2 expression as well (37). We found that

although our differentiated CRTh2^{+} Th2 cells expressed OX40, adding exogenous OX40L to cultures could not rescue the decrease in CRTh2 expression following stimulation.

OX40 signaling may increase IL-4 due to increased NFAT2 nuclear translocation in both naïve and effector T cells (151). Further, OX40 signaling with IL-4 results in increased GATA3 which can also maintain the Th2 phenotype (23, 151). We demonstrated that OX40L had an effect on IL-4 expression **(Figure 27)**, but it did not change CRTh2 expression *in vitro*. Although we demonstrated that exogenous OX40L was effective at the concentrations used, further optimization may be necessary using proliferation assays. The lack of effect on CRTh2 expression may be because the effect seen by Wang *et al* with longer cultures (7 days) or was not caused directly by OX40L. We could investigate this by putting OX40L in our cultures directly or evolving the protocol to use TSLP-DCs.

Although, OX40L did not directly affect CRTh2 expression, there are other transcription factors that are activated by interaction with dendritic cells. One such factor is Notch/RBPJκ, which also has a predicted binding site in the *CRTh2* 5' region and intron **(Figure 28)**. Notch is expressed by T cells and its ligands, Jagged or Delta, are expressed on dendritic cells. Upon ligand binding, the intracellular domain (ICD) of Notch is released by proteolytic cleavage (reviewed in (152)). The Notch ICD targets a transcription factor, RBPJκ, and converts it from a repressor to an activator (reviewed in (153)). There are four members of the Notch family (Notch 1-4) that can bind to five different ligands, Jagged 1-2

and Delta 1, 3 and 4 (153). Studies have shown that activation of Notch through both ligands initiates the same signaling but they may serve different functions. Dendritic cells up-regulated Jagged-1 or Delta-4 in response to Th2 and Th1 polarizing conditions, respectively, and dendritic cells expressing Jagged-1 drive Th2 polarization (154). Amsen *et al* also showed that Notch ligation by Jagged 1 or 2 resulted in Th2 differentiation, (146). This appears to be through increased GATA3 and IL-4 and to be dependent on intact RBPJκ binding sites (146). Therefore, $CRTh2^+$ Th2 cell interactions with Jagged on DCs may be necessary during T cell activation for RBPJκ to become an activator at the *CRTh2* gene. To address this hypothesis, over-expression of the Notch intracellular domain could be assessed in primary *in vitro* differentiated $CRTh2^+$ Th2 cells during stimulation.

In conclusion, although the *CRTh2* proximal promoter shows up-regulation of CRTh2 transcriptional activity following stimulation and GATA3 over-expression, endogenous CRTh2 expression is decreased following stimulation. We found that this reduction correlated with NFAT1 binding to the *CRTh2* proximal promoter region, likely interfering with GATA3 induced activation **(Figure 29)**. Further investigation is needed to understand if the discrepancy in *CRTh2* promoter activation and endogenous CRTh2 expression is due to lack of regulatory regions within the reporter construct studied. Finally, the down-regulation of CRTh2 in response to stimulation still needs to be examined to determine if it occurs *in vivo* or is an artifact of *in vitro* T cell culture in the absence of dendritic cells.

