University of Alberta

The Effect of Interleukin 25 on Human Th2 Lymphocytes

by

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Abstract

CRTh2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) is a marker for Th2 cells and activation through CRTh2 stimulates expression of cytokines that are important for allergic responses such as IL-4, IL-5 and IL-13. We have observed $CRTh2^{+}$ T cells express the IL-25 receptor (IL-25R).

IL-25 is produced by the epithelium in response to allergens, parasites, and viruses. We hypothesized IL-25 would modulate acquisition of the Th2 phenotype. The effect of IL-25 on Th2 differentiation was investigated by culturing naive human CD4 T cells in the presence or absence of IL-25. IL-25 alone induced expression of IL-4, GATA3 and CRTh2. Head to head comparison with the canonical Th2 cytokine, IL-4, showed that IL-25 was equally effective in inducing IL-4 expression. These findings suggest IL-25 initiates Th2 differentiation and amplifies production of Th2 cytokines. Consequently, IL-25 inducing pathogens such as RSV may play a role in initiating and exacerbating allergic diseases.

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List of Abbreviations

Ag	Antigen
APC	Antigen presenting cell
ASM	Airway smooth muscle
CD	Cluster of differentiation
CIKS	Connection to IκB Kinase and Stress-activated protein kinases
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRTh2	Chemoattractant homologous-receptor expressed on Th2 cells
CSR	Class switch recombination
Ct	Cycle threshold
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
Fc	Constant fragment
FcεRI	Fc Epsilon receptor I
FEV_1	Forced expiratory volume in 1 second

GFI1	Growth factor independent 1
GFP	Green fluorescent protein
ICAM1	Intercellular adhesion molecule 1
IFN	Interferon
ILC	Innate lymphoid cell
ILC2	Type 2 innate lymphoid cell
LCR	Locus control region
LPS	Lipopolysaccharide
Ig	Immunoglobulin
iTreg	Induced T regulatory cell
MBP	Major basic protein
MHC	Major histocompatibility complex
NCID	Notch intracellular domain
NFAT	Nuclear factor of activated T cells
NHBE	Normal human bronchial epithelial cells
NK	Natural killer
nTreg	Natural T regulatory cell

PBMC	Peripheral mononuclear cell
RAG	Recombination activating gene
RSV	Respiratory syncytial virus
RT	Room temperature
SCF	Stem cell factor
STAT	Signal transducers and activators of transcription
T1H	Type 1 hypersensitivity
TCR	T cell receptor
T_{CM}	T central memory
T_{EM}	T effector memory
TGF	Transforming growth factor
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor

TSLP	Thymic stromal lymphopoietin
TLR	Toll like receptor
PRR	Pattern recognition receptor
PGD_2	Prostaglandin D_2
VCAM-1	Vascular cell adhesion protein 1
VLA-1	Very late antigen 1

Chapter I: Introduction

1.1 INNATE IMMUNE SYSTEM

1.1.1 Innate immunity mounts initial responses

The innate immune system provides immediate protection from foreign substances and does not require previous pathogen encounter. For example, when a pathogen enters the airways, the epithelial cells act as a mechanical barrier, preventing unrestricted pathogen entry. In addition, pattern recognition receptors (PRR) are present that recognize common structures expressed by pathogens without prior recombination of receptor genes and initiate a general response (reviewed in [1]). The toll like receptors (TLR), a group of ten related PRR, are able to respond to many different foreign substances. More specifically, activation through TLR4, by lipopolysaccharide (LPS) from Gram negative bacteria, has been shown to induce production of inflammatory cytokines, such as TNFα, IL-1β, IL-6, IL-8 and IFNγ (reviewed in [2]). Additionally, antimicrobial proteins, such as defensins, are present in tissue and act to specifically compromise the integrity of microbial membranes [3]. Depending on which PRR is activated, a unique set of mediators are expressed which neutralizes the pathogen. This rapid response comes at the cost of specificity and memory to previously encountered pathogens.

Despite the effectiveness of the initial response, there are instances where these pathogens still pass through the epithelium and enter the tissue. When this occurs,

innate cells such as neutrophils and macrophages, expressing PRR, are able to phagocytose and neutralize pathogen. In addition, natural killer (NK) cells, through the utilization of perforin/granzyme or Fas/FasL pathways [4], are present that terminate pathogen infected cells based on the balance of signals from stimulatory and inhibitory surface receptors expressed by target cells (reviewed in [5]). While T cells are traditionally involved in adaptive immunity, innate T cells such as $CD8^+$ cells that have effector functions prior to antigen (Ag) experience suggest an innate role for certain T cell subsets. (reviewed in [6]). Another subset of innate cells, known as innate lymphoid cells (ILC), are likely present and recognize foreign pathogens and respond with production of cytokines that link to, and may initiate, adaptive immune responses (reviewed in [7]). There are a number of subsets of ILCs, however, three characteristics connect ILCs: lymphoid structure, lack of recombination activating gene (RAG) dependent receptors and lack of lineage markers (reviewed in [7]) and so are classified based on cytokine release (reviewed in [7]).

Pattern recognition, physical barrier and resident cell responses generate the first line of defense against pathogens. However, epithelial cells also initiate a response from the adaptive immune system, which generates a more specific and a memory response to the pathogen.

1.1.2 Epithelial cells are first responders

Epithelial cells line the external (skin) and internal surfaces (airways and gut mucosa) of the human body, functionally separating the external and internal

environments (reviewed in [8]). While the primary role is to act as a physical barrier to the environment, airway epithelial cells serve many other functions necessary for homeostasis, including the facilitation of mucous transport within airways and secretion of various proteins such as chemokines/cytokines and growth factors(reviewed in [8]). In addition to a homeostatic role, the epithelium also provides immunological protection.

Epithelial cells provide a mechanical barrier against entry of foreign substances. For example, tight junctions connecting epithelial cells act to prevent antigens from crossing the epithelium. Break down of the tight junctions between epithelial cells by allergens, such as Der p 1 (a component of house dust mite fecal matter), can lead to increased permeability [9]. Despite these barriers, epithelial cells still mediate antigen transport across the epithelium. For example, specialized epithelial cells have been shown to transport antigens across the epithelium. Specifically, utilizing green fluorescent protein (GFP) labeled antigen and immunohistochemistry, M cells from murine nasal epithelium were shown to effectively transport antigens to the luminal surface [10].

1.2 ADAPTIVE IMMUNE SYSTEM

1.2.1 Adaptive immunity tailors specific immune responses

In contrast to the innate immune response, the adaptive immune system functionally remembers a previously encountered antigen from sources such as virus, parasite, and/or bacteria. Interaction between innate and adaptive cells provide signals that initiate cellular changes such as recombination of receptor

genes on T and B cells that allow specific recognition of antigen upon subsequent exposure. Antigen presenting cells (APC), such as dendritic cells (DC), macrophages and B cells, capture foreign substances and process the antigen resulting in antigen presentation on the cell surface. T helper cells are activated and differentiated by APCs and then provide help to B cells to generate humoral responses by producing antigen specific antibodies. Once the initial response is complete, both memory B and T cells remain as surveillance to monitor for future exposures to the antigen. This memory to the antigen allows a more rapid response upon reencounter. However effective the primary response is, it remains less robust than secondary responses (reviewed in [1]).

1.2.2 Hematopoietic cells of the adaptive immune system

The majority of cell types that comprise the immune system originate in the bone marrow and are produced from hematopoietic stem cells. These stem cells divide and lead to two semipotent stem cells: common lymphoid (CLP) and common myeloid progenitors (CMP). CMP cells can develop into progenitors known as myeloblast that further differentiate into cells carrying vesicles (granules) and that have polymorphic (multisphered) nuclei. These cells are known as granulocytes and include eosinophils, basophils and neutrophils. Other cells originating from the CMP include mast cells, monocytes and dendritic cells (DC) that serve a range of immunoregulatory roles. In contrast, CLP cells give rise to lymphocytes such as T cells, B cells and NK cells. These cells are non-granulocytic and have a mononuclear (simple spherical nucleus) configuration [11-12] (Fig. 1).

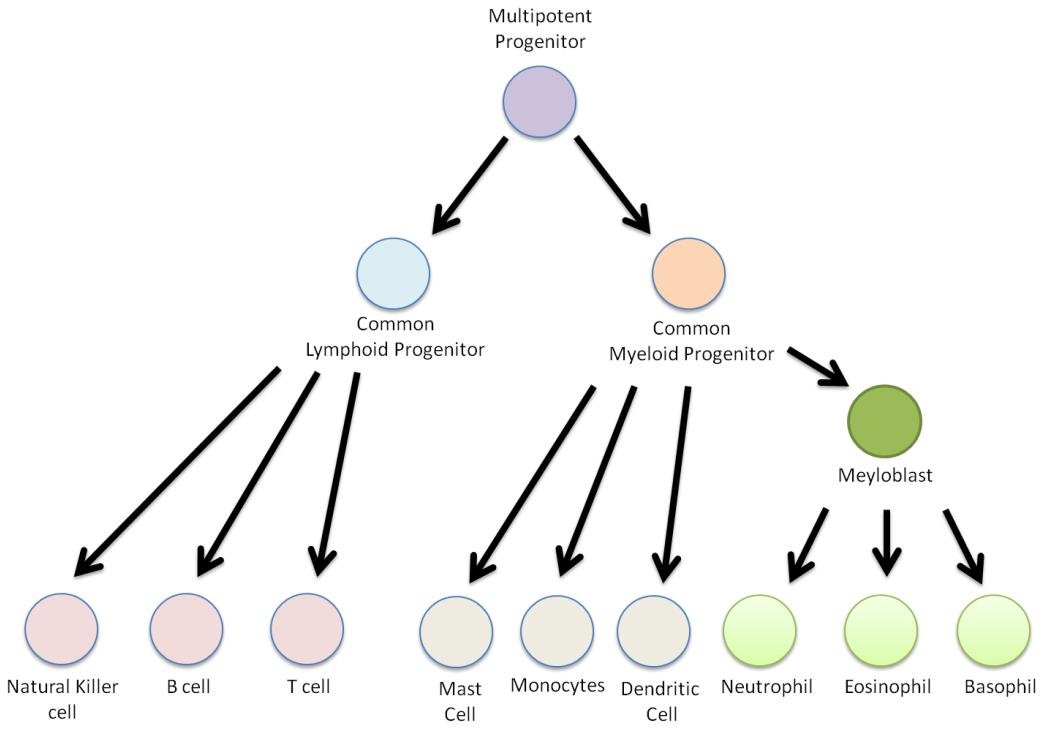

Figure 1 Hematopoietic development of immune cells. A multipotent progenitor gives rise to the common lymphoid (CLP) and common myeloid progenitors (CMP). NK cells, B cells and T cells arise from the CLP. Neutrophils, eosinophils, basophils, monocytes, mast cells and dendritic cells arise from the CMP. Figure adapted from [11].

1.2.3 Dendritic cells are professional antigen presenting cells (APC)

Dendritic cells (DC) develop from CMP (Fig. 1) and in the context of lung immunology, reside in lung submucosa. Once DC acquire antigen, they migrate to lymph nodes [13] where they present antigen to T cells and facilitate their differentiation. DC can obtain antigen in two ways: active antigen transportation through epithelial M cells [10] and through dendrites of dendritic cells that extend into the luminal side of the epithelium and acquire antigen [14]. This dendritic uptake was observed through using confocal microscopy for transepithelial dendritic cell sampling [14].

Dendritic cells uptake antigen by receptor-mediated endocytosis, pinocytosis and phagocytosis. For example, introduction of monoclonal antibody against mannose receptors was shown to reduce antigen uptake by dendritic cells suggesting receptor-mediated endocytosis as a mechanism of uptake [15]. It has been subsequently shown that other receptors, such as Fc receptors and c-type lectins, can mediate endocytosis [16].

Once taken up, antigen processing inside the dendritic cells occurs. Internalized antigen must be first digested into smaller peptides that can then be presented on the cell surface by the major histocompatibility complex class II (MHCII) or MHCI, depending on the antigen (reviewed in [17]). All nucleated cells express MHCI. In contrast, MHCII expression is reserved for APCs, including dendritic cells. Antigens present inside the cell, such as virus, are presented by MHCI. Conversely, extracellular antigens, such as bacteria, are internalized, processed

then presented by MHCII (reviewed in [17]). Following antigen acquisition, the MHCII-antigen complex is then transported and expressed on the surface of the dendritic cell [18]. Other changes, necessary for an effective response, include the increased expression of co-stimulatory molecules, such as CD80 and CD86 (Fig. 2), which can control the adaptive response that ensues [19]. The dendritic cell plays a major role in directing the subsequent adaptive response (Fig. 3A-B).

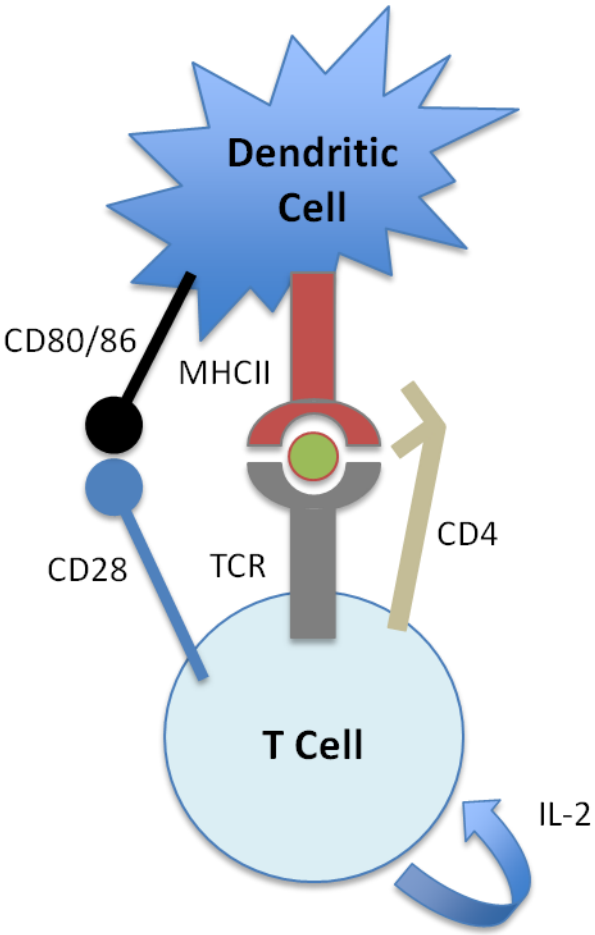

Figure 2: Dendritic cell – T cell interaction. Antigen is presented on MHCII by dendritic cells. T cells are stimulated by antigen through the T cell receptor (TCR). CD4 interacts with MHCII to aid the interaction. Costimulation is provided to the T cell. For example, CD80/86 on dendritic cells interacts with CD28 on T cells. Finally, IL-2 is produced, acting in an autocrine fashion to induce proliferation.

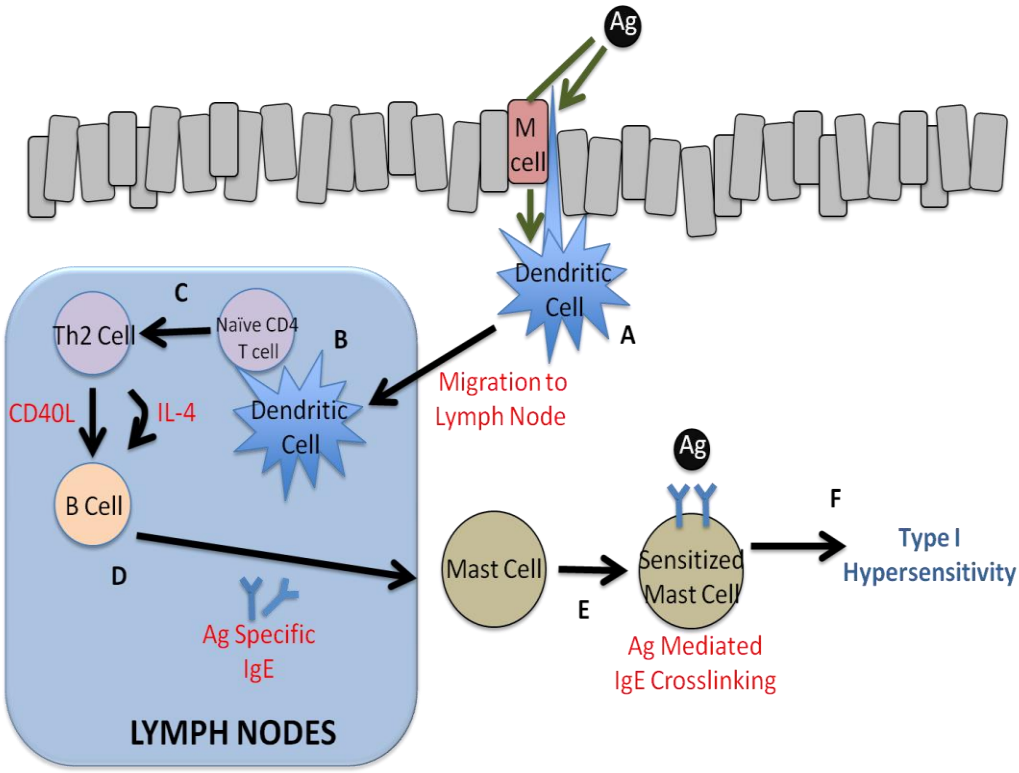

Figure 3: Development of Th2 responses. (A) Dendritic cells acquire antigen either by direct sampling between epithelial cells or by antigen transport through specialized epithelial M cells. (B) Dendritic cells migrate to lymph nodes and present processed antigen on MHCII to naïve CD4 T cells. (C) In a Th2 polarizing environment, IL-4 and CD40L expressing Th2 cells are differentiated. (D) IL-4 in conjunction with CD40L induce isotype switching and subsequent expression of allergen specific IgE. (E) Allergen specific IgE binds to Fc receptors on mast cells functionally sensitizing mast cells to subsequent allergen exposure. Following allergen reexposure, mast cells degranulate releasing mediators into the tissue and initiating type I hypersensitivity (F).

1.2.4 T lymphocytes direct the adaptive response

T cells develop from CLP cells that migrate from the bone marrow to the thymus. Some of these T cells undergo a number of processes to become mature T cells. These processes include expression of the T cell receptor (TCR) as well as the signals that result in single positive CD4 or CD8 T cells [20]. The TCR is made up of three CD3 subunits (CD3γ, CD3ε and CD3δ) that facilitate signaling along with the alpha (α) and beta (β) subunits that recognize antigen presented in the context of MHC. These molecules dictate T cell functions, as CD4 interacts with MHCII [21] (Fig. 3) and 'help' B cells to produce immunoglobulin responses. CD8 assists the TCR in recognizing and interacting with MHCI leading to $CD8^+$ T cells providing cytotoxicity through perforin/granzyme or Fas/FasL pathways [22-23] (Fig. 2C-D). The majority of T cells leave the thymus as naïve cells and will differentiate further in the secondary lymph nodes. However, a subset of CD4 T cells receive differentiation signals in the thymus and exit with regulatory functions. These cells are called natural T regulatory (nTreg) [24].

T cell responses progress in three phases. First is the expansion phase where naïve CD4 T cells encounter antigen presented by DC (Fig. 3) and from there proliferate and differentiate into a specific subset depending on the cytokine mileu. The second phase is the contraction phase where antigen load is reduced and T cell numbers fall. The third phase is development of memory where a small proportion of effector T cells transition into a memory T cell phenotype (reviewed in [25]). Unlike naïve T cells, memory T cells respond rapidly to antigen. Two

distinct classes of memory T cells exist: central (T_{CM}) versus effector (T_{EM}) memory [26]. CCR7, a chemokine receptor that facilitates entry to secondary lymphoid organs, has been shown to phenotypically mark the two memory populations. $CCR7^{+}$ cells coexpressing CD62L, a lymphoid organ homing lectin, are T_{CM} cells that circulate through the blood and monitor secondary lymphoid organs. While being unable to respond as rapidly as $CCR7^{-}$ T_{EM}, upon antigen reencounter they can transition into T_{EM} phenotype marked by a loss of CCR7 expression [27]. In contrast, T_{EM} are $CCR5^{+}CD62L^{lo}CCR7^{-}$ cells allowing localization to nonlymphoid sites and are able to produce cytokines quickly after TCR stimulation [28-29].

In order for an appropriate response to develop in response to a foreign invader, a range of T helper subsets that express unique cytokine profiles are differentiated in lymph nodes. Naïve CD4 T cells when responding to antigen presented by dendritic cells will differentiate into a CD4 T cell subset dictated by the cytokine environment. The inducing cytokines will mediate expression of a transcription factor that drives subsequent helper T cell differentiation [30]. The major peripherally differentiated subtypes are T helper 1 (Th1), T helper 2 (Th2) [31], T helper 17 (Th17) and induced T regulatory cells (iTreg), although other subsets have also been reported (Th9, Th22) (reviewed in [32]).

Th1 cells protect against intracellular pathogens, are characterized by IFNγ expression and develop in response to the transcription factor T-bet [32]. T-bet expression is induced by both the IFNγ/STAT1 [33] and IL-12/STAT4 [34]

signaling pathways. These effects on T-bet are believed to be independent since experiments showed that $STAT4^{-/-}$ T cells treated with IL-12 still express IFNγ and T-bet [35]. However, $STAT4^{+/+}$ T cells treated with IL-12 showed increased proliferation of $IFN\gamma^{+}$ cells through CSFE staining [36], indicating that IL-12 also promotes survival and proliferation of developing Th1 cells (Fig. 4).

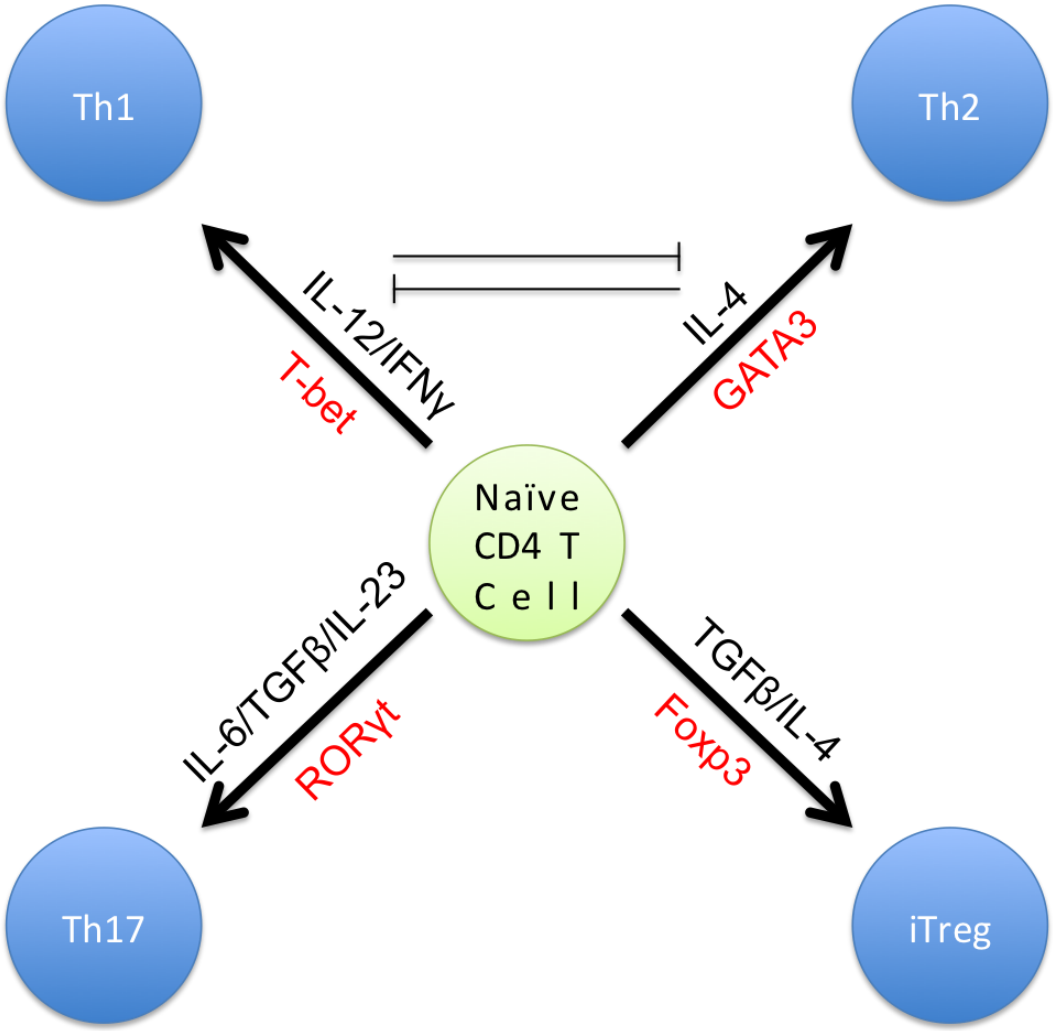

Figure 4: Cytokines and transcription factors drive T cell subset differentiation. The cytokine environment present during antigen presentation to naïve CD4 T cells will determine the T helper subset differentiation. Cytokines, working in conjunction with TCR signals, induce expression of subset specific transcription factors leading to commitment. Furthermore, Th1 and Th2 differentiating cytokines and transcription factors are in competition.

Th2 cells produce IL-4, IL-5, and IL-13 and develop in response to extracellular antigen. GATA3 has been shown to be the Th2 master transcription factor [37] and IL-4, functioning through STAT6, induces expression of GATA3 [38]. Furthermore, lymph node cells from $STAT6^{-/-}$ mice do not proliferate in response to IL-4 and their T cells fail to differentiate into $IL\text{-}4^{+}$ Th2 cells [39]. Also of importance, IL-2, through STAT5, has been shown to be necessary for Th2 differentiation [40]. However, Th2 cells differentiate in competition with Th1. The well accepted model of Th1-Th2 differentiation poses IL-4 and GATA3 in competition with IFNγ and T-bet, inducing reciprocal inhibition on the opposing pathway (Fig. 4).

Th17 cells express IL-17A, IL-17F and IL-21 [41] and develop in response to the transcription factor RORγt (reviewed in [42]). However, Th17 differentiation occurs in response to bacterial challenge [43] as well as hypoxia [44]. IL-6 and IL-23, signaling through STAT3, have been shown to induce RORγt and drive Th17 development [45-46]. TGF-β has also been implicated in Th17 differentiation [47] (Fig 3).

In addition to nTregs differentiated in the thymus, it has been observed that Tregs can also be induced in the periphery. These are called induced T regulatory cells (iTregs). Like nTregs, iTregs function in the periphery to provide tolerance and have been shown to develop in response to Foxp3, the master Treg transcription factor [48] in response to TGF-β [49] (Fig. 3).

Since both Th17 and iTreg differentiation can be induced by TGF-β, a biological mechanism must exist that directs the proper subset. It has been observed that Foxp3 may inhibit RORγt activity [50]. Therefore, the present model is that the balance of transcription factors induced by the cytokine environment will regulate T cell phenotype. Therefore, if TGF-β expression prevails then iTreg differentiation occurs; conversely, if IL-6/IL-23 expression prevails then Th17 differentiation occurs [51].

While the cytokine environment has been shown to play a role in T cell differentiation, dendritic cell expression of specific costimulatory molecules have also been shown to influence T cell differentiation (reviewed in [52]). For example, Notch ligands, expressed by dendritic cells, have been implicated in directing naïve CD4 T cells towards either Th1 or Th2 [53]. Engagement of the notch receptor by one of its ligands induces release of the intracellular domain (ICD) and subsequent ICD binding to and transformation of RBPJκ from a transcriptional repressor to an activator. Ligands of the Notch receptor belong to two families: Delta and Jagged. It has been shown that both ligands can be expressed on dendritic cells and that expression of Jagged1 leads to differentiation towards the Th2 phenotype while Delta leads towards the Th1 phenotype (reviewed in [54]).

1.2.5 B lymphocytes produce immunoglobulins

Although a major role for B cells is immunoglobulin (Ig) production [55], B cells can also function as APCs [56]. Antibodies are immunoglobulins that specifically

recognize and bind antigen. Immunoglobulins with a diverse range of antigen specificities are first generated in the bone marrow where V, D and J genes rearrange to form the antigen binding domain. Mature naive B cells leave the bone marrow expressing IgM and IgD, enter the blood and then migrate to the spleen and lymph nodes (reviewed in [57]). Here B cells undergo further antigen specificity diversification including somatic hypermutation involving mutations in the antigen recognition of immunoglobulins to increase antigen specificity (reviewed in [58]).

B cell immunoglobulins next diversify in a process known as class switch recombination (CSR), where the immunoglobulin subtype expressed by the B cell is determined and enable functional specialization. Naïve B cells, expressing IgM and IgD, switch isotypes and allow specific protection: IgG1 and IgG3 against viruses; IgG2 against bacteria; IgG4 and IgE against parasites or allergen; and IgA1 and IgA2 for mucosal immunity (reviewed in [59]).

1.3 TH2 IMMUNITY

Type 2 immunity is characterized by the development of Th2 cells and the cytokines that they produce. Th2 cytokines are important in protection against both murine [60] and human [61] parasite infestation; mice deficient in various Th2 cytokines display delayed parasite expulsion [62]. However, Th2 effects are not always positive. Th2 cytokines have been implicated in early onset of rheumatoid arthritis though appear to be less abundant in the chronic disease [63]. There is also abundant evidence that Th2 cytokines mediate allergic reactions [64]

and allergic diseases such as allergic rhinitis, atopic dermatitis and asthma pathogenesis [65].

1.3.1 Type 1 hypersensitivity: sensitization

Th2 immunity is important in mediating type I hypersensitivity (TIH). The first step in TIH is sensitization to antigen, after which it is called an allergen. During dendritic cell-Th2-B cell interaction, isotype switching to IgE occurs in a process requiring two signals. First, soluble cytokine directs germline transcription of immunoglobulins. Second, T cell help through cell-cell contact that allows genetic recombination of heavy chain genes. This is called class switch recombination (CSR) (reviewed in [66]). In the case of class switching to IgE, germline transcription is induced by IL-4 and IL-13 [67-68] and physical contact, which is mediated by CD40-CD40L interaction [69-70]. Traditionally, CSR occurs in the lymph nodes. However, observation of class switch loops in the nasal [71] and bronchial mucosa [72] suggest CSR can also occur at the site of allergen exposure. Antigen specific IgE binds to receptors on mast cells thus 'sensitizing' the mast cell to allergen [73].

After an individual is sensitized, subsequent exposure to allergen results in a biphasic response. The early phase starts within an hour of allergen exposure and is followed by the late phase, which commences several hours after exposure and peaks after 24 hours [74].

1.3.2 Type 1 hypersensitvity: early phase

Mast cells originate from hematopoietic precursors in the bone marrow (Fig. 1), circulate in the blood where they mature and migrate into tissues such as intestine and the lungs where the mast cells affect their function [75]. In the early phase response Ag-IgE crosslinking of FcRε on mast cells mediates degranulation and can rapidly release preformed mediators, such as biogenic amines (histamine and serotonin), enzymes that induce pain and tissue damage (trypsin and chymase), and proinflammatory cytokines (TNF). Following preformed mediator release, mast cells release mediators requiring enzymatic processing, such as arachodonic acid metabolites (PGD_2 and leukotriene C_4) and mediators requiring de novo synthesis, such as various cytokines (IL-4, IL-5 and IL-13) (reviewed in [76]).

The early phase of an allergic rhinitis response is physically manifested in rhinorrhea, pruritus, and congestion [77] and is mediated in part by histamine and PGD_2 derived from mast cells. Both these mediators have been shown to cause vasodilation and increase vascular permeability (reviewed in [73, 78]). Together these changes promote a favorable environment for cell infiltration.

PGD2 signals through two unique G protein coupled receptors, D prostanoid 1 (DP1) and CRTh2 (or DP2). DP1, through G alpha stimulatory protein ($G_{\alpha s}$), increases cyclic AMP. In contrast, CRTh2, through G alpha inhibitory protein ($G_{\alpha i}$), leads to an influx of calcium (reviewed in [78]). Physiologically, $PGD_{2,}$ acting through DP_1 in the lungs and has been shown to induce vasodilatation and bronchodilation (reviewed in [78]). It has been suggested the general action

through CRTh2 is pro-inflammatory while through DP_1 is anti-inflammatory. Therefore, the relative expression of CRTh2 to DP_1 may dictate the overall effect of PGD_2 on inflammation (reviewed in [79]). With respect to the early phase of allergic reactions, mast cells play a critical role in initiating recruitment of inflammatory cells. Through release of various lipid mediators, such as PGD_2, mast cells have been suggested to mediate human $CRTh2^+$ cell chemotaxis [80].

1.3.3 Type 1 hypersensitivity: late phase

The late phase of T1H is characterized by the accumulation of inflammatory cells, which are mediated by chemokines. Specifically, the $CRTh2^+$ Th2 cells introduced during the early phase by PGD_2 stimulation begin to produce IL-4 and IL-13. These cytokines create favorable conditions for further cell recruitment by acting on endothelium and inducing production of chemokines.

In order for further cellular accumulation to occur, the induction of transendothelial migration is necessary. In short, inflammatory cells in the blood are induced to slowly roll over the endothelium near sites of inflammation by 'sticky' interaction between endothelium selectins and their respective mucins. This slow rolling is followed by directional movement induced by chemoattractants and aided by additional adhesion interactions, allowing movement into tissue (reviewed in [81]). IL-4 induces endothelial expression of vascular adhesion molecule-1 (VCAM-1) [82], which mediates adhesion of very late antigen-1 (VLA-1) expressing cells, such as lymphocytes, eosinophils and basophils [83]. While IL-4 aids in transendothelial migration of subsequent

inflammatory cells, it has been suggested that IL-13 induces chemokine secretion from dendritic and epithelial cells, which then induce movement of eosinophils (CCL11 and CCL24) and Th2 cells (CCL17 and CCL22) [84]. Additionally, IL-13 has been implicated in the production of CCL17 [85] and CCL5 [86] (Fig. 4).

Following accumulation, the $CRTh2^{+}$ Th2 populations produce Th2 cytokines IL-4, IL-5 and IL-13. IL-4 and IL-13 leads to the production of IgE [87]. This suggests a positive feedback loop on mast cell sensitization, while IL-5 remains an eosinophil differentiation factor [88]. Eosinophils also play a role during late phase response through production of pro-inflammatory mediators, including major basic protein (MBP). MBP has been shown to constrict the airways [89] and this effect appears to be mediated through action on the epithelium [90]. Thus, following early PGD_2 mediated accumulation of Th2 cells, IL-4 and IL-13 mediate the late phase response characterized by further accumulation of inflammatory cells, such as Th2 cells, eosinophils and B cells. The prominent role of Th2 cells in allergic responses has driven research to understand the drivers of Th2 differentiation (Fig. 5).

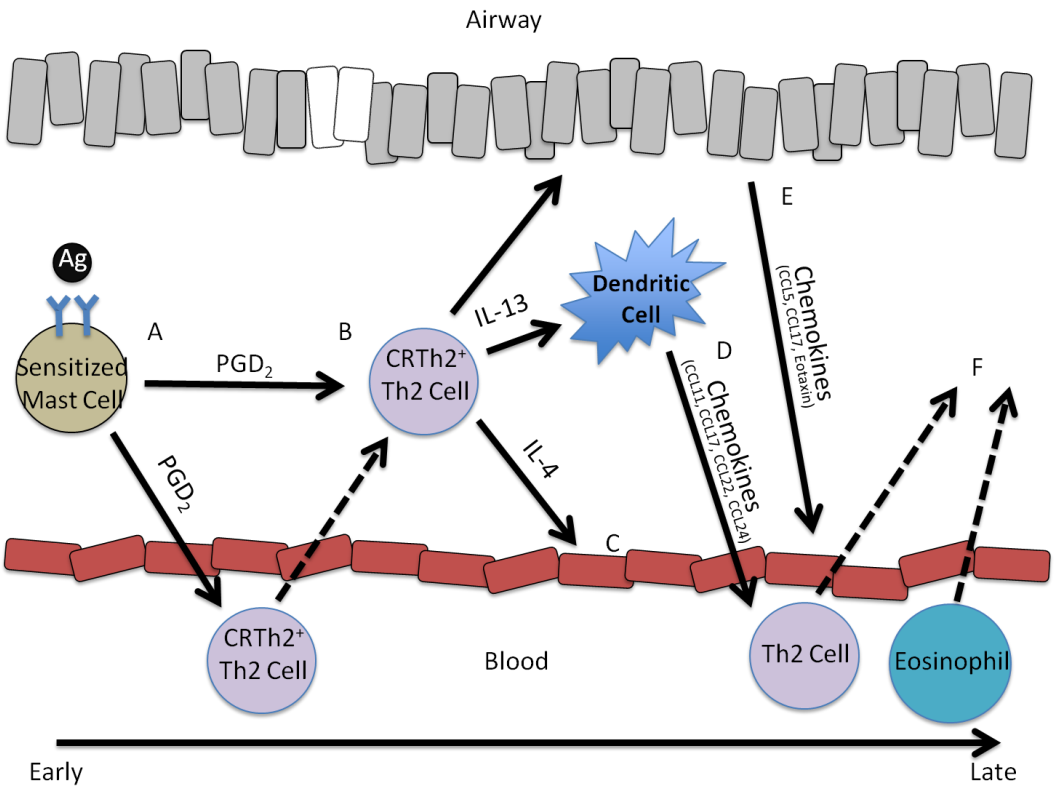

Figure 5: Type 1 hypersensitivity. (A) Allergen-IgE crosslinking on mast cells induces release of PGD_2 causing accumulation of $CRTh2^+$ Th2 cells. (B) Stimulated $CRTh2^+$ Th2 cells release IL-4 and IL-13. CRTh2 Th2 cells can be stimulated by PGD_2 in the tissue. (C) IL-4 acts on the endothelium to increase expression of VCAM-1. (D) IL-13 induces dendritic cells (E) and epithelial cells to release chemokines. (F) Finally, movement of Th2 cells and eosinophils marks further cellular accumulation. Figure adapted from [91].

1.4 TH2 DIFFERENTIATION

Naïve CD4 T cells exhibit low levels of IL-4, IL-13 and IFN-γ mRNA [92], which suggests that they are poised for transcribing either Th1 or Th2 cytokines. Therefore mechanisms must exist that induce upregulation of subset cytokines and drive T cell phenotype commitment. To date, three main mechanisms for driving Th2 differentiation have been described: the cytokine environment; T cell receptor (TCR) engagement; and certain costimulatory molecules from dendritic cells, which all work together to drive Th2 differentiation. While each of these actions is able to induce Th2 factors, such as the Th2 master transcription factor GATA3 [37], it is the combined effect of all three considered to drive differentiation (Fig. 4).

1.4.1 Th2 cytokine environment: IL-2 and IL-4

It is thought that Th2 differentiation unfolds at multiple layers of regulation involving IL-4/STAT6, IL-2/STAT5 signaling and GATA3 (Fig. 3). For example, mouse CD4 T cells, in the presence of IL-4 and IL-2, differentiate into IL-4 and IL-5 producing effector cells [93]. Initially the role of STAT6 in Th2 differentiation was considered to be primarily as a signaling molecule to carry out IL-4 induced transcription [39], leading to upregulation of GATA3 (reviewed in [38]). However, it is now understood that IL-4 not only induces transcription but also epigenetic changes in a STAT6 dependent manner. Epigenetic changes include chromatin remodeling, that alters DNA accessibility and therefore the availability of genes for transcription (reviewed in [94]). To study remodeling,

DNA is treated with DNase I and cleavage indicates areas of DNA that have been made physically accessible by chromatin remodeling. To further understand the role of STAT6 in acquiring IL-4 expression, DNase I hypersensitivity assays were used to examine the IL-4 locus. Unstimulated CD4 T cells from $STAT6^{-/-}$ mice, differentiated in Th2 conditions, had impaired DNA accessibility and showed similar IL-4 locus accessibility to naïve CD4 T cells [95]. Moreover, Rad50, a DNA repair protein, contains a locus control region (LCR) in its gene that has been shown to induce IL-4 promoter activity [96]. Analysis of the Rad50 gene demonstrated that STAT6 is able to induce remodeling more efficiently than GATA3 [97]. Collectively, STAT6 acts at two levels to control Th2 differentiation. First, IL-4, signaling through STAT6, induces chromatin remodeling at the Rad50 and IL-4 locus, leading to a state permissivefor transcription. Second, STAT6 increases GATA3 expression which can bind to the Th2 locus leading to transcription of Th2 cytokines.

Neutralization of IL-2 leads to weakened Th2 differentiation, suggesting IL-2/STAT5 signaling is also important for Th2 differentiation [98]. CD4 T cells expressing a constitutively active STAT5 are able to produce IL-4 in the presence of neutralizing antibody to IL-2. Furthermore, a constitutively active STAT5 and GATA3 mutant showed additive increases in $IL\text{-}4^{+}$ cells compared to single mutants [40]. Since GATA3 is important for Th2 differentiation [37] and IL-2 blockage also leads to reduced Th2 differentiation, it is likely that STAT5 and STAT6/GATA3 are independently important for Th2 differentiation.

Furthermore, other mediators, such as nuclear factor of activated T cells (NFATc1) and cMaf, have been shown to increase IL-4 [99-101].

1.4.2 T cell receptor stimulation induces GATA3

In the absence of T cell receptor engagement T cell differentiation does not occur [102], demonstrating the requirement for TCR engagement during differentiation. While IL-4 functioning through STAT6 induces GATA3, stimulation through the TCR can also induce GATA3 [103]. Specifically, ChIP analysis shows that NFAT binds to the GATA3 promoter in differentiated Th2 as well as naïve CD4 T cells. Further, inhibiting NFAT reduces GATA3 mRNA [104]. NFAT is expressed early after T cell stimulation [105], suggesting GATA3 can be induced early after antigen encounter.

1.4.3 Dendritic cell costimulation induces GATA3

In addition to the above regulators of GATA3, dendritic cell presence may also be necessary for optimal GATA3 expression. The Notch ligand family of costimulatory molecules, expressed by dendritic cells, has been shown to direct T cell subset differentiation. Antigen dose has been suggested to control Th1/Th2 differentiation by regulating costimulatory molecule expression, such as CD40, on dendritic cells [106]. Further, expression of the Notch ligand Jagged1, by low antigen dose, leads to Th2 differentiation while expression of Delta, by high antigen dose, leads to Th1 [54, 107]. Knockout studies have also shown that the

absence of Notch receptors on naive CD4 T cells results in reduced GATA3 expression. Furthermore, activation of the Notch signaling pathway increases GATA3 expression [108]. These results suggest that dendritic cells, supplying the Notch ligand, Jagged1, can influence GATA3 expression.

1.4.4 GATA3 in T lymphoctye development

While GATA3 is considered a Th2 transcription factor, it is present in Th1 cells and is important in T cell development. Mice lacking GATA3 expression in embryonic stages do not survive into birth [109]. Murine embryonic stem cells containing a GATA3 knockout were injected into $RAG^{-/-}$ mice and thymic analysis show absence of $CD4^{+}$ and $CD8^{+}$ cells [109-110], showing GATA3 is necessary during CD4 and CD8 T cell development. In contrast, B cells displayed normal development [110]; suggesting GATA3 may not be required to B cell development. A genome wide murine T cell ChIP-Seq, where DNA segments that bind GATA3 are pulled down then amplified, revealed sites positively and negatively regulated by GATA3 in Th17, iTreg, Th1 and Th2 cells [111]. Collectively, these data show GATA3 is essential for early T cell development and plays a role during subset functioning.

1.4.5 GATA3 in Th2 cells

GATA3 confers two functions to Th2 biology: differentiation and cytokine production [112]. Studies in murine models demonstrate GATA3 is more abundant in Th2 compared to Th1 cells [37]. When naïve CD4 T cells were

differentiated in Th2 conditions GATA3 expression was maintained, however, in Th1 conditions GATA3 is down regulated [37]. While GATA3 knockout murine CD4 T cells have reduced Th2 differentiation, as marked by acquisition of IL-4 expression [113], ectopic expression of GATA3 in B cells is able to induce IL-4 expression [37]. Using conditional GATA3 knockout mice it was observed that knocking GATA3 out early in Th2 differentiation greatly reduces acquisition of IL-4 expression. In contrast, deletion of GATA3 in established Th2 cells did not affect IL-4 but proved to be essential for IL-5 and IL-13 expression [114].