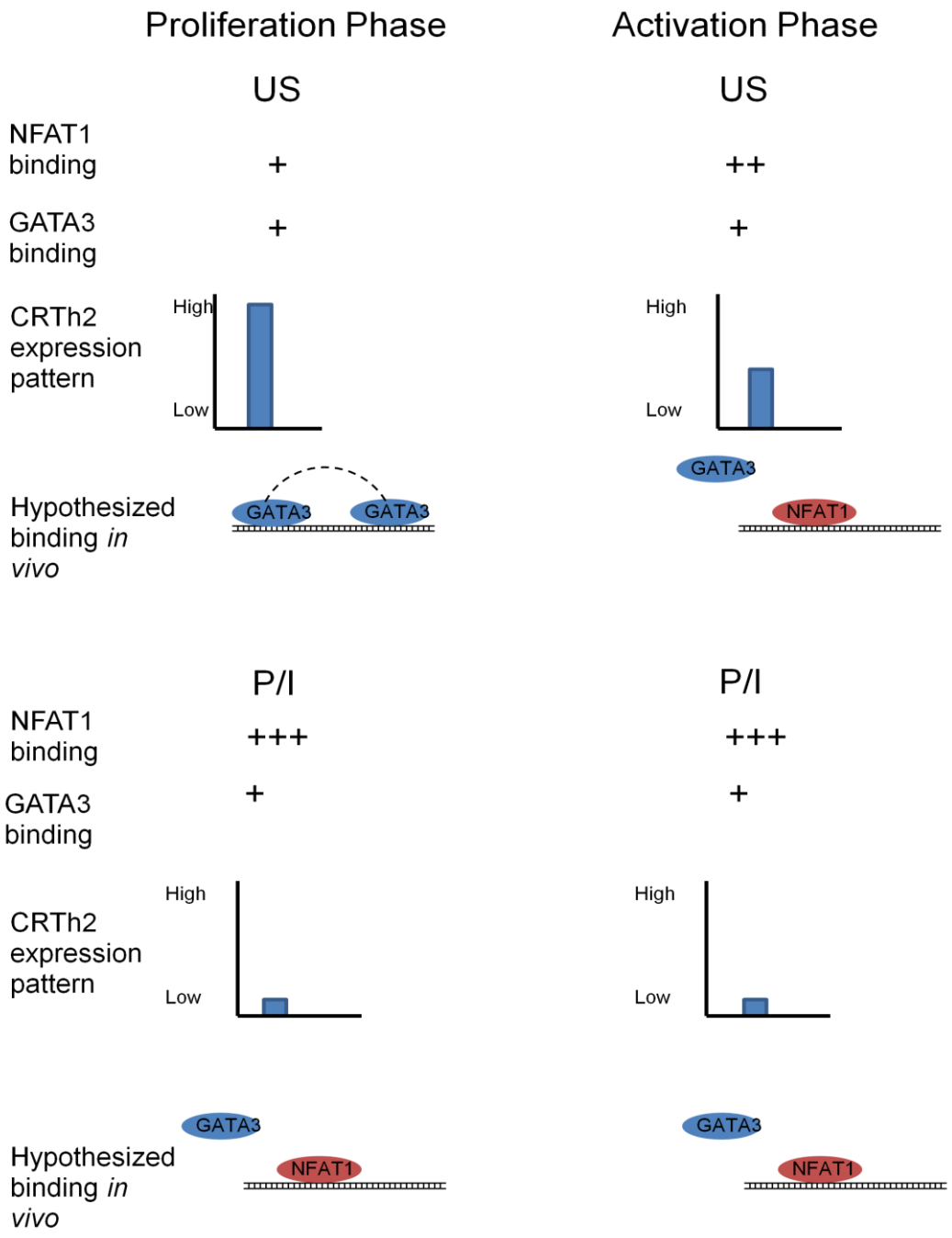

Figure 29. Hypothesized binding of GATA3 and NFAT1 to the *CRTh2* proximal promoter region following proliferation and activation phase

NFAT1, GATA3 binding and CRTh2 expression patterns are based on data shown herein. + refers to binding intensity observed by EMSA. Hypothesized binding *in vivo*

demonstrates what may happen *in vivo*, although GATA3 can bind the *CRTh2* promoter probe, NFAT1 binds with greater intensity by EMSA. Dashed line represents that one or two GATA3 molecules may bind.

References

1. Eder, W., M. J. Ege, and E. von Mutius. 2006. The asthma epidemic. *N Engl J Med* 355:2226-2235.
2. Broide, D. H. 2001. Molecular and cellular mechanisms of allergic disease. *J Allergy Clin Immunol* 108:S65-71.
3. Reinhold, U., S. Kukel, B. Goeden, U. Neumann, and H. W. Kreysel. 1991. Functional characterization of skin-infiltrating lymphocytes in atopic dermatitis. *Clin Exp Immunol* 86:444-448.
4. Broide, D. H. 2010. Allergic rhinitis: Pathophysiology. *Allergy Asthma Proc* 31:370-374.
5. Agrawal, D. K., and Z. Shao. 2010. Pathogenesis of allergic airway inflammation. *Curr Allergy Asthma Rep* 10:39-48.
6. Bingham, C. O., 3rd, and K. F. Austen. 2000. Mast-cell responses in the development of asthma. *J Allergy Clin Immunol* 105:S527-534.
7. Giles, H., P. Leff, M. L. Bolofo, M. G. Kelly, and A. D. Robertson. 1989. The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br J Pharmacol* 96:291-300.
8. Roberts, L. J., 2nd, R. A. Lewis, J. A. Oates, and K. F. Austen. 1979. Prostaglandin thromboxane, and 12-hydroxy-5,8,10,14-eicosatetraenoic acid production by ionophore-stimulated rat serosal mast cells. *Biochim Biophys Acta* 575:185-192.
9. Bach, M. K., K. J. Bloch, and K. F. Austen. 1971. IgE and IgGa antibody-mediated release of histamine from rat peritoneal cells. I. Optimum conditions for in vitro preparation of target cells with antibody and and challenge with antigen. *J Exp Med* 133:752-771.
10. Orange, R. P., D. J. Stechschulte, and K. F. Austen. 1970. Immunochemical and biologic properties of rat IgE. II. Capacity to mediate the immunologic releas of histamine an slow-reacting substance of anaphylaxis (SRS-A). *J Immunol* 105:1087-1095.
11. Arima, M., and T. Fukuda. 2008. Prostaglandin D2 receptors DP and CRTH2 in the pathogenesis of asthma. *Curr Mol Med* 8:365-375.
12. Mucida, D., and H. Cheroutre. 2010. The many face-lifts of CD4 T helper cells. *Adv Immunol* 107:139-152.
13. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.
14. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J Exp Med* 166:1229-1244.
15. Croft, M., L. Carter, S. L. Swain, and R. W. Dutton. 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* 180:1715-1728.
16. Soumelis, V., P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J.