Most studies on GATA3 have been conducted in murine models where it is a strong Th2 marker. However, in human CD4 T cells, the mere presence of GATA3 is not a robust indicator of the Th2 phenotype. To this point, when human naïve CD4 T cells were differentiated in either Th1 or Th2 conditions and total populations were analyzed, no difference in GATA3 levels were observed. However, T-bet was increased in Th1 conditions [115]. Absence of pronounced GATA3 upregulation in humans may be explained by both the downregulation of T-bet in human Th2 cells and the proposed effect of T-bet on GATA3. T-bet has been shown to be phosphorylated and subsequently to physically interact with GATA3, preventing GATA3 from interacting with target DNA [116]. Genome wide analysis demonstrated a role for GATA3 both in positively regulating Th2 differentiation and negatively controlling Th1 and Th17 genes [117]. Thus, the lack of activated and phosphorylated T-bet in Th2 conditions may prevent T-bet

inhibition of GATA3 and allow GATA3 to drive Th2 differentiation through activation of Th2 genes and simultaneous inhibition of other Th lineages.

In efforts to improve our understanding of Th2 cells, , studies comparing Th1 and Th2 subsets revealed selective expression of the chemotactic homologous receptor expressed on Th2 cells (CRTh2) on the surface of human Th2, but not Th1 cells [118]. Subsequent work went on to show that expression of CRTh2 is controlled by GATA3. Specifically, ectopic expression of GATA3 in naïve CD4 T cells demonstrated an increase in CRTh2 surface expression suggesting a direct role for GATA3 in controlling CRTh2 expression [119]. Using a luciferase reporter controlled by the CRTh2 promoter, it was also shown that GATA3 overexpression is able to induce CRTh2 transcription, while NFAT2 and STAT6 were not [120-121]. Furthermore, T-bet overexpression in Th2 cells results in reduced surface CRTh2 [122]. These data show Th2 conditions upregulate CRTh2 while Th1 downregulate CRTh2

CRTh2 has been suggested to be the best marker of Th2 cells. When peripheral human $CD4^+$ T cells were stimulated in vitro, the $CRTh2^+$ cells expressed Th2 cytokines [123]. Further, when human $CRTh2^+$ cells are selected from the circulating memory T cell population ($CD4^+CD45RO^+$), higher GATA3 and IL-4 are observed compared to the $CRTh2^-$ population [115]. This suggests that in vivo differentiated Th2 cells, marked by CRTh2 expression, have upregulated GATA3 and IL-4 compared to other T helper lineages. Early work on CRTh2 demonstrated that isolation of $CRTh2^+$ cells from PBMC cultures results in

enriched IL-4, IL-5 and IL-13 but lower IFNγ expression [124]. In support of this, gating $CD3^+CD4^+$ cells from PBMC cultures also shows that CRTh2 is a better indicator of Th2 cytokine production than CCR3 or CCR4 [125]. Furthermore, these $CRTh2^+$ cells were characterized as a $CD4^+CD45RO^+CD62L^+$ memory T cells [124].

Allergen tetramers are recently developed molecules used to specifically recognize T cells by the MHC-Ag complex.. Recent research, using these tetramers that recognize birch pollen specific T cells, demonstrates that both allergics and non-allergics have allergen specific T cells. However, the vast majority of tetramer positive cells from allergics are $CRTh2^+$ and express IL-4, IL-5 and IL-13. In contrast, the tetramer positive T cells from non-allergics express little CRTh2 or Th2 cytokines [126]. These data suggest that the allergen specific Th2 cells are $CRTh2^+$. In addition to being the best marker for Th2 cells, CRTh2 may mark a non-plastic, polarized CD4 T cell subset. Specifically, Th1 clones placed into Th2 conditions results in some cells producing IL-4 or both IL-4 and IFNγ. In contrast, $CRTh2^+$ isolated Th2 clones placed into Th1 conditions showed no Th1 cytokine production as marked by IFNγ expression [127].

1.4.6 Instructive versus selective Th1/Th2 differentiation

Traditionally T cell differentiation was thought to occur instructively with the cytokine environment acting on T cells to induce T subset specific transcription factors thus driving subset commitment. However, recently the concept of selective differentiaiton, where subset cytokines act to selectively promote survival of the respective subset has emerged [128].

For instance, naïve murine CD4 T cells retrovirally infected with a virus that overexpresses STAT6 demonstrated cell expansion. While naïve CD4 T cells overexpressing GATA3 did not. This may be due to induction of growth factor independent 1 (GFI1) that acts as a transcriptional repressor and inhibits expression of proapoptotic genes thus promoting cell survival [129]. IL-4 has been shown to induce GFI1 in a STAT6 dependent manner. It was shown that Niave CD4 T cells infected with a retrovirus that overexpresses GFI1 have increased expansion [128]. This would suggest that while GATA3 is required for Th2 cytokine expression, stochastically IL-4 expressing cells could be selected through upregulation of the growth factor GFI1. There exists a similar mechanism in Th1 differentiation. Provision of intracellular adhesion molecule 1 (ICAM1), expressed by dendritic cells, has been shown to lead to reduced production of Th2 cytokines [130]. In contrast, stimulation of naive human CD4 T cells with CD3/CD28 and an increasing dose of ICAM1 show a dose dependent increase in number of IFN-γ producing cells. Simultaneously, number of IL-4 producing cells is reduced in a dose dependent manner [131]. Collectively, these data show that

factors independent of the traditional transcription factor inducing cytokines can select for T cell subset commitment. However, it is most likely that a combination of selective and instructive differentiation programs prevail (Fig. 5).

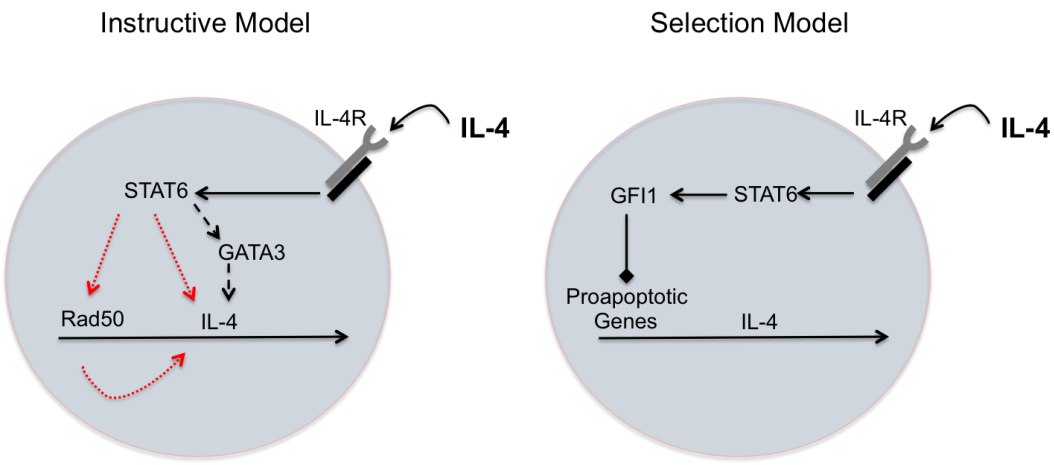

Figure 6: Instructive versus selective Th2 differentiation models. The instructive model proposes that IL-4 remodels both the Rad50 and IL-4 locus in a STAT6 dependent manner. The remodeling allows IL-4 instructed GATA3 transcription and development of IL-4 expressing Th2 cells. In contrast, the selection model suggests IL-4 induces growth factor independent 1 (GFI1) expression in a STAT6 dependent manner. GFI1 acts as a transcriptional repressor and prevents expression of proapoptotic genes thus selecting IL-4 expressing Th2 cells and promoting their expansion. These models are not mutually exclusive and it is likely both functions in maintenance of Th2 immunity. Inhibition(——◆) ,Remodeling (·····►),Transcription(– – ►)

1.5 EPITHELIAL MEDIATED TH2 DIFFERENTIATION

Recent work investigating links between the innate and adaptive immune system show epithelial cells in response to virus, parasites, or antigen can release immunomodulatory signals such as the cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) that have been implicated in the initiation of Th2 differentiation [132]. Stimulation through TLR2, TLR3 and TLR4 have been shown to increase soluble IL-25 from epithelial cells [133]. Similarly, epithelial cells infected with respiratory syncytial virus (RSV) a virus associated with the development of asthma and allergy [134], have been observed to produce IL-25 [135-136]. Collectively, these findings suggest that innate triggers induce IL-25. . Furthermore, ILC2 cells [137] have recently been shown to produce IL-4, IL-5 and IL-13 in response to IL-25 [138]. Therefore, the production of IL-4 by IL-25, following innate activation, could link innate responses with the initiation of Th2 immunity.

1.5.1 Interleukin 25

IL-25 is a cytokine from the IL-17 family and was initially called IL-17E. It was identified in 2001 using the basic local alignment search tool (BLAST) with IL-17A sequence to search for other IL-17 family members [139]. In this initial paper identifying IL-25, it was also shown that intranasal challenge in a murine model resulted in production of Th2 cytokines [139]. Subsequent work revealed IL-25

has been associated with parasite clearance [142], remission in multiple sclerosis [143-144] and selective toxicity to breast cancer cells [145]. However, IL-25 also plays detrimental roles such as mediating allergic asthma [146-148]. While IL-25 appears to play a broad role in both human physiology and patholgy, relatively little is known regarding the mechanisms by which it mediates these functions. In human asthma, IL-25 is increased in bronchial biopsies compared to controls [149] and the expression is increased after 24 hours following allergen challenge [150]. Normal human bronchial epithelial (NHBE) cell cultures have shown resting human epithelial cells store IL-25 but also upregulate IL-25 mRNA as well as release IL-25 upon allergen exposure or protease treatment [133]. However, IL-25 expression is not limited to the epithelium. Monocyte derived dendritic cells (MoDCs) have been shown to express IL-25 both unstimulated and upon LPS treatment. Interestingly, stimulation with LPS but not TSLP increased the number of IL-25 expressing cells by 10% [151]. In order to fully understand the effects of IL-25, physiological location and cellular expression of the protein in human subjects must be understood. Immunohistochemistry demonstrates that IL-25 is present in human bronchial epithelium and human submucosa. Specifically, eosinophils, mast cells and endothelial cells express IL-25. In contrast, macrophages, neutrophils and T cells do not express IL-25 [150]. Additionally, a subset of ILCs, type 2 innate lymphoid cells (ILC2), defined by their ability to produce Th2 cytokines, are more abundant in asthmatics compared

to normal controls [152] and IL-25 induced increase of Th2 cytokines from these cells [138].

Studies also suggest IL-25 has a negative impact on asthma. Increased IL-25 expressing cells in asthmatic airways correlates with reduced forced expiratory volume in 1 second (FEV_1), suggesting a negative impact for IL-25 on disease pathology [153]. Intratracheal administration of IL-25 to mice induces AHR as well as an increase in production of Th2 cytokines in lung cultures compared to PBS treated mice [146]. In an ovalbumin model of asthma,, mice treated intraperitoneally with a neutralizing antibody to IL-25 demonstrated reduced AHR, antigen specific IgE and Th2 cytokines in the lung [154]. Similarly, IL-25 knockout mice showed reduced Th2 cytokine levels in the lung as well as allergen specific IgE. These murine models collectively suggest a systemic role for IL-25 in inducing sensitization [155].

Asthma is characterized by airway remodeling, including an increase in airway smooth muscle (ASM) mass; either by hyperplasia or hypertrophy [156]. ASM cells, innervated by cholinergic receptors, control the diameter of the airways [157]. Therefore, an increased presence of ASM in the airways can lead to greater constriction following allergen challenge. Further, angiogenesis and mucous hypersecretion are also associated with airway remodeling [158]. IL-25 has also been suggested to be involved in these processes. In a house dust mite (HDM) induced murine asthma model neutralization of IL-25 was shown to reduce collagen density and smooth muscle thickness, hallmarks of airway remodeling

[159]. Similarly, endothelial cell lines showed increased vascular endothelial growth factor (VEGF) and increase in endothelial branch points in the presence of IL-25 compared to the absence [153]. These data demonstrate the ability of IL-25 to cause structural changes in the lung.

In a human Th2 co-culture with dendritic cells, IL-25 was shown to induce Th2 cytokines. It was further shown that IL-25 induced the expression of GATA-3 as well as c-MAF, two Th2 transcription factors [149]. However, these experiments were carried out in a co-culture environment and the direct effect of IL-25 on human Th2 cells remains unclear. It has been shown that IL-25R is inducible in human dendritic cells [160] and stimulation by IL-25 through this receptor leads to up-regulation of Jagged1 on dendritic cells in mice [161]. As mentioned previously, Jagged1 has been implicated in dendritic cell mediated T cell commitment towards the Th2 phenotype [53]. Taken together, these studies indicate that IL-25 may play a role in Th2 differentiation.

1.6.2 Interleukin 25 receptor (IL-25R)

IL-25 is a member of the IL-17 cytokine family and is alternately known as IL-17E. The IL-17 receptor (IL-17R) family contains five members: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE [162]. IL-25 signals through a heterodimer of the IL-17 receptor family: IL-17RB and IL-17RA. Microarray data shows that in vivo differentiated CRTh2 cells highly express IL-17RB [163], however, a wide array of cells express IL-17RB [149]. IL-17RB mRNA is upregulated in asthmatic lungs compared to controls [149] and 24 hours after

allergen challenge [150]. Immunohistochemistry shows eosinophils, mast cells, endothelial and T cells in the human bronchial epithelium and submucosa express IL-17RB [150]. In addition, it has been shown that MoDC can express IL-17RB [151, 161], suggesting a role for IL-25 in antigen presentation and T cell priming.

IL-25 has been shown to bind the IL-17RB subunit and induce recruitment of an adaptor molecule, CIKS (Connection to IκB Kinase and Stress-activated protein kinases) also known as Act1, to the SEFIR (similar expression to fibroblast growth factor genes and IL-17Rs) domain of IL-17RA. This Act1/SEFIR interaction was shown to be essential for IL-25 mediated airway inflammation [164]. Further studies showed involvement of TNF receptor-associated factor 6 (TRAF6) in IL-25 signaling. By using immunoprecipitation assays in mice TRAF6 was demonstrated to bind intracellularlly to IL-25R. Furthermore, TRAF6 knockout mice showed its requirement for IL-25 mediated activation of NF-κB, though, activation of ERK, JNK and p38 was independent of TRAF6 [165]. In human peripheral CD45RO T cells blocking IKB-α phosphorylation and p38 MAPK, but not Jun-c, inhibits IL-25 mediated Th2 cytokine expression [166]. While IL-25 has been shown to increase murine JunB and NFATc1 independent of IL-4 [167], these findings have not yet been observed in human studies (Fig. 7).

Mouse CD4 specific Act1 deletion showed CD4 T cells were delayed in IL-25 (without added IL-4) mediated Th2 differentiation. Deletion of Act1 did not however affect Th2 differentiation when IL-4 was added [168]. In another mouse

Th2 model of differentiation, IL-25 effects were shown to be dependent on IL-4/STAT6 signaling [167]. Thus IL-25 may mediate Th2 differentiation by inducing early IL-4 signaling, however, to determine this studies using unmanipulated human cells are required.

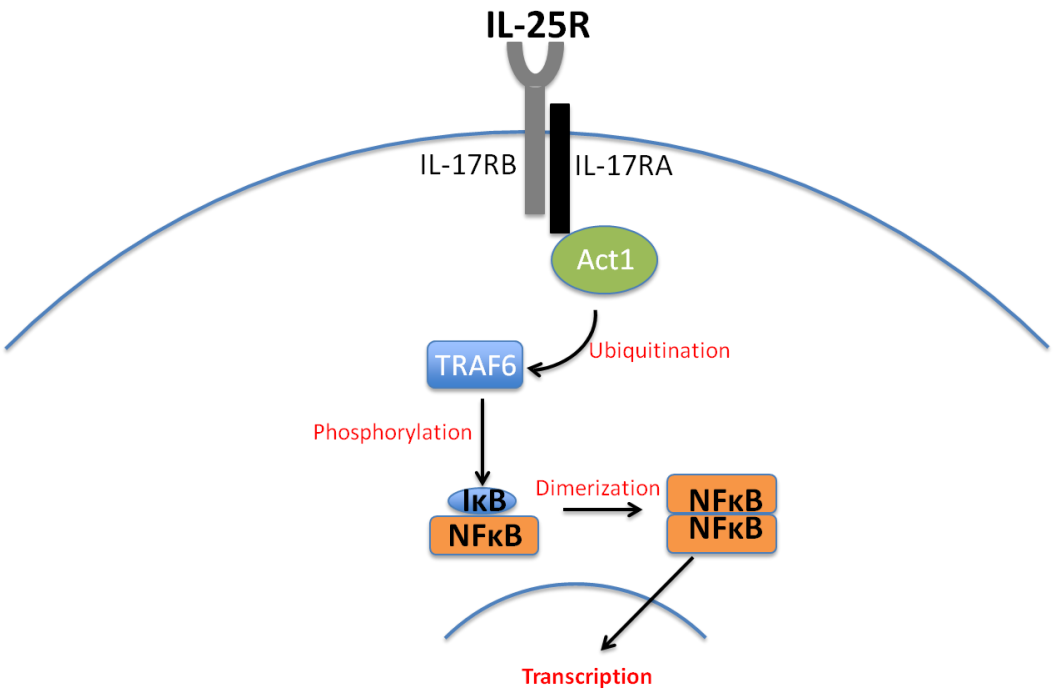

Figure 7: IL-25 interacts with the IL-17RB subunit of the IL-25R inducing SEFIR-SEFIR interaction of the IL-17RA subunit and the adaptor molecule, Act1. Act1 functions as an ubiquitin ligase activation TRAF6. TRAF6 can then go on to phosphorylate IκB releasing inhibition of NFkB following its dimerization, migration into the nucleus and activation of transcription.

1.7 RATIONALE

Th2 immunity, mediated by Th2 cells and the cytokines they produce, mediates positive roles in human physiology, such as clearing helminth infestations [60-62], but also pathology, such as the development of allergic asthma and atopic dermatitis [64-65]. While much is known regarding Th2 cell differentiation, more research is needed to better understand how cytokines such as IL-25 participate in development of Th2 cells. During T cell subset commitment, the cytokine environment is imperative in determining the subset. Th2 differentiation is driven by IL-4 signaling through STAT6 [37, 39] leading to GATA3 expression (reviewed in [38]). These conditions drive Th2 commitment and the expression of characteristic Th2 surface markers such as CRTh2 [115]. However, since IL-4 is the Th2 differentiating cytokine, the *initial* source of IL-4 has always been in question. Recently, the literature has produced a number of reports than shed some light on this longstanding conundrum. Firstly, epithelial cell activation by innate signals such as RSV has been shown to produce IL-25 [135-136] and secondly IL-25 stimulation of ILC2s has been shown to induce expression of IL-4 as well as IL-5 and IL-13 [169]. Collectively, these findings suggest that innate triggers of epilthelial-ILC2 cells, resulting in IL-25 induction of Th2 cytokines, could link the innate and adaptive immune responses. However, since in vivo differentiated $CRTh2^{+}$ Th2 cells have been shown to highly express IL-25R mRNA [163], IL-25 may also be able to circumvent the ILC2 cells and act directly on Th2 cells.

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1.8 STUDY OBJECTIVES

The over-arching hypothesis of this thesis was that IL-25 influences Th2 cells. The specific objectives and questions tested are outlined below.

1.8.1 To understand the effect of IL-25 on Th2 differentiation

We hypothesized that IL-25 can circumvent the ILC2 population and act directly on CD4 T cells, initiating *acquisition* of the Th2 phenotype.

1.8.2 To study the influence of IL-25 on Th2 effector cytokines

We hypothesized that IL-25 can act directly on differentiating Th2 cells and *amplify* expression of Th2 effector cytokines.

1.8.3 To study novel effects of IL-25 on Th2 cells

We hypothesized that IL-25 plays a broad role on Th2 cell functions such as inducing other cytokines/chemokines and mediating chemotaxis.

Chapter II: Methods

2.1 CELL CULTURE

2.1.1 Naïve CD4 T cell isolation

Venous blood (70-100ml) was collected from self reported non-allergic, non-asthmatic human donors using sodium heparin tubes. Dilute blood (1:2 parts PBS) was layered over Ficoll histopaque PLUS (GE Healthcare, Sweden) and separated by centrifugation. Layered blood was spun (30 min, room temperature (RT), 2200 RPM) with no break. Peripheral blood mononuclear cells (PBMC) were collected from the buffy coat. Typical yield was 9-12 x 10^5 PBMC/mL of blood. Naïve human CD4 T cells were isolated from PBMCs using negative selection (naive $CD4^+$ T Cell Isolation Kit II, Cat. #130-094-131, Miltenyi Biotec, Auburn, CA) that contained antibodies selecting against other cell types (CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235a). Additional CD8 microbeads were added to the cocktail to reduce CD8 contamination (Cat. #130-045-201, Miltenyi Biotec, Auburn, CA). Naïve CD4 T cell yield was between 2-3 x 10^5 cells/mL of blood.

2.1.2 Differentiation protocol

Naïve human CD4 T cells were cultured at 2 x 10^6 cells/mL in X-vivo 15 media (Lonza, Cat. #04-744Q) without phenol red and supplemented with 1X penicillin, Streptomycin, Gentamicin (Cat. # 10378-016, Gibco) and 10% HyClone fetal bovine serum (Cat. #SH30070.03, Thermo Scientific). Freshly isolated CD4 T

cells cultured in **Th2 conditions** (rhIL-4, αIFNγ and αIL-12) and cycled between a three day **stimulation stage** (plate bound activating antibodies to CD3 and CD28 with IL-2) followed by a four day **proliferation stage** (IL-2). Culture reagents were purchased as follows: CD3 (1μg/ml, Cat. #MAB100, R&D Systems), CD28 (1ug/ml, Cat. #MAB342, R&D Systems), rhIL-2 (2.5ng/ml, Cat. #202-IL-010, R&D Systems), rhIL-4(20-50ng/ml, Cat. #204-IL, R&D Systems), αIL-12 (1μg/ml, Cat. #16-7129, eBiosciences) and αIFNγ (1μg/ml, Cat. #AF-285-NA, R&D Systems). rhIL-25 (Cat. #1258-IL-025, R&D Systems, MN, USA) was used for experiments where the effect of IL-25 was assessed. Cells were harvested and plates were washed with 0.2 mM PBS-EDTA. Following harvest, cells were pelleted (10 min, 4°C, 300G) and counted. Supernatants and cell pellets were kept for analysis. Cells were recultured at $2x10^6$ cells/ml. Experiments were performed on days when cells were alternated between stages.

2.1.3 CRTh2-isolated Th2 cell lines

To generate a CRTh2 enriched Th2 cell line, the above Th2 differentiation protocol was followed and on day fourteen, anti-CRTh2 antibody coated microbeads cells were used to positively select for CRTh2 (Cat. #130-091-274, Miltenyi Biotec, Auburn, CA). $CRTh2^+$ Th2 cells were alternated between 3 days of priming (rhIL-2 and plate bound CD3/CD28) and 4 days of proliferation (rhIL-2 alone) without Th2 differentiation conditions for up to 49 days (CRTh2 >50%). Experiments with CRTh2 cells were performed either after stimulation or after proliferation when culture media was refreshed. Cells were harvested and plates

were washed with 0.2 mM PBS-EDTA. Following harvest cells were pelleted at (10 min, 300G) and counted. Supernatents and cell pellets were kept for analysis. Cells were recultured at $2x10^6$ cells/ml.

2.2 ASSESSMENT OF PHENOTYPIC CHARACTERISTICS

2.2.1 Extracellular cell staining

Phenotypic characterization of both differentiating CD4 T cells and $CRTh2^-$ isolated Th2 cells was carried out by flow cytometry. Cells were collected after the proliferation stage. Cells were blocked (30 minutes) with either normal rat IgG (Invitrogen, Cat# 10700) or normal mouse IgG (Invitrogen, cat# 10400C) followed by incubation (30 min, 4°C) with either the isotype matched control or marker specific antibody. Finally, cells were placed into paraformaldehyde (2%)/sucrose (0.54%). Cells were stained for CD4 (Clone RPA-T4, isotype mouse IgG1 FITC, AbD serotech) and CD45RA (Clone L48, mouse IgG1κ FITC, BD Pharmagin).

Biotinylated antibodies were used to assess surface CRTh2 and IL-25R (IL-17RB). Cells were blocked (30 minutes, room temperature) with rat IgG (Invitrogen, CA, USA) then stained with primary biotin conjugated CRTh2 antibody (Clone BM16, Miltenyi biotech, CA, USA) or rat IgG2a isotype (AbD serotech, NC, USA) (30 minutes, 4°C). Incubation with streptavidin-APC (30 min, 4°C) (eBioscience, CA, USA) was used as detection. Cells were then fixed with paraformaldehyde (2%)/sucrose (0.54%). Staining for IL-25R (IL-17RB) followed the same protocol but blocking was with goat IgG then stained with

primary biotin conjugated IL-17RB (Cat. #BAF1207, R&D Systems, MN, USA) or isotype (BAF108, R&D Systems, MN, USA). Streptavidin-APC was used for detection.

Staining was read using either FACSCalibur or LSRII (Becton Dickson, ON, Canada). Results were analyzed using FlowJo (Tree Star, OR, USA). Positive signal was determined by setting the gates on the isotype control and expression obtained by antibody shift.

2.2.2 Intracellular cell staining

Intracellular staining for cytokines; IL-4, IL-5, IL-13 and IFNγ was performed after the four day proliferation phase. Cells were collected and stimulated for four hours with PMA (20ng/mL), ionomycin (1μM) and brefaldin A (10 μg/mL). While intracellular cytokine assessment received the four hour stimulation, assessment of intracellular GATA3 and IL-25R was on unstimulated cells. Cells were then fixed (10 min, on ice) with paraformaldehyde (4%) (Sigma Aldrich, On, Canada) then permeabilized (10 min, on ice) with saponin (0.4%) (Sigma Aldrich, On, Canada). For non-permeablized controls saponin was replaced with PBS. Antibodies and isotype controls were added and samples were incubated (30 min, on ice). Cells were stained with IL-4-Alexa-488 (Clone 8D4-8, isotype mouse IgG1κ) IL-5-APC (Clone TRFK5, isotype Rat IgG1), IL-13-PE (Clone JES10-5A2, isotype rat IgG1 PE), IFNγ-Alexa-647 (Clone B27, isotype mouse IgG1κ), GATA3-Alexa-488 (Clone L50-823, isotype mouse IgG1κ) and IL-17RB as described above. Cells were read and analyzed as for extracellular staining.

2.3 POLYMERASE CHAIN REACTION

2.3.1 Quanatative reverse transcription (qRT-PCR)

In order to quantify mRNA from Th2 cells, RNA was extracted using RNAeasy extraction kit (Cat. #74101, ON, Canada) and eluted with 30μL of RNase/DNase free water. RNA concentration was determined by diluting the sample in Tris-EDTA and reading in a spectrophotometer (BioRad SmartSpec 3000). Complimetary DNA (cDNA) was synthesized by incubating 1μg of RNA with oligodT (Invitrogen, CA, USA), dNTPs (Invitrogen, CA, USA) and water (5 min, 65°C). Next 1X Fs Buffer (Invitrogen, CA, USA), DTT (Invitrogen, CA, USA) and RNase out (Invitrogen, CA, USA) were added and incubated (2 min, 42°C). Finally, Superscript II (Invitrogen, CA, USA) was added followed by incubation (50 min, 42°C) then heated (15 min, 70°C). cDNA was stored (-20°C) until PCR was performed. qRT-PCR TaqMan gene expression assays for CRTh2 (Hs00173714_m1), IL-25R (IL-17RB) (Hs00218889_1), IL-4 (Hs00174122_m1), IL-5 (Hs00174200_m1), IL-13 (Hs00174379_m1) and GATA3 (Hs00231122_m1) were purchased from Applied Biosystems (Burlington, On). qRT-PCR was performed with 1μL of cDNA with 19 μL of TaqMan gene expression mastermix (Applied Biosystems, CA, USA). The following program was run: 2 minutes at 50°C then 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C (Eppendorf RealPlex 4, ON, Canada). Samples were conducted in triplicates. If standard deviation was greater than 0.5 ΔCt between triplicates the outlier was removed. Data was analyzed using the

ΔΔCycle Threshold (Ct) compared to GAPDH with a forward primer (5'- CTG AGA ACG GGA AGC TTG TCA-3') and reverse primer (5'-GCA AAT GAG CCC CAG CCT T-3'). Briefly ΔCt is determined by subtracting the Ct of the housekeeping gene from the Ct of the gene of question. The ΔCt from the control condition is then subtracted from experimental conditions to get ΔΔCt for a specific condition. This fold increase is then calculated by using the ΔΔCt as a negative exponent to the base of 2 ($2^{-\Delta\Delta Ct}$).

2.4 ELISA

Supernatants were collected from differentiating Th2 cells after both stimulation and proliferation and stored immediately (-80°C). IL-5 (Cat. #S5000B, R&D Systems) and IL-13 (Cat. #851 630 005, Diaclone) were analyzed as described by manufacturer. Briefly, antibody was coated (4°C, overnight) on to ELISA plates. Following isotype blocking (2 hours, RT) samples were loaded into wells and incubated (2 hours, RT). Enzyme-conjugated antibodies were added and quantity was assayed by color change following substrate addition. Samples were tested in duplicates. ELISA plates were read in Powerwave XS Microplate Reader (Bio-Tek, VT, USA).

2.5 DISCOVERY ASSAY

IL-25 treated CRTh2-isolated Th2 cells were sent for cytokine discovery panel using Multiplex Bead Technology (EVE Technologies, Calgary, Alberta). The multiplex assay utilized two antibodies: one for identification of the analyte and another for quantification. The identification antibody is conjugated to a bead with a unique fluorophore signature. The second antibody is a streptavidin-

phycoerythrin conjugated antibody. One laser activates the identification antibody while a second activates the quantification antibody allowing simultaneous identification and quantification of each analyte.

2.6 CHEMOTAXIS ASSAY

CRTh2-isolated Th2 cell lines were collected, counted, washed and resuspended in phenol red free, serum free X-Vivo medium (unsupplemented). Cells were exposed to Calcein AM (2μm, 30 minutes, 37°C) then washed and resuspended in phenol red free X-Vivodium supplemented with with 1X penicillin, *Streptomycin, Gentamicin* (Cat. # 10378-016, Gibco) and 10% HyClone fetal bovine serum (Cat. #SH30070.03, Thermo Scientific). CCL22 (MDC, Cat. 336-MD-025, R&D systems) or IL-25 was loaded into a ChemoTx 96 well disposable chemotaxis system with 5μm pore size (Cat. #106-5, Neuro Probe, MD, USA). The porous membrane was then overlaid and 30 000 cells in 30 μl were placed over each well. Cells were allowed to chemotax (2 hours, 37°C) then the plate was washed three times with 10 ml of fresh phosphate buffered saline (PBS, Cat. 10010-023, Life Technologies, Burlington, On) and spun (2 min, 200G). The porous membrane was discarded and cells that moved through the membrane into the lower wells were read in a FLx 800 Fluorescence Microplate Reader (Bio-Tek, VT, USA).

2.7 STATISTICS

Experiments done in only the presence or absence of IL-25 were analyzed by student T-tests (paired). Comparing IL-25 vs IL-4 in Th2 differentiation was analyzed by one-way ANOVA and assessed with Tukey comparisons. All

analyses and figures were conducted in GraphPad prism 5 (GraphPad Software, CA,USA).

2.8 OVERVIEW OF MODELS

Throughout this project various models were utilized in order to further understand the role of IL-25 in Th2 biology.

CRTh2-isolated cell lines were established by differentiating naïve human CD4 T cells in Th2 conditions followed by isolation of $CRTh2^+$ CD4 T cells on day 14. After isolation, CRTh2-isolated Th2 cells were maintained in IL-2 ± αCD3/αCD28 and studied until CRTh2 expression dropped below 50% (Fig. 8A).

To understand if IL-25 could initiate Th2 differentiation we established a **short (10 day) model** where naïve human CD4 T cells were isolated and cultured in various conditions (Fig. 8B).

To determine if IL-25 could amplify Th2 cytokine production, we also established a **long (38 day) model** of Th2 differentiation. These experiments tested whether IL-25, when added to the standard Th2 conditions, could amplify the magnitude of Th2 effector cytokine production (Fig 8C).

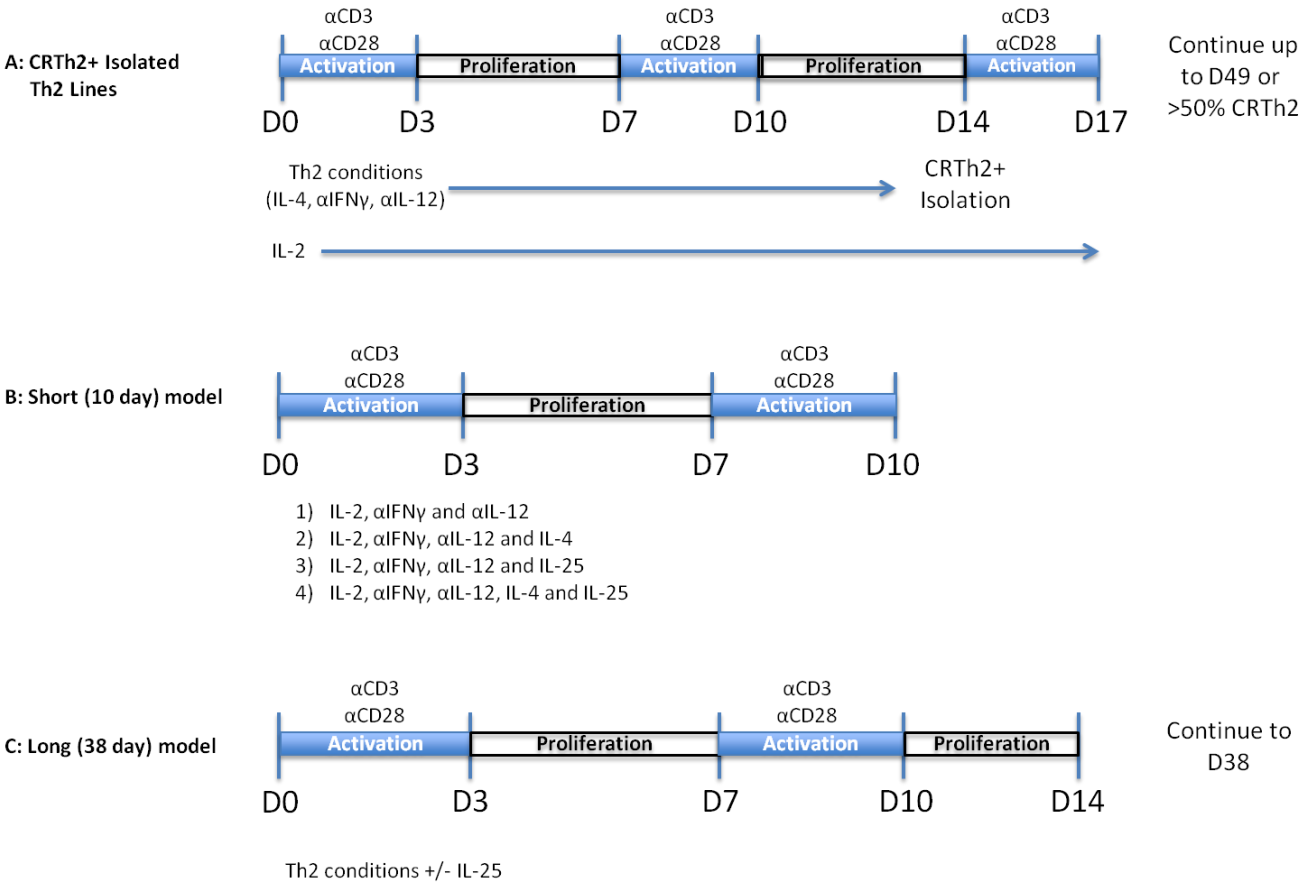

Figure 8: Schematic of models used to assess the effect of IL-25 on Th2 cells. (A) Protocol for differentiation of $CRTh2^{+}$ isolated Th2 cells from a naive CD4 T cell population. (B) Short (10 day) model of Th2 differentiation to assess the potential of IL-25 to initiate differentiation. (C) Long (38 day) model to assess whether IL-25 amplifies Th2 cytokine production.

Chapter III: Results

3.1 DEVELOPMENT AND CHARACTERIZATION OF CELL LINES

3.1.1 Freshly isolated naïve CD4 T cells

Typical yield of cells isolated from peripheral blood was 9-12 x 10^5 PBMCs/mL of blood and 2-3 x 10^5 naïve CD4 T cells/mL of blood. Figure 9 represents the cell surface phenotyping with respect to CD3 (B), CD4 (C) and CD45RA (D). Purity of the isolated population was 96.2±0.3% CD4 and 92.7±0.75 CD45RA (n=7).

3.1.2 Differentiating a Th2 cell line

The purified naïve CD4 T cells were then cultured in Th2 conditions (IL-2, IL-4, αIFNγ and αIL-12) alternating between 3 days of stimulation with plate bound antibodies to CD3 and CD28 followed by 4 days of proliferation (absence of CD3/CD28). Previous work in our lab showed that CRTh2 is highest after resting therefore we quantified CRTh2 on day 14 of differentiation (Fig. 10B), while IL-4 was measured after 4 hours of PMA/Iono stimulation (Fig. 10C). Since GATA3 induces IL-4 and CRTh2 expression, we quantified GATA3 preceding the highest expression of these markers. Therefore GATA3 expression was quantified following stimulation on day 10 (Fig. 10D).

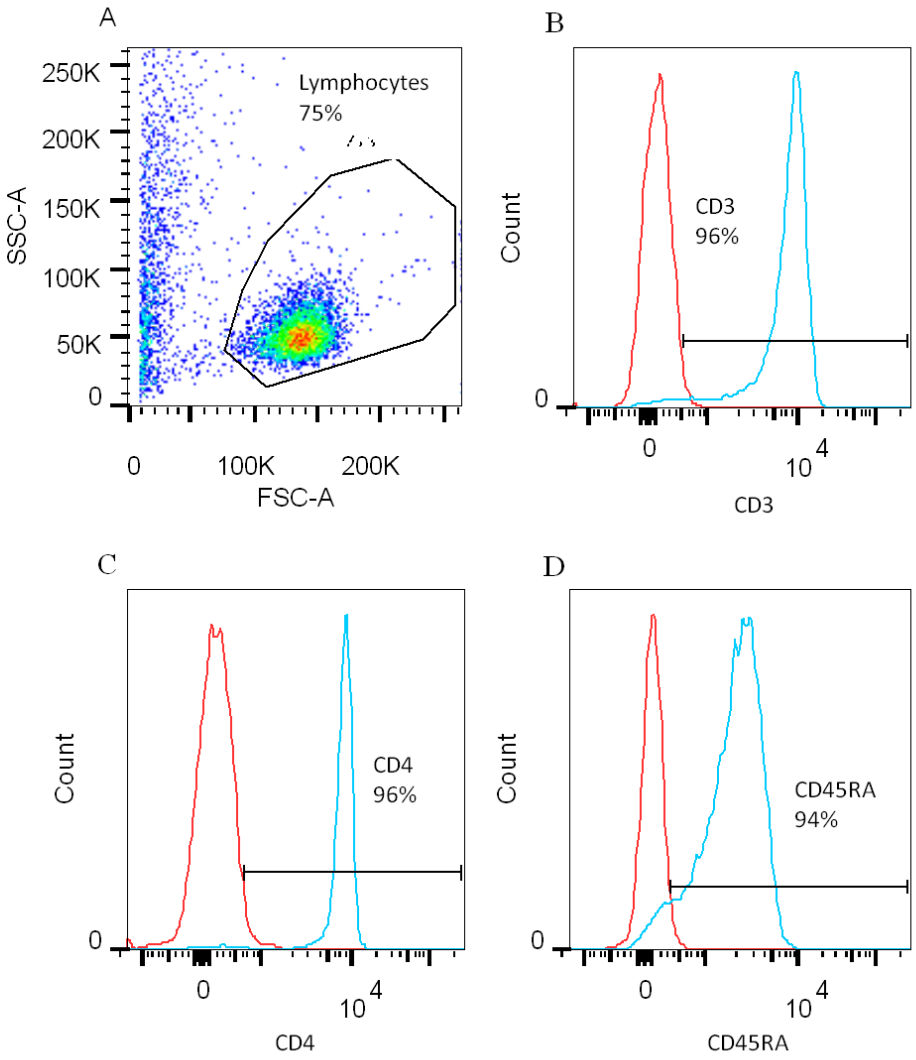

Figure 9: Surface expression of characteristic T cell markers immediately following peripheral blood naive CD4 T cell isolation. Live cells were gated (A) and representative histograms of CD3 (B), CD4 (C) and CD45RA (D) are shown. The data shown is an example of 11 independent isolations.

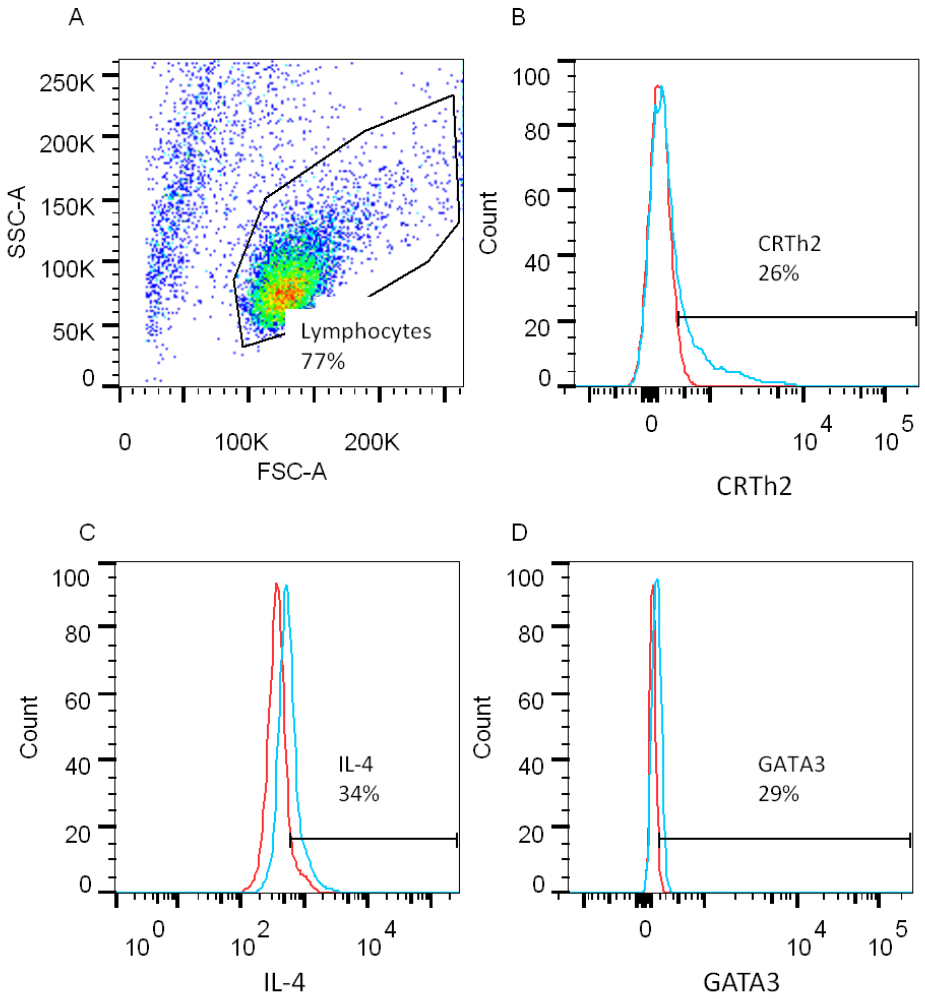

Figure 10: Markers of Th2 differentiation. Live cells were gated (A) and representative histograms of CRTh2 (B), intracellular IL-4 (C) and GATA3 (D) are shown. The data shown is an example of 11 independent isolations.