Abrams, S. Menon, T. McClanahan, R. de Waal-Malefyt Rd, F. Bazan, R. A. Kastelein, and Y. J. Liu. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3:673-680.
17. Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G. M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* 6:507-514.
18. Okayama, Y., S. Okumura, H. Sagara, K. Yuki, T. Sasaki, N. Watanabe, M. Fueki, K. Sugiyama, K. Takeda, T. Fukuda, H. Saito, and C. Ra. 2009. FcepsilonRI-mediated thymic stromal lymphopoietin production by interleukin-4-primed human mast cells. *Eur Respir J* 34:425-435.
19. Swain, S. L., A. D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 145:3796-3806.
20. Manetti, R., F. Gerosa, M. G. Giudizi, R. Biagiotti, P. Parronchi, M. P. Piccinni, S. Sampognaro, E. Maggi, S. Romagnani, G. Trinchieri, and et al. 1994. Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. *J Exp Med* 179:1273-1283.
21. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-669.
22. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787-793.
23. De Fanis, U., F. Mori, R. J. Kurnat, W. K. Lee, M. Bova, N. F. Adkinson, and V. Casolaro. 2007. GATA3 up-regulation associated with surface expression of CD294/CRTH2: a unique feature of human Th cells. *Blood* 109:4343-4350.
24. Cosmi, L., F. Annunziato, M. I. G. Galli, R. M. E. Maggi, K. Nagata, and S. Romagnani. 2000. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol* 30:2972-2979.
25. Nagata, K., H. Hirai, K. Tanaka, K. Ogawa, T. Aso, K. Sugamura, M. Nakamura, and S. Takano. 1999. CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Lett* 459:195-199.
26. Shapira, S. K., H. H. Jabara, C. P. Thienes, D. J. Ahern, D. Vercelli, H. J. Gould, and R. S. Geha. 1991. Deletional switch recombination occurs in interleukin-4-induced isotype switching to IgE expression by human B cells. *Proc Natl Acad Sci U S A* 88:7528-7532.
27. Lebman, D. A., and R. L. Coffman. 1988. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J Exp Med* 168:853-862.
28. Zhang, M., G. Srivastava, and L. Lu. 2004. The pre-B cell receptor and its function during B cell development. *Cell Mol Immunol* 1:89-94.
29. Griebel, P., T. Beskorwayne, A. Van den Broeke, and G. Ferrari. 1999. CD40 signaling induces B cell responsiveness to multiple members of the gamma chain-common cytokine family. *Int Immunol* 11:1139-1147.
30. Kracker, S., and A. Durandy. 2011. Insights into the B cell specific process of immunoglobulin class switch recombination. *Immunol Lett*.
31. Mills, F. C., G. Thyphronitis, F. D. Finkelman, and E. E. Max. 1992. Ig mu-epsilon isotype switch in IL-4-treated human B lymphoblastoid cells. Evidence for a sequential switch. *J Immunol* 149:1075-1085.

32. Jabara, H. H., R. Loh, N. Ramesh, D. Vercelli, and R. S. Geha. 1993. Sequential switching from mu to epsilon via gamma 4 in human B cells stimulated with IL-4 and hydrocortisone. *J Immunol* 151:4528-4533.
33. Punnonen, J., G. Aversa, B. G. Cocks, A. N. McKenzie, S. Menon, G. Zurawski, R. de Waal Malefyt, and J. E. de Vries. 1993. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci U S A* 90:3730-3734.
34. Cameron, L., A. S. Gounni, S. Frenkiel, F. Lavigne, D. Vercelli, and Q. Hamid. 2003. S epsilon S mu and S epsilon S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. *J Immunol* 171:3816-3822.
35. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 22:745-763.
36. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
37. Wang, Y. H., T. Ito, Y. H. Wang, B. Homey, N. Watanabe, R. Martin, C. J. Barnes, B. W. McIntyre, M. Gilliet, R. Kumar, Z. Yao, and Y. J. Liu. 2006. Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. *Immunity* 24:827-838.
38. Shirasaki, H., M. Kikuchi, E. Kanaizumi, and T. Himi. 2009. Accumulation of CRTH2-positive leukocytes in human allergic nasal mucosa. *Ann Allergy Asthma Immunol* 102:110-115.
39. van Reijsen, F. C., C. A. Bruijnzeel-Koomen, R. A. de Weger, and G. C. Mudde. 1997. Retention of long-lived, allergen-specific T cells in atopic dermatitis skin - letter. *J Invest Dermatol* 108:530.
40. Ito, T., Y. H. Wang, O. Duramad, T. Hori, G. J. Delespesse, N. Watanabe, F. X. Qin, Z. Yao, W. Cao, and Y. J. Liu. 2005. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 202:1213-1223.
41. Calder, P. C. 2009. Fatty acids and immune function: relevance to inflammatory bowel diseases. *Int Rev Immunol* 28:506-534.
42. Pettipher, R. 2008. The roles of the prostaglandin D(2) receptors DP(1) and CRTH2 in promoting allergic responses. *Br J Pharmacol* 153 Suppl 1:S191-199.
43. Nagata, K., K. Tanaka, K. Ogawa, K. Kemmotsu, T. Imai, O. Yoshie, H. Abe, K. Tada, M. Nakamura, K. Sugamura, and S. Takano. 1999. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol* 162:1278-1286.
44. Hirai, H., K. Tanaka, O. Yoshie, K. Ogawa, K. Kenmotsu, Y. Takamori, M. Ichimasa, K. Sugamura, M. Nakamura, S. Takano, and K. Nagata. 2001. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 193:255-261.
45. Sawyer, N., E. Cauchon, A. Chateauneuf, R. P. Cruz, D. W. Nicholson, K. M. Metters, G. P. O'Neill, and F. G. Gervais. 2002. Molecular pharmacology of the human prostaglandin D2 receptor, CRTH2. *Br J Pharmacol* 137:1163-1172.
46. Gazi, L., S. Gyles, J. Rose, S. Lees, C. Allan, L. Xue, R. Jassal, G. Speight, V. Gamble, and R. Pettipher. 2005. Delta12-prostaglandin D2 is a potent and selective CRTH2 receptor agonist and causes activation of human eosinophils and Th2 lymphocytes. *Prostaglandins Other Lipid Mediat* 75:153-167.

47. Heinemann, A., R. Schuligoi, I. Sabroe, A. Hartnell, and B. A. Peskar. 2003. Delta 12-prostaglandin J2, a plasma metabolite of prostaglandin D2, causes eosinophil mobilization from the bone marrow and primes eosinophils for chemotaxis. *J Immunol* 170:4752-4758.
48. Monneret, G., H. Li, J. Vasilescu, J. Rokach, and W. S. Powell. 2002. 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. *J Immunol* 168:3563-3569.
49. Hata, A. N., and R. M. Breyer. 2004. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Ther* 103:147-166.
50. Boehme, S. A., K. Franz-Bacon, E. P. Chen, T. W. Ly, Y. Kawakami, and K. B. Bacon. 2009. Murine bone marrow-derived mast cells express chemoattractant receptor-homologous molecule expressed on T-helper class 2 cells (CRTh2). *Int Immunol* 21:621-632.
51. Huang, S. K., R. A. Mathias, E. Ehrlich, B. Plunkett, X. Liu, G. R. Cutting, X. J. Wang, X. D. Li, A. Togias, K. C. Barnes, F. Malveaux, S. Rich, B. Mellen, E. Lange, and T. H. Beaty. 2003. Evidence for asthma susceptibility genes on chromosome 11 in an African-American population. *Hum Genet* 113:71-75.
52. Cameron, L., M. Depner, M. Kormann, N. Klopp, T. Illig, E. von Mutius, and M. Kabesch. 2009. Genetic variation in CRTh2 influences development of allergic phenotypes. *Allergy* 64:1478-1485.
53. Huang, J. L., P. S. Gao, R. A. Mathias, T. C. Yao, L. C. Chen, M. L. Kuo, S. C. Hsu, B. Plunkett, A. Togias, K. C. Barnes, C. Stellato, T. H. Beaty, and S. K. Huang. 2004. Sequence variants of the gene encoding chemoattractant receptor expressed on Th2 cells (CRTH2) are associated with asthma and differentially influence mRNA stability. *Hum Mol Genet* 13:2691-2697.
54. Matsuoka, T., M. Hirata, H. Tanaka, Y. Takahashi, T. Murata, K. Kabashima, Y. Sugimoto, T. Kobayashi, F. Ushikubi, Y. Aze, N. Eguchi, Y. Urade, N. Yoshida, K. Kimura, A. Mizoguchi, Y. Honda, H. Nagai, and S. Narumiya. 2000. Prostaglandin D2 as a mediator of allergic asthma. *Science* 287:2013-2017.
55. Hammad, H., M. Kool, T. Soullie, S. Narumiya, F. Trottein, H. C. Hoogsteden, and B. N. Lambrecht. 2007. Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. *J Exp Med* 204:357-367.
56. Tanaka, K., H. Hirai, S. Takano, M. Nakamura, and K. Nagata. 2004. Effects of prostaglandin D2 on helper T cell functions. *Biochem Biophys Res Commun* 316:1009-1014.
57. Mutalithas, K., C. Guillen, C. Day, C. E. Brightling, I. D. Pavord, and A. J. Wardlaw. 2010. CRTH2 expression on T cells in asthma. *Clin Exp Immunol* 161:34-40.
58. Mutalithas, K., C. Guillen, C. Raport, R. Kolbeck, D. Soler, C. E. Brightling, I. D. Pavord, and A. J. Wardlaw. 2010. Expression of CCR8 is increased in asthma. *Clin Exp Allergy* 40:1175-1185.
59. Iwasaki, M., K. Nagata, S. Takano, K. Takahashi, N. Ishii, and Z. Ikezawa. 2002. Association of a new-type prostaglandin D2 receptor CRTH2 with circulating T helper 2 cells in patients with atopic dermatitis. *J Invest Dermatol* 119:609-616.
60. Xue, L., A. Barrow, and R. Pettipher. 2009. Interaction between prostaglandin D and chemoattractant receptor-homologous molecule expressed on Th2 cells mediates cytokine production by Th2 lymphocytes in response to activated mast cells. *Clin Exp Immunol* 156:126-133.