3.1.3 Culturing a CRTh2-isolated Th2 cell line

Following 14 days of the above differentiation protocol in Th2 conditions, Th2 cells were positively selected for CRTh2. These CRTh2-isolated Th2 cell lines were alternated between 3 days of stimulation and 4 days of proliferation without the need of added IL-4. These populations highly expressed CRTh2, IL-4 and IL-13 while expressing low IFNγ. A representative surface (Fig. 11) and intracellular (Fig. 12) flow experiment are shown below. These representative stains show our in vitro cultured CRTh2 cells remaining highly polarized for Th2 markers, such as IL-4, IL-13 and CRTh2, even after 42 days of our culture protocol. While culturing these CRTh2-isolated Th2 cell lines, we monitored surface CRTh2 expression and observed CRTh2 is highly expressed following proliferation and downregulated after stimulation (Fig. 13).

3.1.4 IL-25R on CRTh2-isolated Th2 cell lines

After establishing a CRTh2-isolated Th2 cell line, we examined the expression profile of these cells using whole genome microarray. We observed that IL-25R mRNA was significantly higher in CRTh2-isolated Th2 cells (day 45) compared to non-polarized CD4 T cells (from the same donor) primed for three days. This analysis showed an average 300 fold increase in expression of IL-25R mRNA in CRTh2 cells compared to CD4 T cells and was substantiated with quantitative RT-PCR which showed a 450 fold increase (Fig. 14A). Protein expression was assayed following stimulation using flow cytometry and these experiments confirmed that $CRTh2^{+}$ CD4 T cells express IL-25R protein (Fig. 14B). These

data are consistent with a similar microarray showing human in vivo differentiated CRTh2^{+} T cells express ~1000 fold higher IL-25R mRNA compared to naïve CD4 T cells [170].

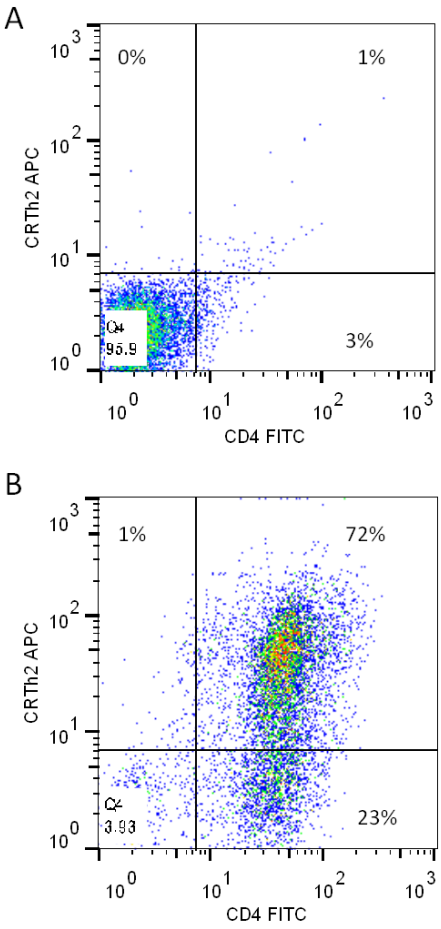

Figure 11: CRTh2-isolated Th2 cells (Shown is a line on day 42). (A) Isotype control and (B) antibody.

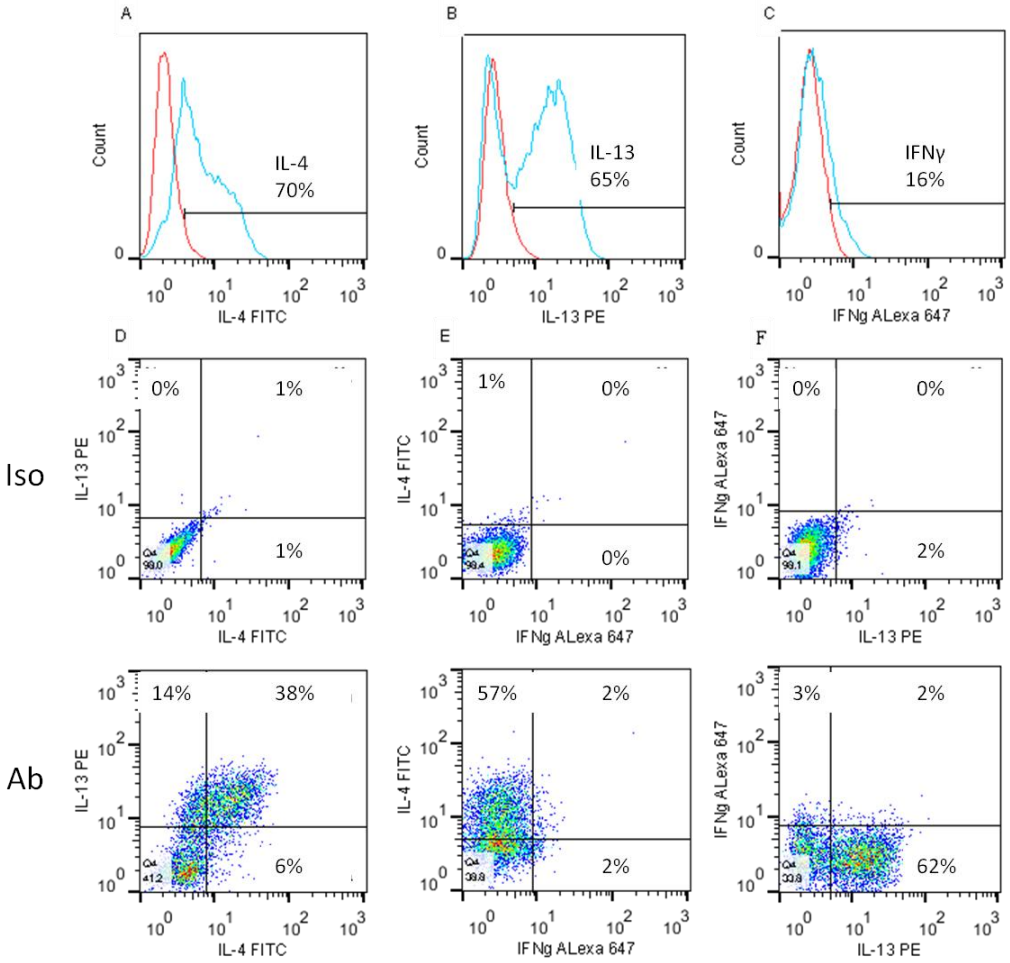

Figure 12: Cytokine expression by CRTh2-isolated Th2 cell lines. Single stains show high positivity for IL-4 (A) and IL-13 (B) and low for IFNγ (C). Double stains for IL-4/IL-13 (D), IL-4/IFNγ (E) and IL-13/IFNγ (F).

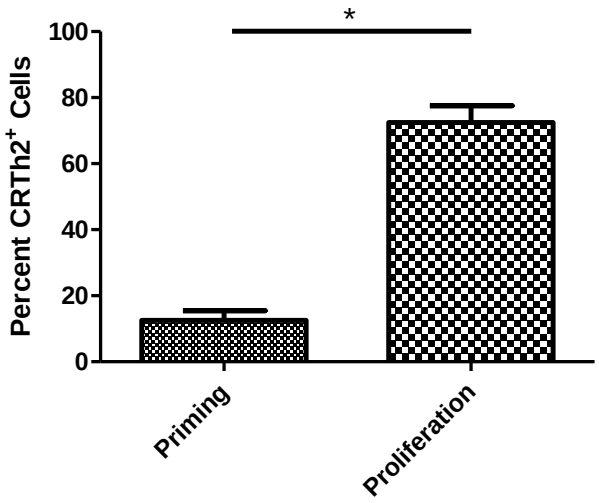

Figure 13: CRTh2 expression is higher after proliferation compared to priming. (n=10, *p<0.05).

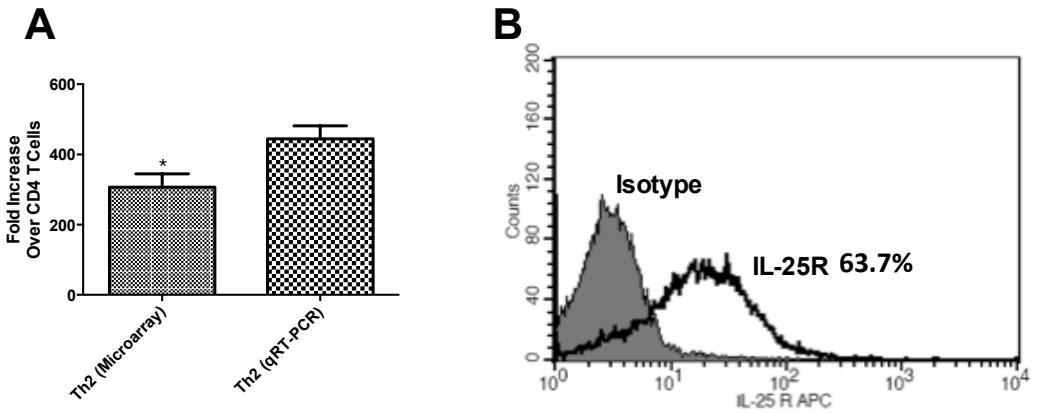

Figure 14: IL-25R is highly expressed by CRTh2-isolated Th2 cell lines. (A) Using microarray (n=3) and qRT-PCR (n=2). (B) Flow cytometry histogram of IL-25R positivity in a $CRTh2^+$ isolated line. Data represents 14 experiments (*$p<0.05$).

3.1.5 The effect of IL-25 on CRTh2-isolated Th2 cell lines

After observing that IL-25R is highly expressed in CRTh2-isolated Th2 cells, we wondered how IL-25 treatment would affect IL-25R and CRTh2 surface expression. CRTh2-isolated Th2 cell lines (Fig. 8A) described above (Fig. 11 and 12) were treated with IL-25 and after 1 and 2 days of stimulation, surface IL-25R was lower in the presence of IL-25, suggesting IL-25R internalization (Fig 15A). In addition, after 3 days of stimulation IL-25R mRNA levels were also lower (Fig. 15B). There were no changes to the number of total IL-25R^{+} cells (intracellular and surface), further supporting IL-25 mediated receptor internalization (Fig. 15C). Collectively these data suggest Th2 cells highly express IL-25R and show a response quickly following IL-25 stimulation.

Since CRTh2 expression decreases with stimulation (Fig. 13) we examined whether IL-25 could influence this loss of expression. Figure 15D shows that IL-25 had no effect on the rate or magnitude of surface CRTh2 expression loss. Since we did not observe an IL-25 effect on surface expression of CRTh2 in highly polarized Th2 cells, we next hypothesized that IL-25 could initiate Th2 differentiation.

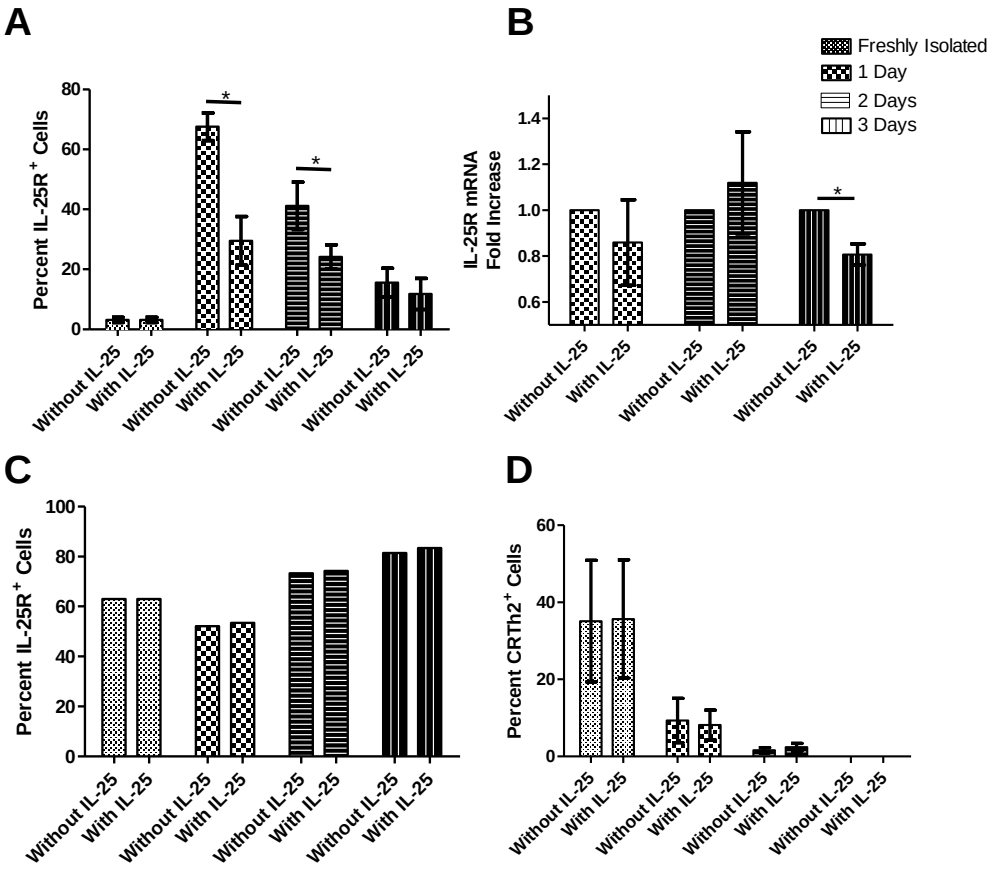

Figure 15: CRTh2 and IL-25R regulation on CRTh2 cells. CRTh2-isolated Th2 cell lines were stained for (A) IL-25R (n=5), (B), IL-25R mRNA levels (n=3), (C) total receptor levels (n=1) and (D) surface CRTh2 (n=4) were assayed (*$p<0.05$).

3.2 IL-25 INCREASES TH2 DIFFERENTIATION: SHORT (10 DAY) MODEL

3.2.1 IL-25R is expressed early during Th2 differentiation

While our previous data showed IL-25R mRNA is increased in differentiated Th2 cells (~Ct = 22.63), CD4 T cells also express IL-25R mRNA, although in lower abundance (~Ct = 29.88). In order for IL-25 to have an effect on Th2 differentiation, IL-25R protein must be expressed by naïve CD4 T cells or shortly after antigen encounter. To examine IL-25R expression, freshly isolated naïve CD4 T cells were compared to cells primed in Th2 conditions for 1, 2, or 3 days. Surface and total IL-25R expression were assayed by flow cytometry. Freshly isolated naïve CD4 T cells primed for 1 day expressed low surface IL-25R, however, after 2 days of stimulation surface IL-25R could be detected, but was lost after 3 days of stimulation (Fig. 16A). However, we also found that freshly isolated naïve CD4 T cells highly express intracellular IL-25R and after 2 and 3 days of stimulation total IL-25R expression was still appreciable (>20%, Fig 16B). To understand the relative magnitude of IL-25R during differentiation we compared early surface expression (three days) to polarized cells (day seventeen). Cells differentiated for seventeen days showed elevated surface IL-25R expression (Fig. 16C). These data demonstrated that IL-25R is present inside naïve CD4 T cells and the protein is expressed on the surface shortly after stimulation and continues to increase during Th2 differentiation.

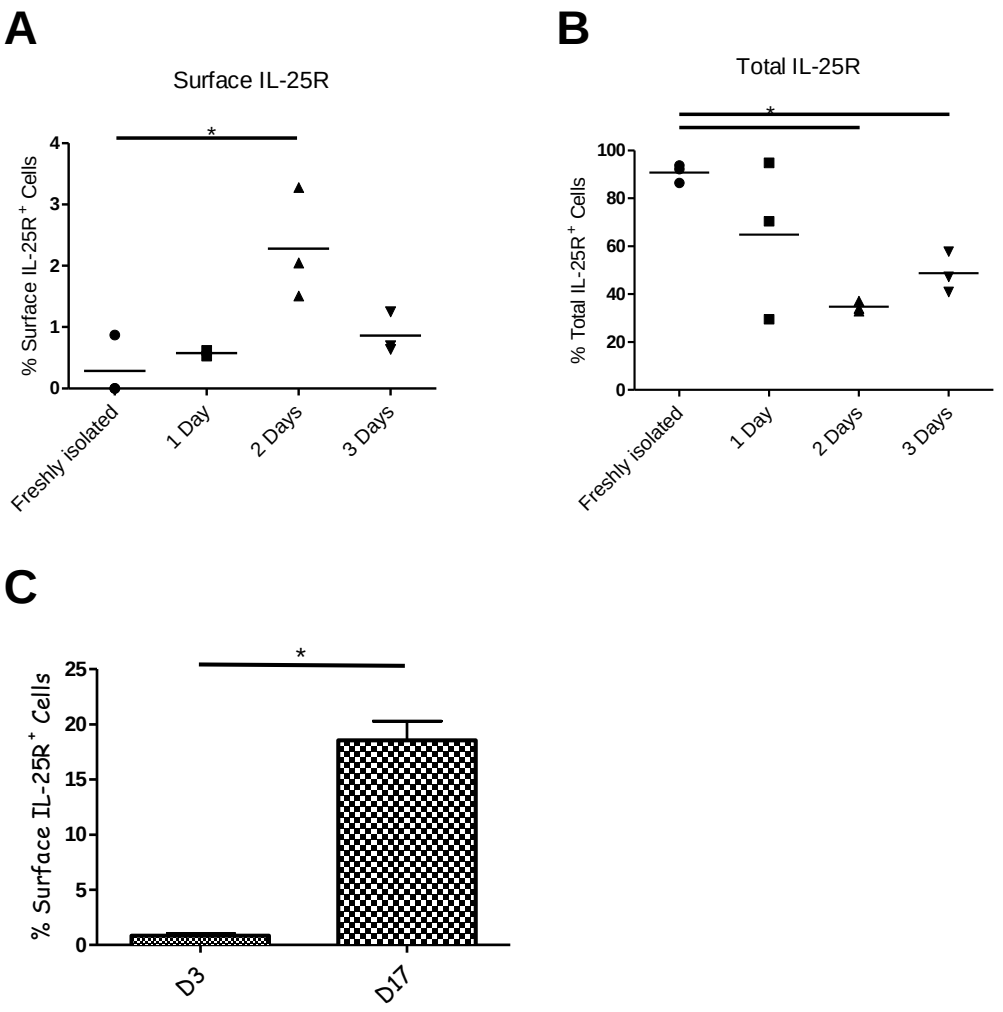

Figure 16: IL-25R is expressed early in Th2 differentiation. (A) Flow cytometry of surface IL-25R (n=2-3) and (B) total IL-25R (n=3). (C) IL-25R levels were compared after three days of stimulation: early (day 3, n=3) versus late (day 17, n=3) in differentiation (*$p<0.05$).

3.2.2 IL-25 is able to initiate Th2 differentiation

Since IL-25R is expressed in naïve CD4 T cells and on the surface of Th2 cells during differentiation (Fig. 16C), we hypothesized that IL-25 can meditate acquisition of the Th2 phenotype. First, we monitored the surface expression of CRTh2 in response to stimulation and proliferation and found that differentiating Th2 cell lines show increased surface CRTh2 following proliferation and reduced levels following stimulation (Fig 17), similar to CRTh2-isolated Th2 cell lines (Fig. 13). These data indicated that assessment of CRTh2 after the proliferation phase was optimal.

To test whether IL-25 influences initiation of Th2 differentiation, naïve CD4 T cells were cultured with IL-2, αIFNγ and αIL-12 with or without IL-25 (Fig. 8B). We found that IL-25 (in the absence of added IL-4) could induce expression of intracellular IL-4 (Fig. 18A) and GATA3 (Fig. 18B) as well as surface CRTh2 (Fig. 18B).

To understand the initiating strength of IL-25, we compared IL-25 to IL-4 during Th2 differentiation. We hypothesized IL-25 would increase Th2 differentiation as effectively and additively with IL-4. In order to optimize a seven day differentaiton various IL-4 concentrations (0-50ng/ml) were compared. Titration of IL-4 showed little difference across conditions for CRTh2 (Fig. 19A), GATA3 (Fig. 19B) or T cell growth (Fig. 19C), so we chose 50ng/ml of IL-4.

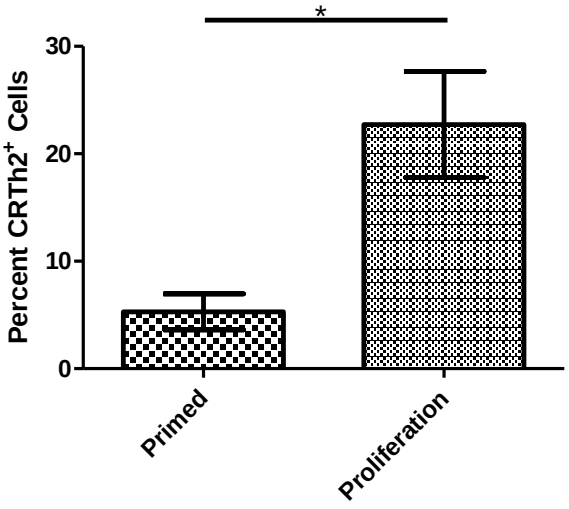

Figure 17: Surface CRTh2 on differentiating CD4 T cells. Surface CRTh2 was compared between stimulation and proliferation (n=8, $p<0.05$).