61. Xue, L., S. L. Gyles, F. R. Wettey, L. Gazi, E. Townsend, M. G. Hunter, and R. Pettipher. 2005. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. *J Immunol* 175:6531-6536.
62. Vinall, S. L., E. R. Townsend, and R. Pettipher. 2007. A paracrine role for chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) in mediating chemotactic activation of CRTH2+ CD4+ T helper type 2 lymphocytes. *Immunology* 121:577-584.
63. Xue, L., S. L. Gyles, A. Barrow, and R. Pettipher. 2007. Inhibition of PI3K and calcineurin suppresses chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)-dependent responses of Th2 lymphocytes to prostaglandin D(2). *Biochem Pharmacol* 73:843-853.
64. Xue, L., A. Barrow, and R. Pettipher. 2009. Novel function of CRTH2 in preventing apoptosis of human Th2 cells through activation of the phosphatidylinositol 3-kinase pathway. *J Immunol* 182:7580-7586.
65. Nantel, F., C. Fong, S. Lamontagne, D. H. Wright, A. Giaid, M. Desrosiers, K. M. Metters, G. P. O'Neill, and F. G. Gervais. 2004. Expression of prostaglandin D synthase and the prostaglandin D2 receptors DP and CRTH2 in human nasal mucosa. *Prostaglandins Other Lipid Mediat* 73:87-101.
66. Perez-Novo, C. A., G. Holtappels, S. L. Vinall, L. Xue, N. Zhang, C. Bachert, and R. Pettipher. 2010. CRTH2 mediates the activation of human Th2 cells in response to PGD(2) released from IgE/anti-IgE treated nasal polyp tissue. *Allergy* 65:304-310.
67. Wenzel, S. E., J. Y. Westcott, and G. L. Larsen. 1991. Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: relationship to the development of late asthmatic responses. *J Allergy Clin Immunol* 87:540-548.
68. Balzar, S., M. L. Fajt, S. A. Comhair, S. C. Erzurum, E. Bleecker, W. W. Busse, M. Castro, B. Gaston, E. Israel, L. B. Schwartz, D. Curran-Everett, C. G. Moore, and S. E. Wenzel. 2011. Mast cell phenotype, location, and activation in severe asthma: data from the severe asthma research program. *Am J Respir Crit Care Med* 183:299-309.
69. Hijnen, D., E. Nijhuis, M. Bruin-Weller, F. Holstege, M. G. Koerkamp, I. Kok, C. Bruijnzeel-Koomen, and E. Knol. 2005. Differential expression of genes involved in skin homing, proliferation, and apoptosis in CD4+ T cells of patients with atopic dermatitis. *J Invest Dermatol* 125:1149-1155.
70. Spik, I., C. Brenuchon, V. Angeli, D. Staumont, S. Fleury, M. Capron, F. Trottein, and D. Dombrowicz. 2005. Activation of the prostaglandin D2 receptor DP2/CRTH2 increases allergic inflammation in mouse. *J Immunol* 174:3703-3708.
71. Lukacs, N. W., A. A. Berlin, K. Franz-Bacon, R. Sasik, L. J. Sprague, T. W. Ly, G. Hardiman, S. A. Boehme, and K. B. Bacon. 2008. CRTH2 antagonism significantly ameliorates airway hyperreactivity and downregulates inflammation-induced genes in a mouse model of airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 295:L767-779.
72. Satoh, T., R. Moroi, K. Aritake, Y. Urade, Y. Kanai, K. Sumi, H. Yokozeki, H. Hirai, K. Nagata, T. Hara, M. Utsuyama, K. Hirokawa, K. Sugamura, K. Nishioka, and M. Nakamura. 2006. Prostaglandin D2 plays an essential role in chronic allergic inflammation of the skin via CRTH2 receptor. *J Immunol* 177:2621-2629.