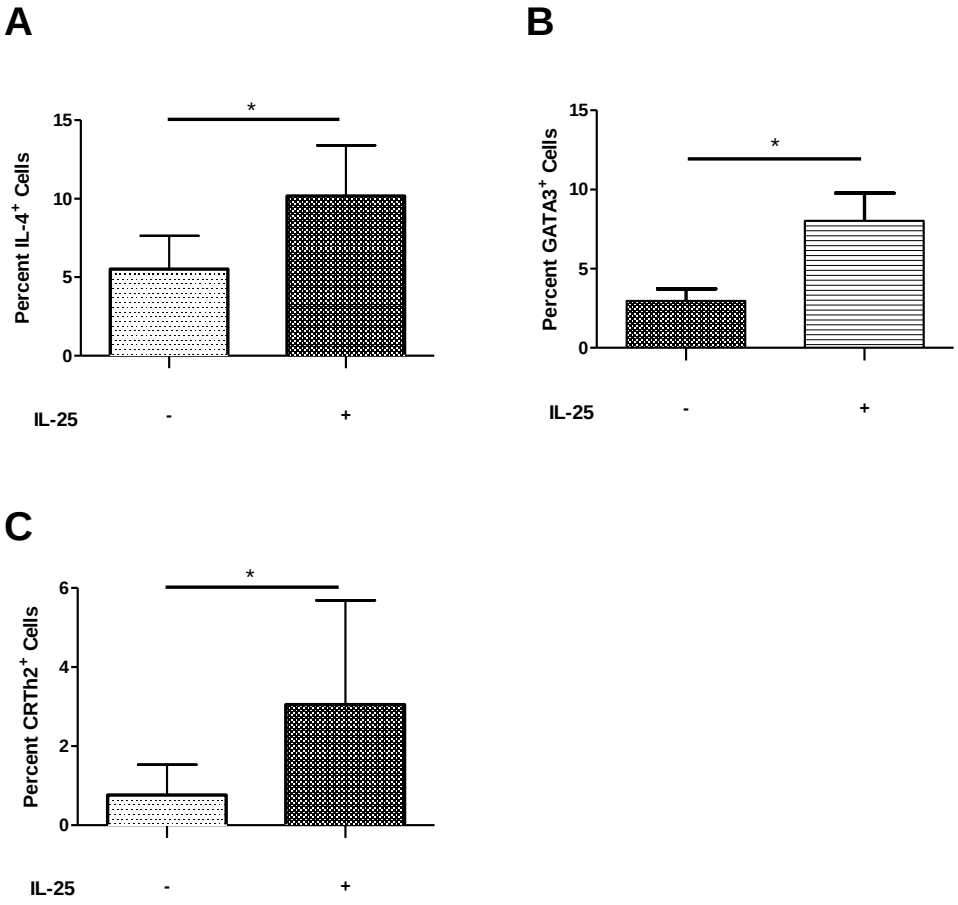

Figure 18: IL-25 increases acquisition of the Th2 phenotype. (A) Intracellular IL-4 and (C) surface CRTh2 was assayed after proliferation. (B) GATA3 was assayed after stimulation (n=7, *$p<0.05$).

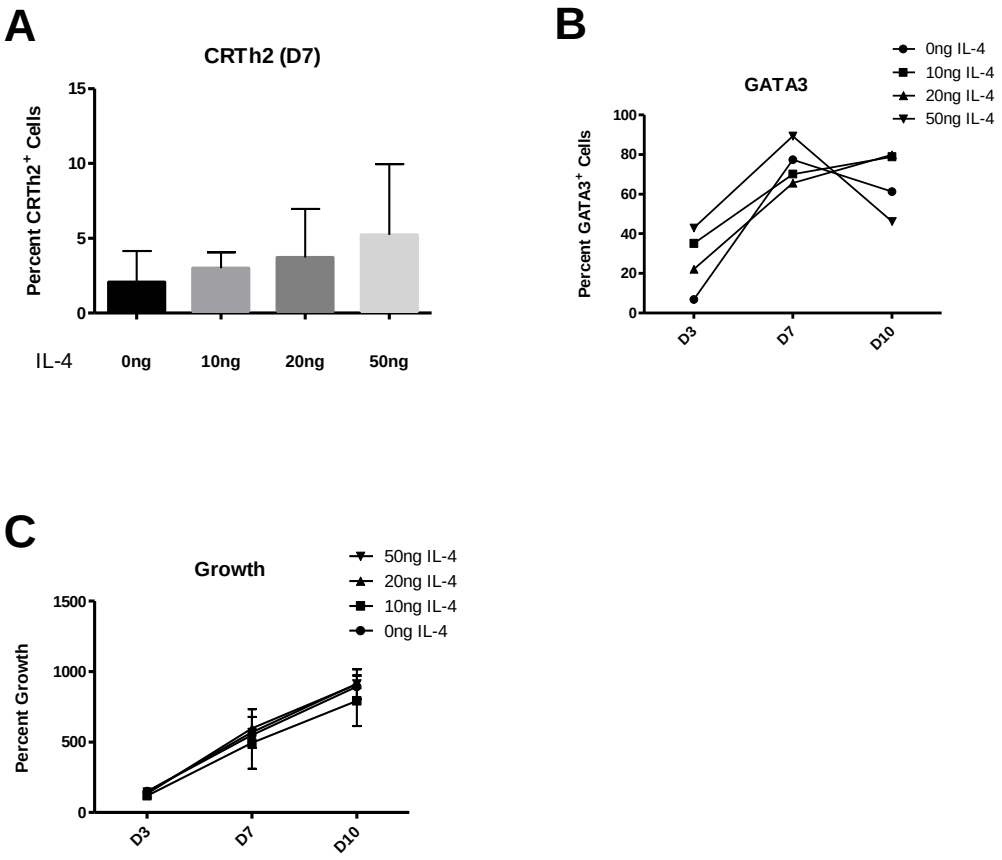

Figure 19: Optimization of Th2 differentiation. (A) CRTh2 after seven days of differentiation (n=2). (B) GATA3 and (C) T cell growth were followed after three, seven and ten days of differentiation (n=1).

Head to head comparison between IL-4 and IL-25 showed intracellular IL-4, GATA3 and surface CRTh2 were significantly induced in the presence of IL-4 alone (Fig 20A, B and C), while IL-25 alone significantly increased IL-4^+ cells (Fig. 20A), but not $GATA3^+$ or $CRTh2^+$ cells (Fig. 20B and C respectively). IL-4 and IL-25 together had an additive effect on both IL-4^+ (Fig 20A) and $GATA3^+$ (Fig 20B) cells, while a trend was apparent for $CRTh2^+$ cells (Fig. 20C).

After assessing the initiating capacity of IL-25 in head to head comparison with 50ng/ml of IL-4, we wondered if our inability to observe an additive effect on CRTh2 was due to using a high IL-4 concentration (50ng/ml). Since IL-4 can induce CRTh2 expression, we speculated if this concentration of IL-4 overshadowed a weaker IL-25 effect. Therefore we performed experiments using 20ng/ml of IL-4 in an effort to uncover an additive effect on CRTh2. However, we found that the trends were the same (Fig. 21).

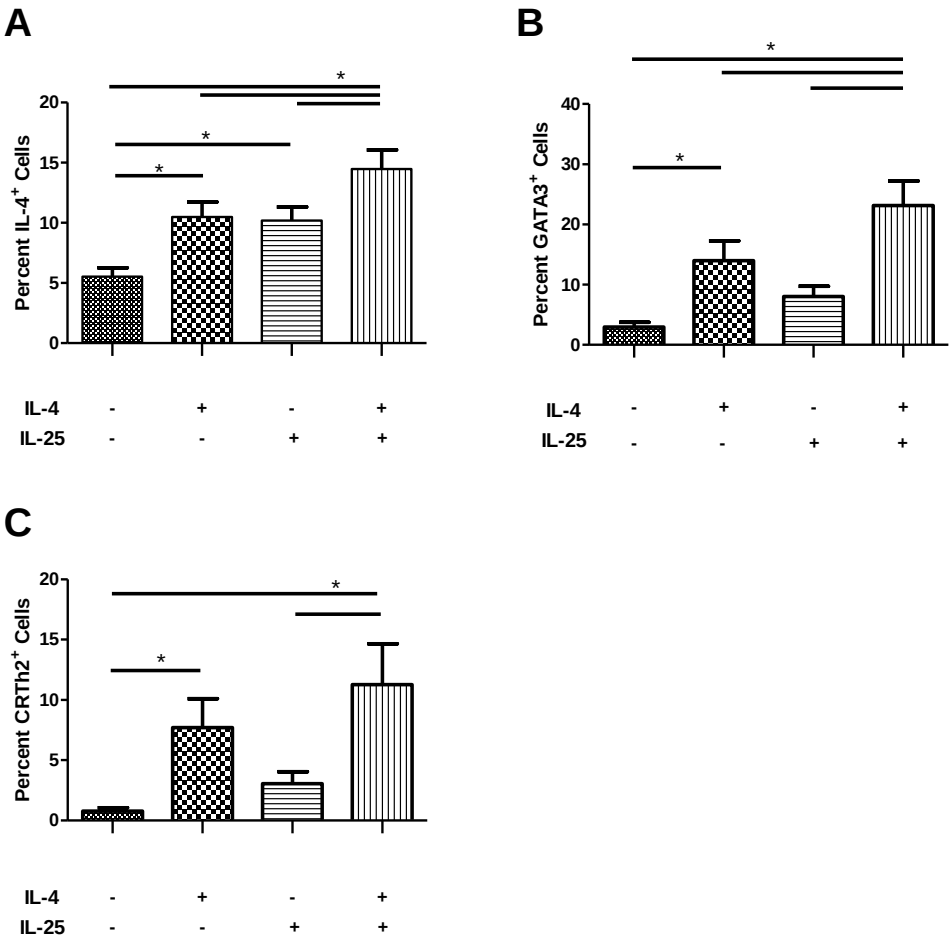

Figure 20: IL-25 increases acquisition of the Th2 phenotype. (A) Intracellular IL-4 and (C) surface CRTh2 were assayed following proliferation. (B) GATA3 was assayed after stimulation (n=7, *$p<0.05$).

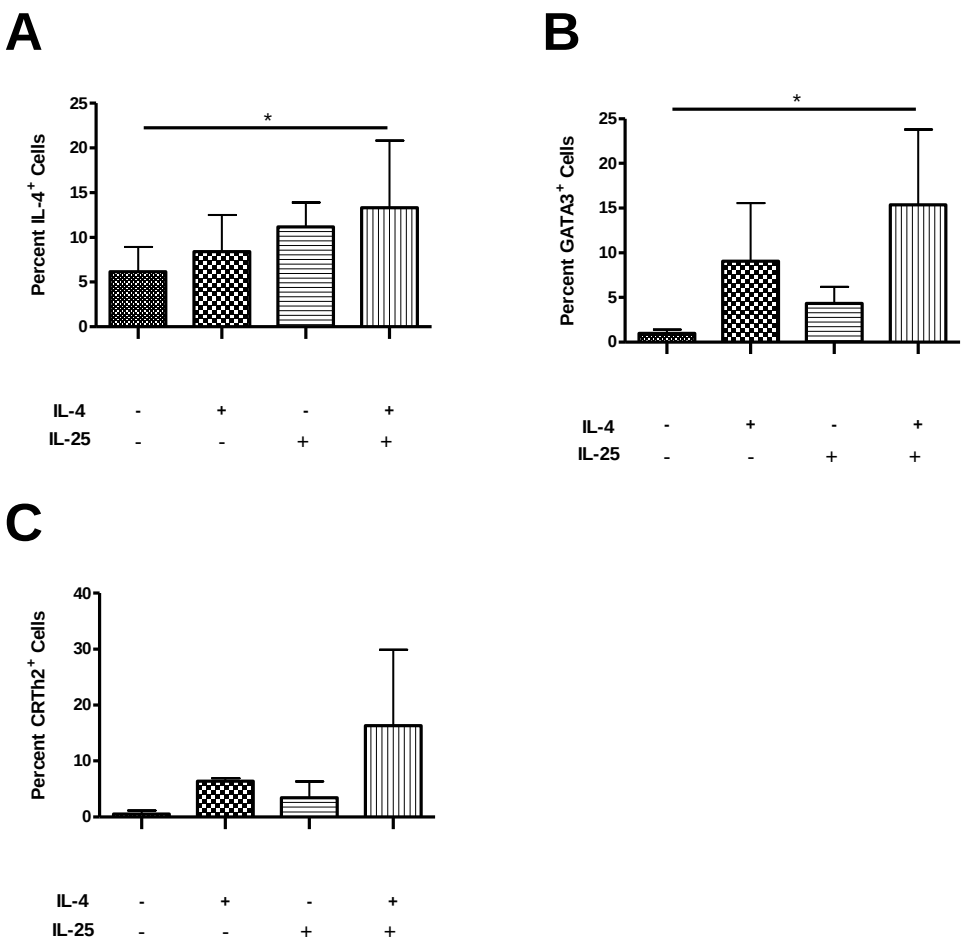

Figure 21 Decreased IL-4 (20ng/ml) does not change IL-25 initiated Th2 differentiation. (A) Intracellular IL-4 and (C) surface CRTh2 were assayed following proliferation. (B) GATA3 was assayed after stimulation (n=7, *$p<0.05$).

3.2.3 IL-25 influences IL-25R expression

Surface staining of naïve CD4 T cells showed relatively low IL-25R expression compared to CD4 T cells following 17 days of Th2 differentiation (Fig 16C). Therefore we sought to further understand the acquisition of IL-25R expression during Th2 differentiation. The presence of both cytokines increased IL-25R mRNA compared to the absence of both cytokines, though, IL-4 alone or IL-25 alone were unable to induce a significant increase (Fig 22A). There was no significant effect on surface (Fig. 22B) or total IL-25R (Fig. 22C) following stimulation.

3.2.4 IL-25 does not directly induce CD4 T cell growth

IL-25 has been suggested to play a role in inducing proliferation in TSLP stimulated human dendritic cell-Th2 co-culture [149], however this has not been substantiated by others. To determine if IL-25 acts directly on CD4 T cells to induce growth we compared cell counts of differentiating Th2 cells (Fig. 8B). No effects were seen after three, seven, or ten days of differentiation. However, after fourteen days of differentiation the presence of both cytokines as well as IL-4 alone induced more growth compared to the absence of both cytokines (Fig 23).

These data from the short (10 day) model show IL-25 may initiate the acquisition of IL-4 expressing CD4 T cells (Fig 18A and 20A). As well, IL-25 was seen to induce higher GATA3 (Fig. 18B and 20B) and CRTh2 (Fig. 18C). Collectively,

these date demonstrate that IL-25 is able to initiate Th2 differentiation, albeit to a lesser extent that IL-4

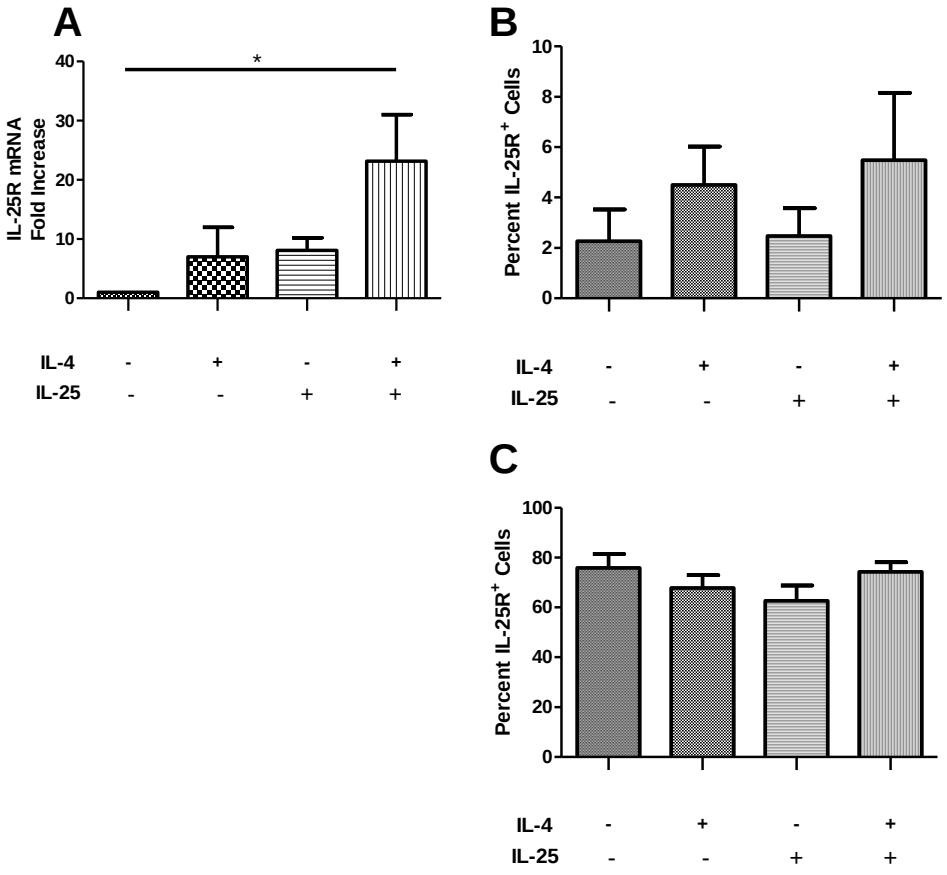

Figure 22: IL-25 has no effect on IL-25R expression. (A) IL-25R mRNA was assayed on day three, seven and ten of differentiation (n=10). (B) Surface IL-25R (n=6) and (C) total IL-25R (n=6) were assayed on day ten of differentiation. Data represents seven independent cell lines (*$p<0.05$)

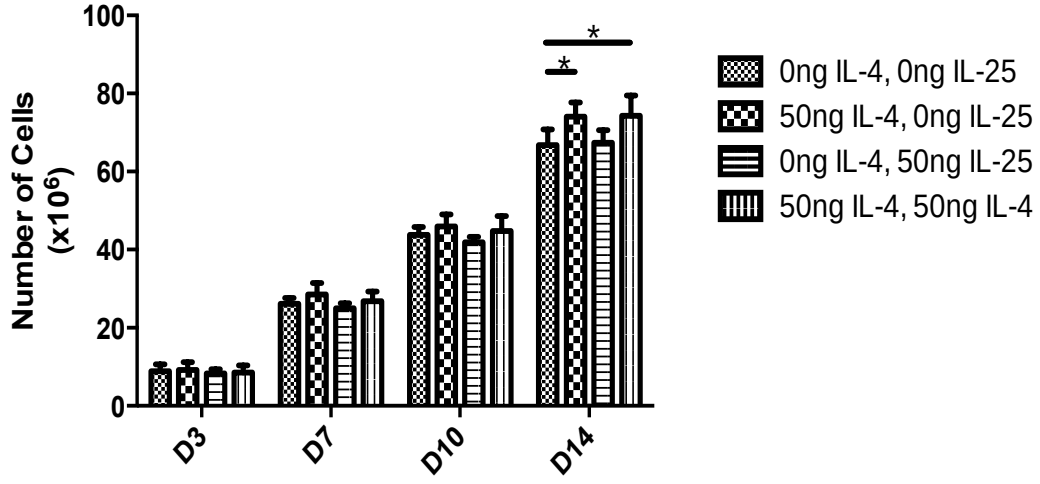

Figure 23: IL-25 does not directly induce CD4 T cell growth. Cell growth was assessed by cell count (n=6). Data represents six independent cell ($p^{*}<0.05$).

3.3 IL-25 AMPLIFIES TH2 CYTOKINES: LONG (38 DAY) MODEL

3.3.1 IL-25 amplifies Th2 effector cytokines

Since we observed IL-25 is able to initiate Th2 differentiation, we next hypothesized IL-25 would amplify Th2 effector cytokine production. To do this we cultured naïve CD4 T cells in Th2 conditions in the presence or absence of IL-25 (Fig. 9C). The absolute amount of cytokine produced in both conditions gradually increased and ranged from 140 – 65,000 pg/ml. While a similar trend for the effect IL-25 was observed in all differentiations, the data was generated from cells from xx human subjects and therefore there was variability in the absolute amount of cytokine across the lines. For this reason, the data is presented as fold increase. Figure 24 shows that in the presence of IL-25, IL-5 and IL-13 mRNA (Fig. 24A and C respectively) and protein (Fig 24B and D respectively), were significantly increased compared to Th2 conditions without IL-25. The absolute amount of cytokine secreted by differentiating Th2 cells is higher following stimulation than proliferation (Fig 25A and B).

3.3.2 Influence of IL-25 on CRTh2 and IL-25R expression

We observed an increase in surface CRTh2 expression in the presence of IL-25 compared to the absence of IL-25 (Fig. 18C). To test whether longer term culture in IL-25 would influence CRTh2 and/or IL-25R expression, we analyzed CRTh2 and IL-25R over 38 day cultures (Fig. 8C). Figure 24A shows that CRTh2 mRNA but not surface CRTh2 (Fig. 26B) is upregulated by IL-25. Similarly, IL-25R

mRNA (Fig. 24C) but not surface IL-25R (Fig. 26D) is higher in the presence of IL-25.

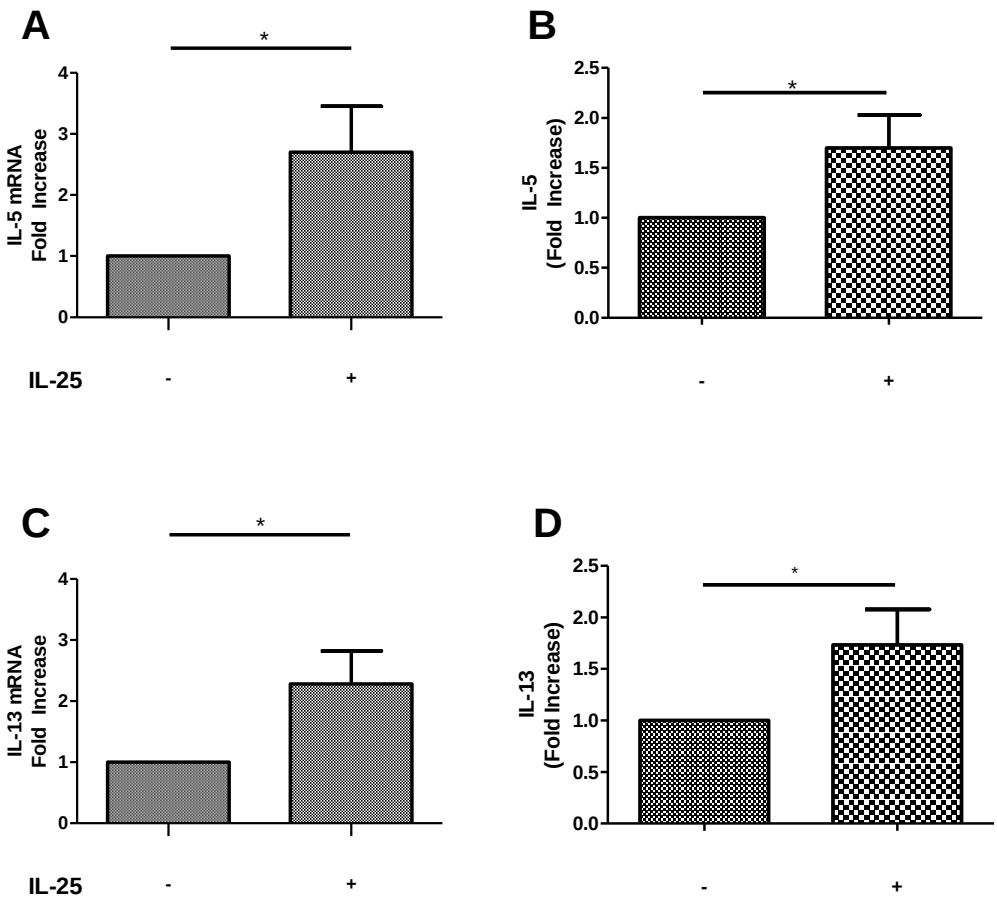

Figure 24: IL-25 increases Th2 effector cytokine expression. Samples were collected following stimulation and analyzed for (A) IL-5 mRNA (n=10) and (C) IL-13 mRNA (n=10). (B) IL-5 protein (n=10) and (D) IL-13 protein (n=13) were analyzed by ELISA. Data represents four independent cell lines (*$p<0.05$).