73. He, R., M. K. Oyoshi, J. Y. Wang, M. R. Hodge, H. Jin, and R. S. Geha. 2010. The prostaglandin D receptor CRTH2 is important for allergic skin inflammation after epicutaneous antigen challenge. *J Allergy Clin Immunol* 126:784-790.
74. Nomiya, R., M. Okano, T. Fujiwara, M. Maeda, Y. Kimura, K. Kino, M. Yokoyama, H. Hirai, K. Nagata, T. Hara, K. Nishizaki, and M. Nakamura. 2008. CRTH2 plays an essential role in the pathophysiology of Cry j 1-induced pollinosis in mice. *J Immunol* 180:5680-5688.
75. Fujitani, Y., Y. Kanaoka, K. Aritake, N. Uodome, K. Okazaki-Hatake, and Y. Urade. 2002. Pronounced eosinophilic lung inflammation and Th2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. *J Immunol* 168:443-449.
76. Almishri, W., C. Cossette, J. Rokach, J. G. Martin, Q. Hamid, and W. S. Powell. 2005. Effects of prostaglandin D2, 15-deoxy-Delta12,14-prostaglandin J2, and selective DP1 and DP2 receptor agonists on pulmonary infiltration of eosinophils in Brown Norway rats. *J Pharmacol Exp Ther* 313:64-69.
77. Monneret, G., C. Cossette, S. Gravel, J. Rokach, and W. S. Powell. 2003. 15R-methyl-prostaglandin D2 is a potent and selective CRTH2/DP2 receptor agonist in human eosinophils. *J Pharmacol Exp Ther* 304:349-355.
78. Boehme, S. A., K. Franz-Bacon, E. P. Chen, R. Sasik, L. J. Sprague, T. W. Ly, G. Hardiman, and K. B. Bacon. 2009. A small molecule CRTH2 antagonist inhibits FITC-induced allergic cutaneous inflammation. *Int Immunol* 21:81-93.
79. Oiwa, M., T. Satoh, M. Watanabe, H. Niwa, H. Hirai, M. Nakamura, and H. Yokozeki. 2008. CRTH2-dependent, STAT6-independent induction of cedar pollen dermatitis. *Clin Exp Allergy* 38:1357-1366.
80. Chevalier, E., J. Stock, T. Fisher, M. Dupont, M. Fric, H. Fargeau, M. Leport, S. Soler, S. Fabien, M. P. Pruniaux, M. Fink, C. P. Bertrand, J. McNeish, and B. Li. 2005. Cutting edge: chemoattractant receptor-homologous molecule expressed on Th2 cells plays a restricting role on IL-5 production and eosinophil recruitment. *J Immunol* 175:2056-2060.
81. Noguchi, J., E. Kuroda, and U. Yamashita. 2005. Strain difference of murine bone marrow-derived mast cell functions. *J Leukoc Biol* 78:605-611.
82. Kuroda, E., and U. Yamashita. 2003. Mechanisms of enhanced macrophage-mediated prostaglandin E2 production and its suppressive role in Th1 activation in Th2-dominant BALB/c mice. *J Immunol* 170:757-764.
83. Abe, H., T. Takeshita, K. Nagata, T. Arita, Y. Endo, T. Fujita, H. Takayama, M. Kubo, and K. Sugamura. 1999. Molecular cloning, chromosome mapping and characterization of the mouse CRTH2 gene, a putative member of the leukocyte chemoattractant receptor family. *Gene* 227:71-77.
84. Aizawa, H., M. Shigyo, H. Nogami, T. Hirose, and N. Hara. 1996. BAY u3405, a thromboxane A2 antagonist, reduces bronchial hyperresponsiveness in asthmatics. *Chest* 109:338-342.
85. Sugimoto, H., M. Shichijo, M. Okano, and K. B. Bacon. 2005. CRTH2-specific binding characteristics of [3H]ramatroban and its effects on PGD2-, 15-deoxy-Delta12, 14-PGJ2- and indomethacin-induced agonist responses. *Eur J Pharmacol* 524:30-37.
86. Sugimoto, H., M. Shichijo, T. Iino, Y. Manabe, A. Watanabe, M. Shimazaki, F. Gantner, and K. B. Bacon. 2003. An orally bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits prostaglandin D2-induced eosinophil migration in vitro. *J Pharmacol Exp Ther* 305:347-352.

87. Uller, L., J. M. Mathiesen, L. Alenmyr, M. Korsgren, T. Ulven, T. Hogberg, G. Andersson, C. G. Persson, and E. Kostenis. 2007. Antagonism of the prostaglandin D2 receptor CRTH2 attenuates asthma pathology in mouse eosinophilic airway inflammation. *Respir Res* 8:16.
88. Gallant, M., C. Beaulieu, C. Berthelette, J. Colucci, M. A. Crackower, C. Dalton, D. Denis, Y. Ducharme, R. W. Friesen, D. Guay, F. G. Gervais, M. Hamel, R. Houle, C. M. Krawczyk, B. Kosjek, S. Lau, Y. Leblanc, E. E. Lee, J. F. Levesque, C. Mellon, C. Molinaro, W. Mullet, G. P. O'Neill, P. O'Shea, N. Sawyer, S. Sillaots, D. Simard, D. Slipetz, R. Stocco, D. Sorensen, V. L. Truong, E. Wong, J. Wu, H. Zaghdane, and Z. Wang. 2011. Discovery of MK-7246, a selective CRTH2 antagonist for the treatment of respiratory diseases. *Bioorg Med Chem Lett* 21:288-293.
89. Gervais, F. G., N. Sawyer, R. Stocco, M. Hamel, C. Krawczyk, S. Sillaots, D. Denis, E. Wong, Z. Wang, M. Gallant, W. M. Abraham, D. Slipetz, M. A. Crackower, and G. P. O'Neill. 2011. Pharmacological characterization of MK-7246, a potent and selective CRTH2 (chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells) antagonist. *Mol Pharmacol* 79:69-76.
90. Stock, N., D. Volkots, K. Stebbins, A. Broadhead, B. Stearns, J. Roppe, T. Parr, C. Baccei, G. Bain, C. Chapman, L. Correa, J. Darlington, C. King, C. Lee, D. S. Lorrain, P. Prodanovich, A. Santini, J. F. Evans, J. H. Hutchinson, and P. Prasit. 2011. Sodium [2'-[(cyclopropanecarbonyl-ethyl-amino)-methyl]-4'-(6-ethoxy-pyridin-3-yl) -6-methoxy-biphenyl-3-yl]-acetate (AM432): a potent, selective prostaglandin D2 receptor antagonist. *Bioorg Med Chem Lett* 21:1036-1040.
91. Royer, J. F., P. Schratl, J. J. Carrillo, R. Jupp, J. Barker, C. Weyman-Jones, R. Beri, C. Sargent, J. A. Schmidt, D. Lang-Loidolt, and A. Heinemann. 2008. A novel antagonist of prostaglandin D2 blocks the locomotion of eosinophils and basophils. *Eur J Clin Invest* 38:663-671.
92. Norman, P. 2010. DP(2) receptor antagonists in development. *Expert Opin Investig Drugs* 19:947-961.
93. Vaissiere, T., C. Sawan, and Z. Herceg. 2008. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutat Res* 659:40-48.
94. Jaenisch, R., and A. Bird. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 33 Suppl:245-254.
95. Newell-Price, J., A. J. Clark, and P. King. 2000. DNA methylation and silencing of gene expression. *Trends Endocrinol Metab* 11:142-148.
96. Bruniquel, D., and R. H. Schwartz. 2003. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat Immunol* 4:235-240.
97. Lee, D. U., S. Agarwal, and A. Rao. 2002. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* 16:649-660.
98. Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* 16:6-21.
99. Schubeler, D., M. C. Lorincz, D. M. Cimbora, A. Telling, Y. Q. Feng, E. E. Bouhassira, and M. Groudine. 2000. Genomic targeting of methylated DNA: influence of methylation on transcription, replication, chromatin structure, and histone acetylation. *Mol Cell Biol* 20:9103-9112.

100. Bonasio, R., S. Tu, and D. Reinberg. 2010. Molecular signals of epigenetic states. *Science* 330:612-616.
101. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9:765-775.
102. Takemoto, N., Y. Kamogawa, H. Jun Lee, H. Kurata, K. I. Arai, A. O'Garra, N. Arai, and S. Miyatake. 2000. Cutting edge: chromatin remodeling at the IL-4/IL-13 intergenic regulatory region for Th2-specific cytokine gene cluster. *J Immunol* 165:6687-6691.
103. Kaneko, T., H. Hosokawa, M. Yamashita, C. R. Wang, A. Hasegawa, M. Y. Kimura, M. Kitajiama, F. Kimura, M. Miyazaki, and T. Nakayama. 2007. Chromatin remodeling at the Th2 cytokine gene loci in human type 2 helper T cells. *Mol Immunol* 44:2249-2256.
104. Yamashita, M., M. Ukai-Tadenuma, T. Miyamoto, K. Sugaya, H. Hosokawa, A. Hasegawa, M. Kimura, M. Taniguchi, J. DeGregori, and T. Nakayama. 2004. Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. *J Biol Chem* 279:26983-26990.
105. Fields, P. E., S. T. Kim, and R. A. Flavell. 2002. Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol* 169:647-650.
106. Avni, O., D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher, and A. Rao. 2002. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol* 3:643-651.
107. Pai, S. Y., M. L. Truitt, and I. C. Ho. 2004. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci U S A* 101:1993-1998.
108. Lee, H. J., N. Takemoto, H. Kurata, Y. Kamogawa, S. Miyatake, A. O'Garra, and N. Arai. 2000. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J Exp Med* 192:105-115.
109. Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, Jr., L. Guo, and W. E. Paul. 2004. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol* 5:1157-1165.
110. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596.
111. Zhu, J., L. Guo, C. J. Watson, J. Hu-Li, and W. E. Paul. 2001. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* 166:7276-7281.
112. Kurata, H., H. J. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 11:677-688.
113. Scheinman, E. J., and O. Avni. 2009. Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor. *J Biol Chem* 284:3037-3048.
114. Maneechotesuwan, K., Y. Xin, K. Ito, E. Jazrawi, K. Y. Lee, O. S. Usmani, P. J. Barnes, and I. M. Adcock. 2007. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J Immunol* 178:2491-2498.
115. Skapenko, A., J. Leipe, U. Niesner, K. Devriendt, R. Beetz, A. Radbruch, J. R. Kalden, P. E. Lipsky, and H. Schulze-Koops. 2004. GATA-3 in human T cell helper type 2 development. *J Exp Med* 199:423-428.

116. Cousins, D. J., T. H. Lee, and D. Z. Staynov. 2002. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. *J Immunol* 169:2498-2506.
117. Sundrud, M. S., S. M. Grill, D. Ni, K. Nagata, S. S. Alkan, A. Subramaniam, and D. Unutmaz. 2003. Genetic reprogramming of primary human T cells reveals functional plasticity in Th cell differentiation. *J Immunol* 171:3542-3549.
118. Lyakh, L., P. Ghosh, and N. R. Rice. 1997. Expression of NFAT-family proteins in normal human T cells. *Mol Cell Biol* 17:2475-2484.
119. Zhou, B., R. Q. Cron, B. Wu, A. Genin, Z. Wang, S. Liu, P. Robson, and H. S. Baldwin. 2002. Regulation of the murine Nfatc1 gene by NFATc2. *J Biol Chem* 277:10704-10711.
120. Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree. 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* 369:497-502.
121. Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 5:472-484.
122. Macian, F., C. Garcia-Rodriguez, and A. Rao. 2000. Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *EMBO J* 19:4783-4795.
123. Cron, R. Q., S. J. Bort, Y. Wang, M. W. Brunvand, and D. B. Lewis. 1999. T cell priming enhances IL-4 gene expression by increasing nuclear factor of activated T cells. *J Immunol* 162:860-870.
124. Agarwal, S., O. Avni, and A. Rao. 2000. Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. *Immunity* 12:643-652.
125. Yoshida, H., H. Nishina, H. Takimoto, L. E. Marengere, A. C. Wakeham, D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, P. S. Ohashi, J. M. Penninger, G. R. Crabtree, and T. W. Mak. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity* 8:115-124.
126. Ranger, A. M., M. R. Hodge, E. M. Gravallese, M. Oukka, L. Davidson, F. W. Alt, F. C. de la Brousse, T. Hoey, M. Grusby, and L. H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. *Immunity* 8:125-134.
127. Ansel, K. M., I. Djuretic, B. Tanasa, and A. Rao. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol* 24:607-656.
128. Hermann-Kleiter, N., and G. Baier. 2010. NFAT pulls the strings during CD4+ T helper cell effector functions. *Blood* 115:2989-2997.
129. Kiani, A., J. P. Viola, A. H. Lichtman, and A. Rao. 1997. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity* 7:849-860.
130. Hodge, M. R., A. M. Ranger, F. Charles de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity* 4:397-405.
131. Xanthoudakis, S., J. P. Viola, K. T. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, D. C. Luk, T. Curran, and A. Rao. 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science* 272:892-895.
132. Rengarajan, J., B. Tang, and L. H. Glimcher. 2002. NFATc2 and NFATc3 regulate T(H)2 differentiation and modulate TCR-responsiveness of naive T(H)cells. *Nat Immunol* 3:48-54.