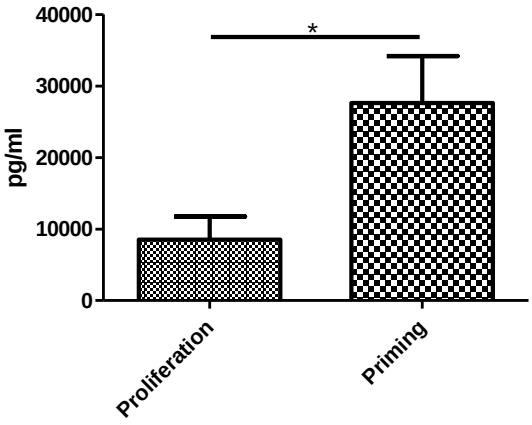

Figure 25: Th2 cytokines are increased following stimulation. (A) IL-5 (n=10) and (B) IL-13 (n=13).

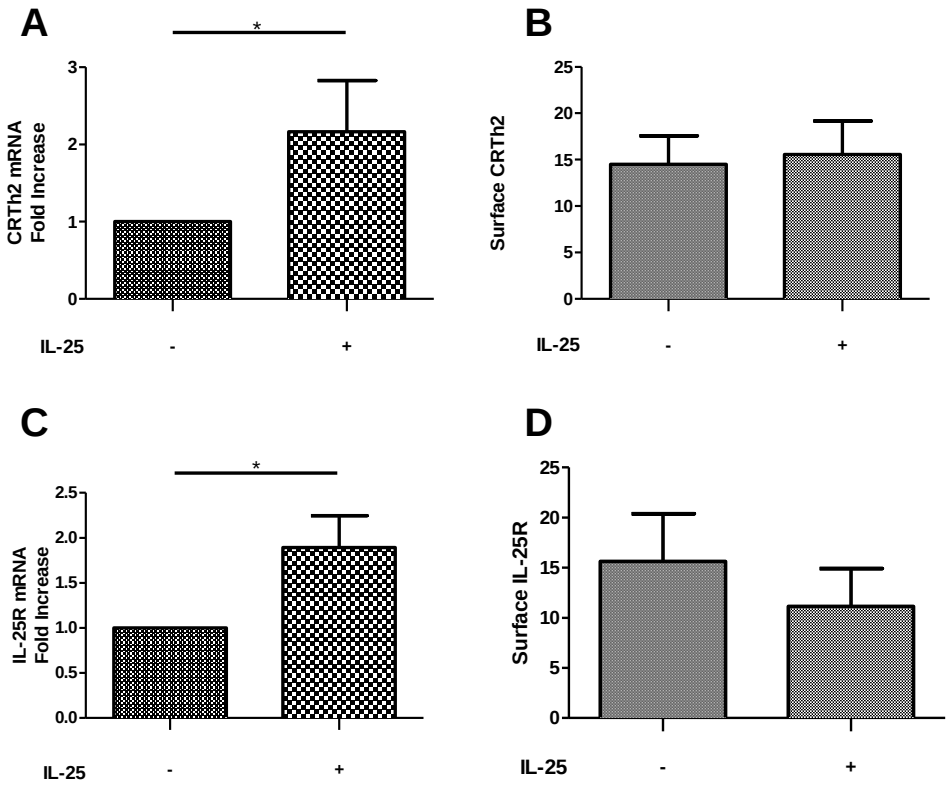

Figure 26: The effect of IL-25 on Th2 surface receptors. (A) CRTh2 mRNA (n=18) and (B) surface expression (n=14) after proliferation. (C) IL-25R mRNA (n=11) and (D) surface expression (n=20) after stimulation**.** Data is from two independent cell lines (*$p<0.05$).

3.4 IL-25 EFFECTS A RANGE OF TH2 FUNCTIONS

3.4.1 IL-25 increases mediators released from CRTh2$^+$ cells

In order to further understand the broader role of IL-25 on Th2 cells, supernatants from CRTh2-isolated Th2 cells treated with various concentrations of IL-25 were analyzed (Fig. 9A) using multiplex bead technology. Table 1 shows that IL-25 (50ng/ml) increased sIL-2Ra (1.5 fold, $p<0.05$). However, when the results were compiled as 'with' or 'without' IL-25 the results showed a significant fold increase in the amount of sIL-2Rα, IL-9 and sCD40L (Fig. 27).

3.4.2 IL-25 does not directly mediate CRTh2-isolated Th2 cell chemotaxis

Since other IL-25 family members have been show to induce chemotaxis [171] and IL-25 transgenic mice overexpressing IL-25 in the lungs showed an accumlulation of CD4 T cells [7], we hypothesized IL-25 directly induces Th2 cell chemotaxis. CRTh2-isolated Th2 cells (Fig. 9A) cells were assayed using the modified boyden chamber. CCL22 (a Th2 chemokine), was used as a positive control. Acknowledging that surface IL-25R is high on Th2 cells after stimulation, chemotaxis was first assayed after 3 days of stimulation. Under these conditions, we did not observe IL-25 mediated migration, however, there was a trend for CCL22 (Fig. 28A).

Since we observed IL-25R was highest after 1 day of stimulation (Fig. 14B), we next hypothesized that IL-25 would induce chemotaxis after 1 day of stimulation.

However, we did not notice CCL22 or IL-25 mediated movement (Fig. 28B). Since these conditions did not seem optimal even for our positive control, we also tested cells after 5 days of proliferation. While we did not see an IL-25 meditated effect, we did notice a dose dependent response to CCL22 (~2.4 fold increase, Fig. 28C). This data suggests IL-25R is expressed at a time when Th2 cells may not be receptive to chemotactic signals.

3.4.3 IL-25 does not affect CCR4

When IL-25 was overexpressed in the murine lungs, $CD4^+$ T cells accumulated [171]. Therefore, we hypothesized IL-25 mediates a change in CCR4 expression, the receptor for the Th2 chemokine CCL22. The number of cells expressing CCR4 was not significantly affected by IL-25 (Fig. 27A) nor was mean fluorescent intensity (Fig. 27B). Therefore, these data show IL-25 does not alter the number of cells expressing CCR4 or the amount of CCR4 each cell is expressing. These data suggest that IL-25 does not alter Th2 responsiveness to CCL22.

Concentration of IL-25	0ng/ml	50ng/ml	100ng/ml	150ng/ml
IL-9 (pg/ml)	103.6 ± 63.6	128.9 ± 68.1	129.48 ± 55.8	141.9 ± 64.2
sCD40L (pg/ml)	1202.8 ± 980.9	2365.8 ± 1846.3	1419.68 ± 852.9	1244.9 ± 706.6
sIL-2Rα (pg/ml)	858.3 ± 304.9	1176.0 ± 606.5*	1146.5 ± 446.8	1140.3 ± 414.7

Table 1: Effect of IL-25 on novel Th2 derived soluble mediators. Absolute amount of mediator (n=3) is shown as average (pg/ml) ± standard error of the mean. Statistical significant by ANOVA (*$p<0.05$).

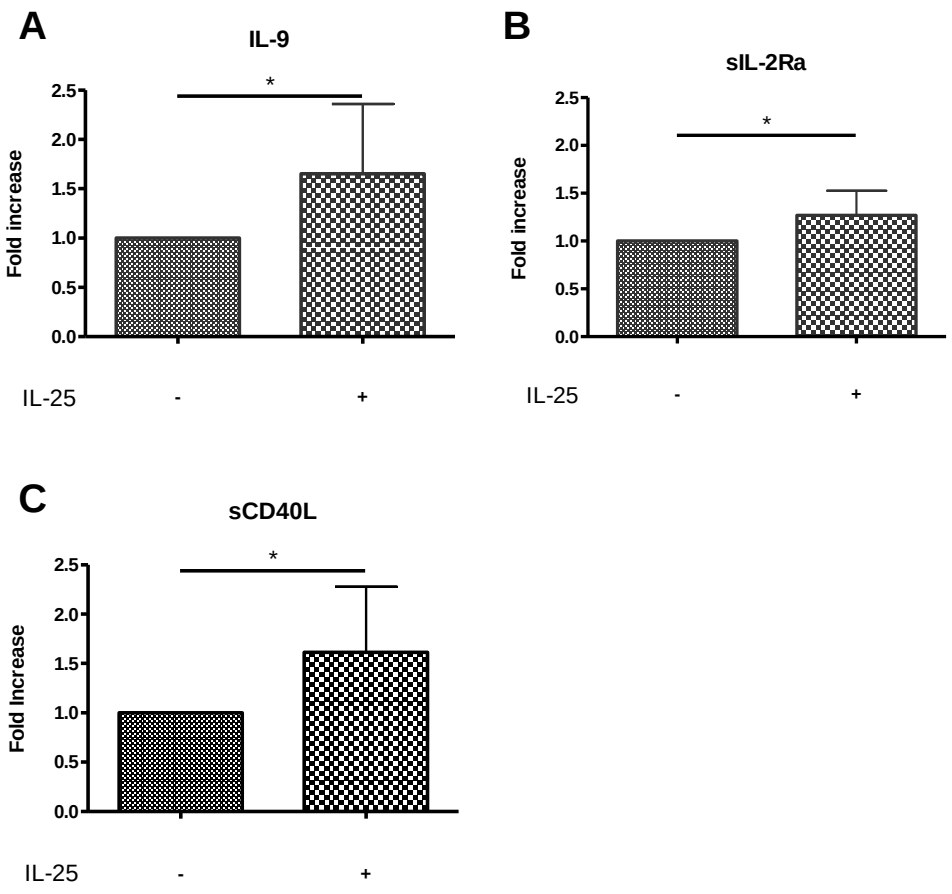

Figure 27: IL-25 increases expression of soluble mediators. (A through C) CRTh2-isolated Th2 cells (Fig 8A) were primed and assessed by presence vs. absence of IL-25. Data represents three independent cell lines (*$p<0.05$).

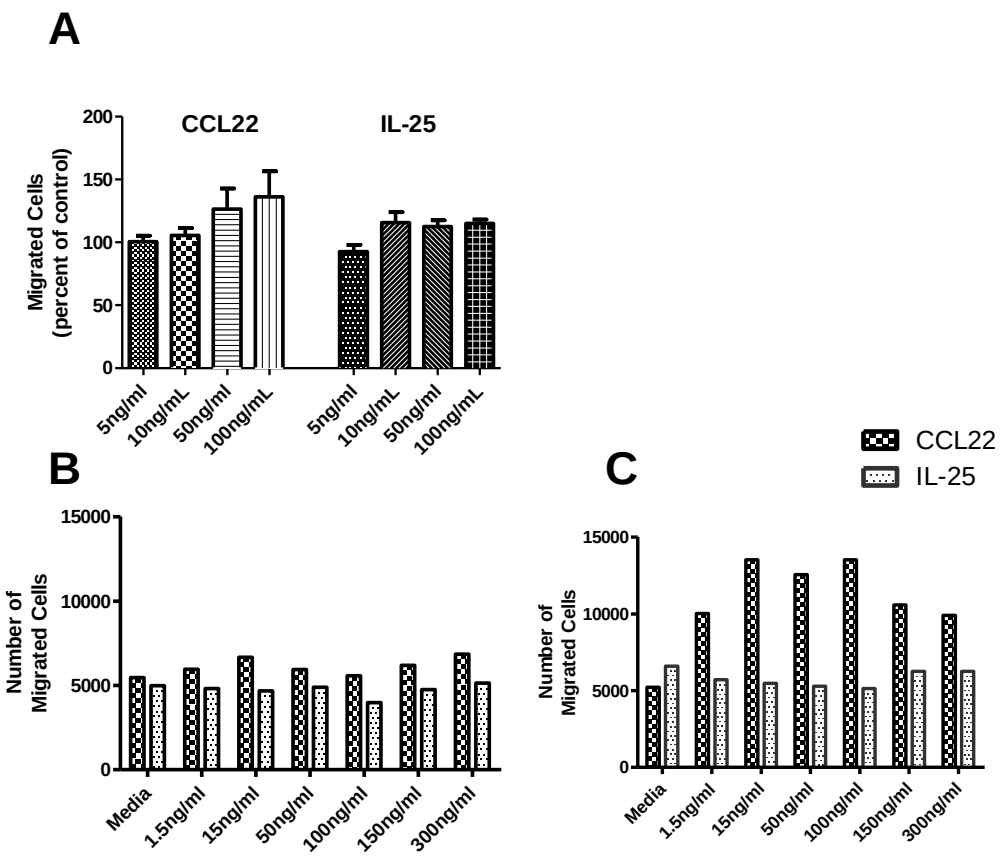

Figure 28: IL-25 does not appear to directly mediate CRTh2^{+} Th2 chemotaxis. (A) CRTh2-isolated Th2 cell lines were assayed following 3 days of stimulation (n=3), (B) 1 day of stimulation (n=1) and (C) five days of proliferation (n=1). CCL22 was used as a positive control.

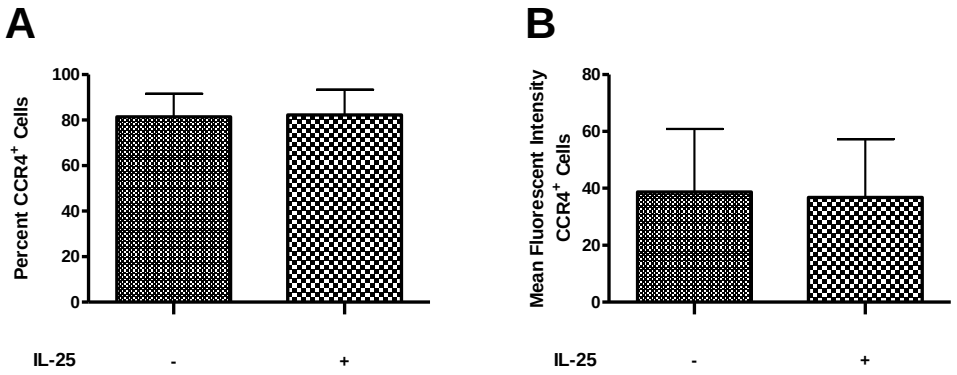

Figure 29: IL-25 does not affect CCR4 expression. Differentiating CD4 T cells were stained for CCR4 expression. (A) Number of cells expressing CCR4 and (B) amount of CCR4 expressed by each cell were unchanged (n=7).

Chapter IV: Discussion

4.1 SUMMARY

Our results showed that IL-25R is highly expressed on polarized CRTh2$^+$ Th2 cells compared to nonpolarized CD4 T cells. We also observed that naïve CD4 T cells highly express intracellular IL-25R, which comes to the surface shortly after CD3/CD28 stimulation. Knowing this, we subsequently examined how IL-25 influences Th2 cells. We showed that IL-25 initiates acquisition of the Th2 phenotype by increasing the proportion of IL-4$^+$, GATA3$^+$ and CRTh2$^+$ cells. We also demonstrated that IL-25 enhances production of Th2 effector cytokines IL-5 and IL-13. Lastly, we explored the possibility of further IL-25 mediated Th2 cell regulation. Discovery cytokine assays showed IL-25 may increase expression of mediators such as sIL-2Rα, IL-9 and sCD40L. Therefore, IL-25 has the potential to mediate effects directly onto Th2 cells.

4.2 PURITY OF CELL CULTURES

Since we were focused on assessing the effect of IL-25 directly on Th2 differentiation, we started with a highly purified naïve CD4 T cells population (Fig. 9). This approach ensured that differentiation effects were not due to contaminating cells, including in vivo differentiated Th1 or Th2, and that the differentiation we observed was induced by our experimental conditions. For the short model (10 days), we typically observed ~5-10% Th2 cells marked by surface CRTh2 (Fig. 20), while after fourteen days of Th2 differentiation ~30% of the population were Th2 cells marked by surface CRTh2 (Fig. 10). After

positively selecting for CRTh2 our cultures were highly enriched for $CD4^{+}CRTh2^{+}$ Th2 cells (~70%, Fig. 11) as well as IL-4 and IL-13, with low IFNγ (Fig. 12).

Importantly, we believe our cultures do not contain ILC2, which also express CRTh2 and have been shown to produce Th2 cytokines [169, 172]. The naïve CD4 T cell isolation column we used contains antibodies to CD25, which is expressed by ILC2s. As a result, our isolation protocol removed them from the naïve T cell population, thoughwe did not confirm the absence by flow cytometry. These data confirm that we had a sound isolation and differentiation protocol as well as a highly purified Th2 cell culture.

4.3 IL-25R EXPRESSION BY TH2 CELLS

Along with others [149] we have shown that IL-25R mRNA is more abundantly expressed by $CRTh2^{+}$ Th2 cells compared to naive CD4 T cells. That said, a number of reports have shown that IL-25R expression is not limited to Th2 cells. Specifically, a panel of human cDNA templates from various cell types revealed eosinophils, basophils, and dendritic cells [149], as well mast cells and endothelial cells from skin biopsies, can also express IL-25R [150]. On the other hand, monocyte derived dendritic cells were shown to express both IL-25 and IL-25R [151]. The presence of IL-25R on immune surveillance and effector cells suggest a diverse role for IL-25 in immunity. Specifically, our observation of the abundance of IL-25R expression by naïve CD4 T cells and Th2 differentiating cells suggests IL-25 plays a role in developing Th2 responses.

While our results show that IL-25R is not on the surface of naïve CD4 T cells, it is abundantly expressed inside these T cells (Fig. 16). Freshly isolated naive CD4 T cells highly express intracellular IL-25R (~90%). Furthermore, after 2 days of stimulation, IL-25R is expressed on the surface (Fig. 16A). While this seems like a high positive signal, similar protocols were used for staining IL-25R and CRTh2 but reported no intracellular CRTh2. Both antibodies are biotinylated and our biotin alone and strep alone controls did not show any background (data not shown). Future work should use a biological control, such as CD8 T cells that are known to be IL-25R negative, in order to verify and understand these data. Despite, the abundance of intracellular IL-25R, the emergence onto the surface after 2 days of stimulation supports a role for IL-25 early in T cell subset commitment. Intracellular IL-4R has also been observed on granules inside eosinophils [173] showing cytokine receptors can exist inside cells. However, to our knowledge, there are no reports of cytokine receptors within CD4 T cells. It is unlikely that IL-25 would be able to enter the cystol and there do not appear to be reports of this in the literature. However, it is possible the IL-25R requires other signals to be fully shuttle to the surface.

To monitor the acquisition of IL-25R during Th2 development, we first studied CD4 T cells developing in Th2 conditions in both the presence and absence of IL-25 (Fig. 8C). We observed an increase in IL-25R mRNA; however, these effects were not reflected by surface expression (Fig. 26). It is possible that we did not observe an effect on the level of IL-25R protein due to the time after stimulation

the staining was conducted. IL-25R is highest after 1 day of stimulation (Fig. 15B) but we assayed IL-25R after 3 days of stimulation in order not to disturb our culture protocol. Alternatively, the increased IL-25R mRNA may have been translated into protein but the addition of IL-25 to the culture caused internalization.

In further effort to understand the regulation of IL-25R on Th2 cells, we stained surface IL-25R after proliferation and stimulation. This anaylsis showed that Th2 cells exposed to mimicked antigen stimulation (CD3 and CD28) upregulate surface IL-25R (Fig. 15B). This suggests that when dendritic cells present antigen to Th2 cells, surface expression of IL-25R is increased. We next treated CRTh2^{+} isolated Th2 cell lines (Fig. 8A) with IL-25 and monitored IL-25R after 1, 2 and 3 days of stimulation. We noticed a reduction in surface IL-25R in the presence of IL-25 after 1 and 2 days (Fig. 15B) but no difference in total IL-25R protein expression (Fig. 15D). While there was a statistically significant reduction in IL-25R mRNA following 3 days of stimulation, it was small and may not be biologically significant (Fig. 15C). We suspect that IL-25 interaction with IL-25R induces internalization of the receptor, but our data are not conclusive. It is possible the exogenously added IL-25 protein binds to IL-25R and activates the receptor while remaining at the surface. However, the bound IL-25 may prevent our IL-25R antibody from detecting surface IL-25R, thus report a false reduction in surface IL-25R. In order to ascertain the true IL-25 mediated regulation of IL-25R, an antibody to IL-25 that recognizes the protein while it binds to IL-25R

must be used. An antibody like this would allow identification of surface bound IL-25; however, to our knowledge, it does not exist.

The IL-25R is made up of two subunits IL-17RB and IL-17RA. Throughout the literature antibodies used to detect the 'IL-25R' are actually specific for the IL-17RB subunit [149, 174]. In this thesis, antibody to IL-17RB was used to detect IL-25R and as such, our data comes with the caveat that while we detected changes in surface expression of IL-17RB we have not taken the levels of IL-17RA into account. While we did perform extracellular staining for IL-17RA we were unable to detect any expression. This may be due to using a directly conjugated antibody, since our directly conjugated IL-17RB antibody failed to detect receptor. Conversely, it is possible that when the receptor subunits dimerize, the IL-17RA epitope is hidden. Since we saw functional effects after adding IL-25 (Fig. 18 and 23), we feel the IL-17RB antibody does represent expression of a functional IL-25R. However, further characterization with better Il-17RA antibodies and/or one that recognizes both subunits would aid in understanding the regulation of IL-25R on Th2 cells. Unfortunately, these antibodies do not yet exist.