133. Ranger, A. M., M. Oukka, J. Rengarajan, and L. H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* 9:627-635.
134. Kim-Saijo, M., E. M. Janssen, and K. Sugie. 2008. CD4 cell-secreted, posttranslationally modified cytokine GIF suppresses Th2 responses by inhibiting the initiation of IL-4 production. *Proc Natl Acad Sci U S A* 105:19402-19407.
135. Quapp, R., N. Madsen, and L. Cameron. 2007. Characterization of the promoter of human CRTh2, a prostaglandin D2 receptor. *Biochem Biophys Res Commun* 363:948-953.
136. Messi, M., I. Giacchetto, K. Nagata, A. Lanzavecchia, G. Natoli, and F. Sallusto. 2003. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat Immunol* 4:78-86.
137. Gramaglia, I., A. D. Weinberg, M. Lemon, and M. Croft. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 161:6510-6517.
138. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14:205-215.
139. Lavenu-Bombled, C., C. D. Trainor, I. Makeh, P. H. Romeo, and I. Max-Audit. 2002. Interleukin-13 gene expression is regulated by GATA-3 in T cells: role of a critical association of a GATA and two GATG motifs. *J Biol Chem* 277:18313-18321.
140. Bates, D. L., Y. Chen, G. Kim, L. Guo, and L. Chen. 2008. Crystal structures of multiple GATA zinc fingers bound to DNA reveal new insights into DNA recognition and self-association by GATA. *J Mol Biol* 381:1292-1306.
141. Tan, A. H., S. Y. Goh, S. C. Wong, and K. P. Lam. 2008. T helper cell-specific regulation of inducible costimulator expression via distinct mechanisms mediated by T-bet and GATA-3. *J Biol Chem* 283:128-136.
142. Yellaturu, C. R., S. K. Ghosh, R. K. Rao, L. K. Jennings, A. Hassid, and G. N. Rao. 2002. A potential role for nuclear factor of activated T-cells in receptor tyrosine kinase and G-protein-coupled receptor agonist-induced cell proliferation. *Biochem J* 368:183-190.
143. Ahlfors, H., A. Limaye, L. L. Elo, S. Tuomela, M. Burute, K. V. Gottimukkala, D. Notani, O. Rasool, S. Galande, and R. Lahesmaa. 2010. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. *Blood* 116:1443-1453.
144. Yao, X., Y. Yang, H. Y. He, M. Wang, K. S. Yin, and M. Huang. 2010. GATA3 siRNA inhibits the binding of NFAT1 to interleukin-13 promoter in human T cells. *Chin Med J (Engl)* 123:739-744.
145. Strempel, J. M., R. Grenningloh, I. C. Ho, and D. Vercelli. 2010. Phylogenetic and functional analysis identifies Ets-1 as a novel regulator of the Th2 cytokine gene locus. *J Immunol* 184:1309-1316.
146. Amsen, D., J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515-526.
147. Constant, S. L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 15:297-322.
148. Brogdon, J. L., D. Leitenberg, and K. Bottomly. 2002. The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive CD4+ T cells. *J Immunol* 168:3825-3832.

149. Song, J., S. Salek-Ardakani, P. R. Rogers, M. Cheng, L. Van Parijs, and M. Croft. 2004. The costimulation-regulated duration of PKB activation controls T cell longevity. *Nat Immunol* 5:150-158.
150. Song, J., T. So, M. Cheng, X. Tang, and M. Croft. 2005. Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. *Immunity* 22:621-631.
151. So, T., J. Song, K. Sugie, A. Altman, and M. Croft. 2006. Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment. *Proc Natl Acad Sci U S A* 103:3740-3745.
152. Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science* 284:770-776.
153. Maillard, I., S. H. Adler, and W. S. Pear. 2003. Notch and the immune system. *Immunity* 19:781-791.
154. Liotta, F., F. Frosali, V. Querci, A. Mantei, L. Fili, L. Maggi, B. Mazzinghi, R. Angeli, E. Ronconi, V. Santarlasci, T. Biagioli, L. Lasagni, C. Ballerini, P. Parronchi, A. Scheffold, L. Cosmi, E. Maggi, S. Romagnani, and F. Annunziato. 2008. Human immature myeloid dendritic cells trigger a TH2-polarizing program via Jagged-1/Notch interaction. *J Allergy Clin Immunol* 121:1000-1005 e1008.