4.4 ROLE OF IL-25 IN TH2 DIFFERENTIATION

4.4.1 IL-25 initiates Th2 differentiation

Since IL-25R is highly expressed by in vitro differentiated Th2 cells lines we first examined its effect on CRTh2, but found it did not increase CRTh2 expression (Fig. 15D). This may be due to the cells already having been polarized to express

CRTh2. To determine this we next examined whether IL-25 could initiate Th2 differentiation. Our first attempt to study the effect of IL-25 on Th2 differentiation was to add IL-25 to the usual Th2 differentiation protocol. We studied this effect for 5 weeks, a time after which most cultures showed waning levels of CRTh2.

Over these 38 days we noticed that CRTh2 mRNA was increased by the presence of IL-25, but did not change surface expression (Fig. 26A and B respectively). PGD_2 production by Th2 cells has been reported [175] and we have observed that $CRTh2^+$ Th2 cells produce PGD_2 (data not shown). It is therefore possible that as the T cells differentiated towards a Th2 phenotype, they were releasing PGD_2 leading to CRTh2 internalization.

Despite the lack of increased surface protein, the data suggested IL-25 may increase Th2 acquisition. However, this model did not allow us to distinguish between whether IL-25 played a role in selectively proliferating and/or promoting survival of Th2 cells or whether it actively instructed naïve T cells to differentiate toward the Th2 phenotype. In order to test this, we developed a short (10 day) model of differentiation that would allow us to better monitor the influence of IL-25 on Th2 differentiation.

We first compared the ability of IL-25 to initiate Th2 differentiation in the absence of the canonical differentiation factor IL-4 (Fig. 8B). Interestingly, we found that IL-25 increased intracellular IL-4, GATA3 and surface CRTh2 (Fig.

18). These data showed that IL-25 is able to initiate Th2 differentiation, but since it also increased IL-4 it is possible the effect is through elevating IL-4 production. Experiments with αIL-4 could be performed to test this further.

We also examined the effect of IL-25 versus IL-4 in head to head comparison. We found that IL-25 was able to induce intracellular IL-4 expression as effectively as IL-4 itself (Fig. 20A). This effect may be through an independent mechanism since an additive effect with IL-4 was observed. Alternatively, it is also possible that our culture conditions had not saturated the cells ability to respond to IL-4, allowing IL-25 to induce further differentiating signal through IL-4 production. In these multi-condition experiments (Fig. 8B), in which statistics were assessed by ANOVA, IL-25 alone did not show significant effects on GATA3 or CRTh2, though IL-4 and IL-25 together induced significantly more GATA3 than IL-4 alone (Fig. 20B). Collectively these data show IL-25 can increase expression of the canonical Th2 markers, IL-4, GATA3 and CRTh2 (Fig. 18), but to a lesser extent than IL-4 (Fig. 20). The significant increase in surface CRTh2 in the short model (Fig. 18C) but absence in the long model (Fig. 26B) indicates that IL-25 plays a role in acquisition of the Th2 phenotype but, its effects may be overshadowed by IL-4, a more potent inducer of Th2 differentiation.

It has been shown that dendritic cells can express IL-25 during antigen presentation; and therefore is the likely in vivo source of IL-25 influencing Th2 differentiation. Here, we focused on understanding the role of IL-25 directly on T

cells, but when provided in conjunction with the other Th2 inducing signals from dendritic cells in vivo, IL-25 may have a much a larger role.

However, in vivo evidence in supporting our findings is provided by Angkasekwinai et. al. They showed, that in mice, IL-25 induced Th2 differentiation could be blocked by a neutralizing antibody to IL-4 [176]. Whether IL-4 production is the mechanism inducing IL-25 mediated Th2 differentiation is unclear. However, it is reasonable to hypothesize that dendritic cell derived IL-25 can act directly on naïve CD4 T cells to induce expression of IL-4. This IL-4 could then act in an autocrine fashion to induce further Th2 acquisition and polarization. Further experiments, determining if neutralization of IL-4 can abrogate IL-25 initiated Th2 differentiation will help us understand the mechanism underlying the effects of IL-25 (Fig. 30).

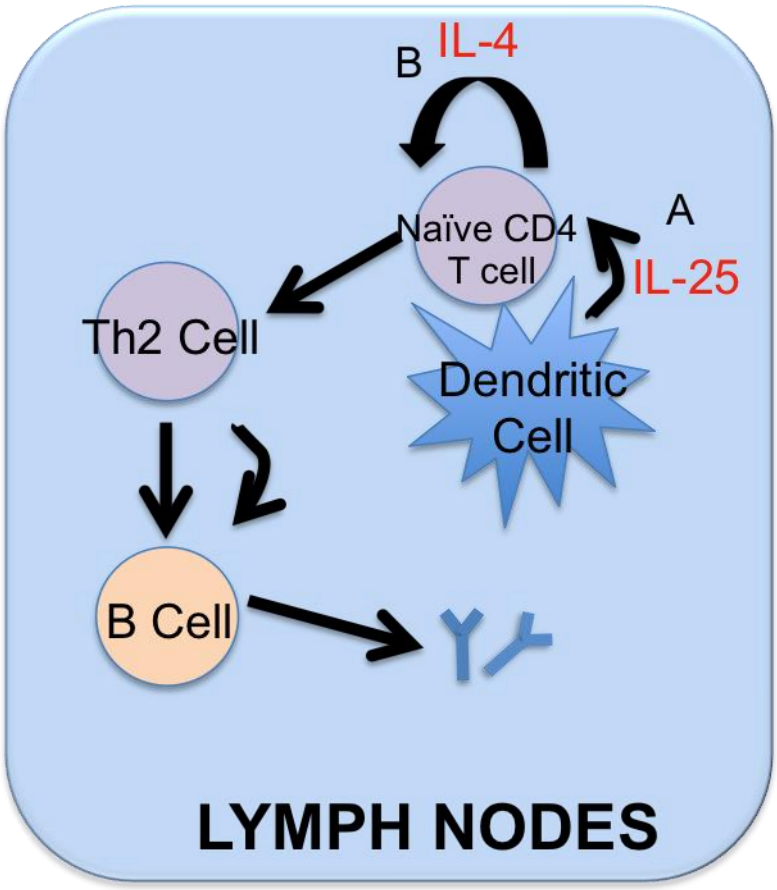

Figure 30: The role of IL-25 during sensitization. (A) Antigen is acquired by dendritic cells and presented to naïve CD4 T cells in the lymph nodes. As a result, IL-25 can be supplied to naïve CD4 T cells by dendritic cells. (B) IL-25 stimulates Th2 differentiation potentially through production of IL-4 from the differentiating T cells; therefore, driving IL-25 initiated Th2 differentiation. These Th2 cells lead to antigen specific IgE and mast cell sensitization.

4.4.2 Disparity between IL-4, GATA3, and CRTh2 expression

Our results showed that IL-25 alone could induce IL-4, GATA3 and CRTh2 (Fig. 18), and that the IL-25 effect on intercellular IL-4 was similar to adding IL-4 alone (Fig. 20A). However, the IL-25-induced GATA3 and CRTh2 were not as robust as when IL-4 alone was added (Fig. 20B and C respectively), not showing significance under ANOVA analysis across all four conditions (Fig. 8B). This was surprising since IL-4 has been shown to increase GATA3 [39], which then increases CRTh2 expression [122]. While a significant effect may have been noticed if more experiments were performed, other explanations for this divergence may be possible. First, the concentration of IL-25 (50ng/mL) may not have been sufficient. Increasing IL-25 may induce GATA3 over a threshold required for CRTh2 protein expression allowing a difference to be distinguished in GATA3 and CRTh2 expression. Others have shown that Th2 differentiated cultures do not show upregulated GATA3, however, when CRTh2 cells are isolated, GATA3 is significantly upregulated [115]. This suggests that CRTh2 cells do express higher GATA3 and may be a more polarized Th2 subset. In support, other groups have used up to 100ng/ml of IL-25 [149]. While this was a possibility for our experiments, we did not notice a difference between 50ng/ml, 100ng/ml or 150ng/ml in our discovery assay (Table 1), therefore, we went with the lower concentration.

A second hypothesis is that differentiating Th2 cells require another signal, possibly provided by dendritic cells, to upregulate CRTh2. Observations in

dendritic cell-T cell cocultures suggest IL-25 mediates T cell growth [149]. However, our data show IL-25 does not directly mediate proliferation (Fig. 23), suggesting this effect of IL-25 relies on cosignaling from dendritic cells. Indeed OX40L-OX40 interactions between dendritic cells and Th2 cells have been shown to be important for maintaining the Th2 phenotype [163] and data from our lab has shown OX40 is increased on Th2 cells after stimulation (Maclean, in preparation). Another possible signal is Notch as IL-25 has been shown to induce the Th2 polarizing Notch ligand, Jagged1, on dendritic cells [136]. When an activated Notch allele, the Notch intracellular domain (NCID), is introduced into CD4 T cells expression of GATA3 mRNA is increased [108]. Thus, the lack of Notch signaling provided by our T cell culture conditions may suggest that in co-culture/in vivo IL-25 may act through dendritic cells to induce higher GATA3 levels and have a greater impact on Th2 differentiation. It is possible that T cells, differentiating in the presence of IL-25, increase the number of IL-4 expressing cells, sufficient to induce some, but not maximal signals for Th2 differentiation.

Finally, IL-25 may act to induce IL-4 expression in a GATA3 independent fashion. GATA3 is traditionally known to be the canonical driver of IL-4 [37]. In contrast, NFATc1 deficient T cells were unable to develop into IL-4 producing cells [99-100] and ectopic expression of c-MAF in Th1 or B cells was able to induce expression of IL-4 [101]. Therefore, GATA3 does not appear to be the only controller of IL-4 expression. IL-25 mediated activation of NF-κB has been well documented [165-166], and, IL-25 has also been shown to upregulate

NFATc1 and JunB in Th2 cells [176]. Thus, IL-25 may mediate an increase in IL-4 expressing cells that may not be completely reliant on GATA3.

4.5 IL-25 MEDITATES TH2 EFFECTOR FUNCTION

While IL-25 has been shown to have a systemic role in increasing Th2 pathologies we show IL-25 can act directly to increase production of Th2 cytokine effector. Indeed naïve CD4 T cells, differentiated in Th2 favoring conditions, showed increased expression of both IL-5 and IL-13 in response to IL-25 (Fig 24B and D). These factors have been shown to be independently important for allergic inflammation. IL-13 mediates isotype switching, production of IgE [87, 177], mucous production and AHR [178]. Additionally IL-5 is a known eosinophil differentiation factor [88] and eosionphils are imperative in type 1 hypersensitivity (T1H) [179]. Others have reported the ability of IL-25 to induce Th2 cytokine production in a Th2-dendritic cell coculture [149], here we have shown that IL-25 can directly amplify Th2 production.

4.5.1 Unique IL-25 regulated Th2 functions

In addition to Th2 cytokine production, we wondered if IL-25 could also effect expression of other soluble mediators from Th2 cells. When CRTh2- isolated Th2 cells were cultured with IL-25 we noticed an increase in IL-9, sCD40L and sIL-2Rα. The lack of dose response could be due to two factors. First, our range may not have been wide enough to reveal a dose response. Second, we may have started at a maxiamal dose that was already at saturation. However, since these

are prelimanray data we analyzised the effect as with or without IL-25 and found significant effects (Fig. 27).

The observed increased IL-9 production (Fig. 27B) ,is interesting but others have also shown that naïve CD4 T cells stimulated with CD3/CD28, TGF-β and IL-4 produce IL-9 in response to IL-25 [174, 180]. IL-9, similar to IL-25, has been observed to play a protective role in clearing helminths [181] and to increase eosinophil, neutrophil and lymphocyte numbers as well as Th2 cytokine production in the lungs [182]. Taken together these data suggest that IL-25 may meditate some of its protective and/or detrimental effects through IL-9 production.

In addition to IL-9, we also found IL-25 stimulates release of sIL-2Rα and sCD40L. When T cells activated with PHA were incubated with MMP-9 surface IL-2Ra was downregulated, suggesting MMP-9 mediates cleavage [183]. Similarly others have shown a role for MMP-9 in increasing sCD40L from platlets [184]. Since IL-25 increased sCD40L as well as sIL-2Ra from Th2, cells these data suggest that IL-25 may increase MMP9 activity or expression. However, there have been no reports of this kind. sCD40L can activate B cell proliferation [185] and has been shown to be necessary for otpimal IgE production from B cells [70]. Therefore, since our data shows IL-25 acts directly on Th2 cells and increases IL-4 (Fig 18A and 20A) and sCD40L (Fig 27), it suggests IL-25 may play a role in orchestrating IgE responses.

4.5.4 T cell growth

When cell counts were compared in dendritic cell-Th2 cells cocultures, IL-25 was shown to increase cell cell number, suggesting IL-25 may mediate Th2 cell growth [149]. While the authors of this paper contend that IL-25 increases proliferation, cell counts do not ascertain proliferative effects but rather total growth effects. This is because cell counts do not account for cell death and potential effects IL-25 may play in promoting survival or death. However, we asked whether this increased growth was due to direct effects on Th2 cells themselves (Fig. 8B). While we observed IL-4 induced a difference (day 14), there was no growth effect for conditions with IL-25 alone (Fig. 23). Therefore, we conclude that IL-25 does not directly affect Th2 cell growth. However, to truly understand the role of IL-25 on Th2 cell growth/proliferationa carboxyfluorescein succinimidyl ester (CSFE) assay could be performed.

An explanation for this may be that in order for IL-25 to induce T cell growth an additional stimulus is required, such as dendritic cell expression of the Notch ligand Jagged1[186] or OX40L [163]. Therefore, it can be hypothesized that IL-25 can increase necessary costimulatory molecules from a second cell group such as dendritic cells. However, it is imperative to acknowledge that our experimental conditions were not optimized for assessing T cell growth. IL-2, CD3 and CD28 were all included in our cultures and are known to induce T cell growth [187]. Therefore, it is possible that inclusion of these necessary growth factors masked any effect of IL-25. To better understand the role IL-25 plays in Th2 growth, T

cell counts and/or CSFE experiments should be performed under reduced concentrations of IL-2, CD3 and CD28.

4.5.5 Chemotaxis

IL-17A, a family member to IL-25, was shown to induce smooth muscle chemotaxis [171]. Furthermore, lymphocytes have been shown to accumulate in the asthmatic lung of mice and localization is abrogated when IL-25 is neutralized [155]. Therefore, we hypothesized that IL-25 directly induces T cell chemotaxis. However, our data indicates that IL-25 does not mediate Th2 cell chemotaxis. The differential ability of IL-17A versus IL-25 to induce chemotaxis may be due to differences in receptor signaling since IL-17A, but not IL-25, binding to its receptor induces recruitment of TRAF2 and TRAF5 [164, 188]. It has been shown that downstream effects of TRAF2/5 can lead to mRNA stabilization [188] and possibly lead to selective chemokine expression. However, IL-17A was shown to induce chemotaxis in smooth muscle cells, not T cells [189] Therefore, while our data indicates that IL-25 does not mediate Th2 cell chemotaxis, it may have a chemotactic effect on other cell types.

However, IL-25 has been shown to play some role in the accumulation of T cells in the lung. Our data suggest this may be due to its ability to induce expression of IL-13 and/or IL-9. IL-13 treated epithelial cells were shown to release CCL17 [85], CCL5 and eotaxin [86]. Additionally, it was shown that $STAT6^{-/-}$ mice cannot produce CCL17 and CCL22 [190], suggesting a role for IL-4 and/or IL-13 in this expression. Supernatents of IL-9 stimulated epithelial cell lines and

primary epithelial cell lines are able to induce T cell chemotaxis by producing IL-16 and CCL5 [191]. These experiments suggest IL-13 and IL-9 can induce T cell chemokine expression from epithelial and dendritic cells leading to T cell chemotaxis.

We also found that Th2 cells did not show a robust response to the Th2 chemokine, CCL22 after stimulation with CD3/CD28, though they did seem to respond after proliferation (Fig. 28). This suggests that when Th2 cells are encountering antigen they become unresponsive to signals endorsing movement. We also examined whether IL-25 could influence CCL22 mediated chemotaxis by examining expression of it receptor, CCR4. We found that the surface expression of CCR4, in the presence or absence of IL-25, was not affected since CCR4 MFI or number of cells expressing CCR4 were the same (Fig. 29).

Collectively, these findings suggest a potential role for IL-25 in type 1 hypersensitivity (T1H). Since we noticed that CRTh2-isolated Th2 cell lines do not respond to chemotactic signals following stimulation (Fig. 28B) it implies that once Th2 cells have entered the tissue they remain there until antigen stimulation wanes. We have also shown that CRTh2 is downregulated following stimulation (Fig. 13 and 17) that IL-25 could not influence this loss of CRTh2 expression (Fig. 15D). Therefore, in addition to Th2 differentiation, during T1H and allergic reaction IL-25 may exaggerate inflammatory cell accumulation through induction of chemokines and Th2 cytokines such as IL-13 and IL-9 leading to Th2 chemokine production from epithelial and/or dendritic cells (Fig. 31).

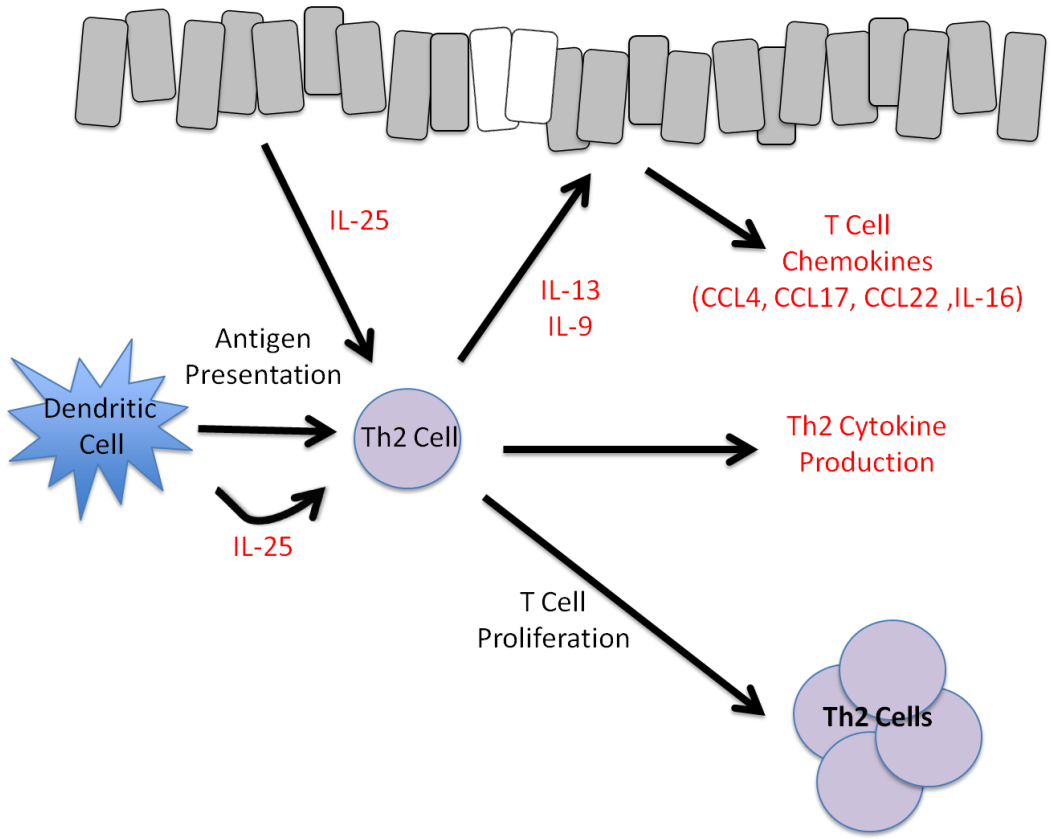

Figure 31: Range of IL-25 effects. During antigen presentation IL-25 can be supplied to Th2 cells by dendritic or epithelial cells. Stimulation of Th2 cells directly leads to Th2 cytokine production, IL-9 production and, in conjunction with dendritic cell signals, T cell proliferation. IL-13 and IL-9 can stimulate production of chemokines (IL-16, CCL5, CCL17, and CCL22) leading to T cell accumulation.

CONCLUSION

We have shown that IL-25 acts directly on naïve CD4 T cells to induce Th2 cell differentiation, marked by IL-4, GATA3 and CRTh2, as well as directly on differentiating Th2 cells to stimulate effector cytokine production. However, the mechanism(s) underlying these effects remains largely unknown. Future work could focus on determining how IL-25 mediates Th2 differentiation. Experiments using neutralizing IL-4 antibodies and monitoring the effect on differentiation would allow us to determine whether IL-25 acts independently of IL-4. In addition to differentiation effects, we observed that IL-25 directly stimulates Th2 cells to produce effector cytokines. While IL-25 has been shown to induce Th2 cytokines in vivo and in cocultures [149, 154], our data indicate that this is at least partly due to direct effect on Th2 cells. Additionally we observed that IL-25 increases the cytokine IL-9 that may lead to the expression of epithelial derived chemokines and subsequent cellular accumulation in the lung.

Studies that strive to further understand the signaling cascades that mediate the IL-25 effects are required. Examining nuclear import of molecules such as NFκB, and cJun after IL-25 treatment by western blot would determine the involvement of these factors. Transcription factor ELISAs to indentify novel factors mediating IL-25 signaling and electromobility shift/chromatin immunoprecipitation assays to examine binding of these transcription factors on the genes of interest (IL-4, IL-5, IL-13, CRTh2, and GATA3) would help uncover IL-25 mechanism(s) of action. In addition to these mechanistic studies further work examining the effect

of IL-25 on soluble mediators such as sCD40L and sIL-2Rα are necessary to understanding the broader role of IL-25.

Collectively, the data presented within this thesis has shown that IL-25 mediates development of Th2 cells, stimulates Th2 effector cytokine production and potentially furthers the inflammatory cascade by mediating recruitment of inflammatory cells to sites of allergic response. The fact that IL-25 is released from the epithelial cells in response to innate immune signals such as TLR activation [192] and virus infection [136] indicates IL-25 provide a link between innate and adaptive immune responses.

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