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Characterization of Il-22-Producing Cells in the Human Thymus and the Mechanism by Which Il-22 Expression Is Modulated by Thymic Epithelial Cells.

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CHARACTERIZATION OF IL-22-PRODUCING CELLS IN THE HUMAN THYMUS AND THE MECHANISM BY WHICH IL-22 EXPRESSION IS MODULATED BY THYMIC EPITHELIAL CELLS.

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LIST OF ABBREVIATIONS AND CELL SURFACE MARKERS

AHR	Aryl hydrocarbon receptor
APC	Allophycocyanin
AF750	Alexa-Fluorochrome 750
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic acid
DL1	Delta-like 1
DL4	Delta-like 4
DSS	Dextran sodium sulfate
eF450	e-Fluorochrome 450
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hr	Hours
IBD	Inflammatory bowel disease
ID2	Inhibitor of DNA binding-2

IFN-γ	Interferon-gamma
ILCs	Innate Lymphoid Cells
ILC1	Group 1 innate lymphoid cells
ILC2	Group 2 innate lymphoid cells
ILC3	Group 3 innate lymphoid cells
IL	Interleukin
IL-7R	IL7 receptor
IL-22R	IL22 receptor
JAK	Janus kinase
LIN-	Lineage negative
LPS	Lipopolysaccaride
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
NKT	Natural Killer cells
NCR	Natural cytoxicity triggering receptor
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCPeF710	Peridinin chlorophyll protein 710
PE-Cy7	phycoerythrin-Cy7
RNA	Ribonucleic acid

RT-PCR	Reverse transcriptase-PCR
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TEC	Thymic epithelial cell
Th	T helper cell
CCR7	C-C chemokine receptor 7
CD3	Cell surface marker for T cell
CD4	Co-receptor for helper T cells
CD8	Co-receptor for cytotoxic T cells
CD56	Cell surface marker for NK Cells

ABSTRACT

Thymic epithelial cells (TECs) are indispensable for T cell development and maturation. Therefore, damages to the thymic epithelial cells are detrimental to thymic function and immune response. In young healthy individuals, TECs have a high regenerative potential and are capable of renewal from serious damage; however, the molecular mechanism of this recovery is unclear. A recent study has shown that IL-22-producing ILCs are present in the mouse thymus and can regenerate thymic epithelial cells following radiation induced injuries (7). However, it is unknown whether IL-22-producing cells are also found in the human thymus. Thus, the goal of this study is to identify IL-22 producing cells in the human thymus and to determine the mechanism by which IL-22 production is modulated. IL-22 is an effector cytokine that has been linked to promoting epithelial survival and proliferation within several secondary lymphoid tissues organs of mice and humans (53). To investigate the presence of IL-22-producing cells in the human thymus, we used flow cytometry and Reverse Transcriptase-PCR (RT-PCR) to analyze IL-22 expression in human pediatric thymocytes.

We reported that the highest percentage of IL-22-producing cells found in *ex vivo* thymocytes are the LIN-RORγt+IL-22+ cells and CD4+ RORγt+IL-22+ T cell subsets. We also reported that the production of IL-22 by the LIN-RORγt+IL-22+ cells and the CD4+ RORγt+IL-22+ T cells are not solely modulated by TECs. Furthermore, although the frequency of circulating RORγt-expressing ILC3-derived IL-22 increased following

PBMC co-cultured with TECs, we could not rule out whether this effect is likely due to allogenic T cell response and/or cytokines produced by the activated allogenic T cells. Since so much is still unknown about IL-22 biology, particularly in the human thymus, the results from study contribute to our current understanding of the biology of IL-22 with the expectation that the new findings provide opportunities towards elucidating the mechanism of thymus regeneration, repair and maintenance.

CHAPTER ONE

INTRODUCTION – LITERATURE REVIEW

Thymic function and thymic epithelial cells

The thymus is an essential lymphoid organ in which T cell development occurs in mice and humans. Following T cell maturation, single positive CD4+ and CD8+ T cells are exported from the thymus into the peripheral and made up the T cell pool with T cell repertoire required for an effective immune response against non-self as well as pathogens (1). Even though age dependent thymic involution affects T cell development and the output of naïve T cells, more evidences are emerging to suggest that a normal thymus can export mature T cells throughout life, although with reduced capacity (1, 2, 3).

One crucial component of the thymic microenvironment is the thymic epithelial cells (TECs) which through cell-cell interactions, cytokine productions and chemokine secretions induce the development and differentiation of functional T cells from hematopoietic stem cells, a process term thymopoiesis (1-3). The thymic epithelium is susceptible to deleterious factors such as stress, inflammation, chronic infections and immunosuppressive drugs, which can lead to impaired thymopoiesis (2-5). Harmful factors such as stress and inflammation can cause tissue injuries. The thymus is believed to be able to recover from these tissue injuries (2-3). However, a more extensive tissue damage such as that caused by myeloabative conditioning for organ transplantation can cause stromal cell injury and thymocyte death leading to lymphopenia (2, 3-6). Previous studies have shown that lethal or sublethal total body irradiation in mice lead to a

dramatic shrinkage of thymic volume and a significant decrease in thymic cellularity (7, 8). For individuals like patients with chronic infections such as HIV, or patients with complications after transplantation such as GVHD, the regeneration of TECs is crucial to maintain thymus homeostasis and thymopoiesis. However, factors that contribute to the proliferation of TECs following damage are not well defined. In fact, there are limited studies investigating the role of TECs in prolonging/restoring thymic function (3). Since the thymus is such an important organ in host defense, therapeutic strategies are needed to regenerate the thymic epithelium to restore thymic function and to re-establish the T cell repertoire following T-cell immunodeficiency. In this context, it is necessary to investigate factors that contribute to the maintenance of homeostasis within the thymic microenvironment and particularly their role in triggering the regeneration program of TECs.

Interleukin 22

IL-22 is a newly characterized class 2 α-helical cytokine that is a member of the interleukin-10 family of cytokines (8-12). The human IL-22 shares 79% homology with the mouse IL-22; the *IL22* gene is located on human chromosome 12 (9, 12, 14). IL-22 signals through a heterodimer receptor complex (IL-22R) which comprised of the subunits IL-22R1 (IL22Rα1) and the IL-10R2 (IL-10Rβ2) (9-16). IL-22 binds first to IL-22R1, and then the IL-22/IL-22R1 complex binds to the IL-10R2 to generate downstream signals (9-16). IL-22 uses the Jak-STAT signal transduction pathway to

induce the phosphorylation of kinase JAK1 and Tyk2 and the STAT1, 3, and 5 transcription factors which then induce proliferative and anti-apoptotic pathways (9, 12, and 13). IL-22 has been shown to also induce MAP kinase, MER/ERK, JNK and p38 signaling pathway (9, 13, and 16). The IL-22R receptor expression is restricted to non-immune cells with epithelial origin such as intestinal epithelial cells, respiratory epithelial cells, keratinocytes, acinar cells and hepatocytes; IL-10R is ubiquitously expressed (9-16). Wolk et al. showed that there is basal level of IL-22Rα1 expression in the skin, pancreas, intestine, liver, lungs and the kidneys (13.14).

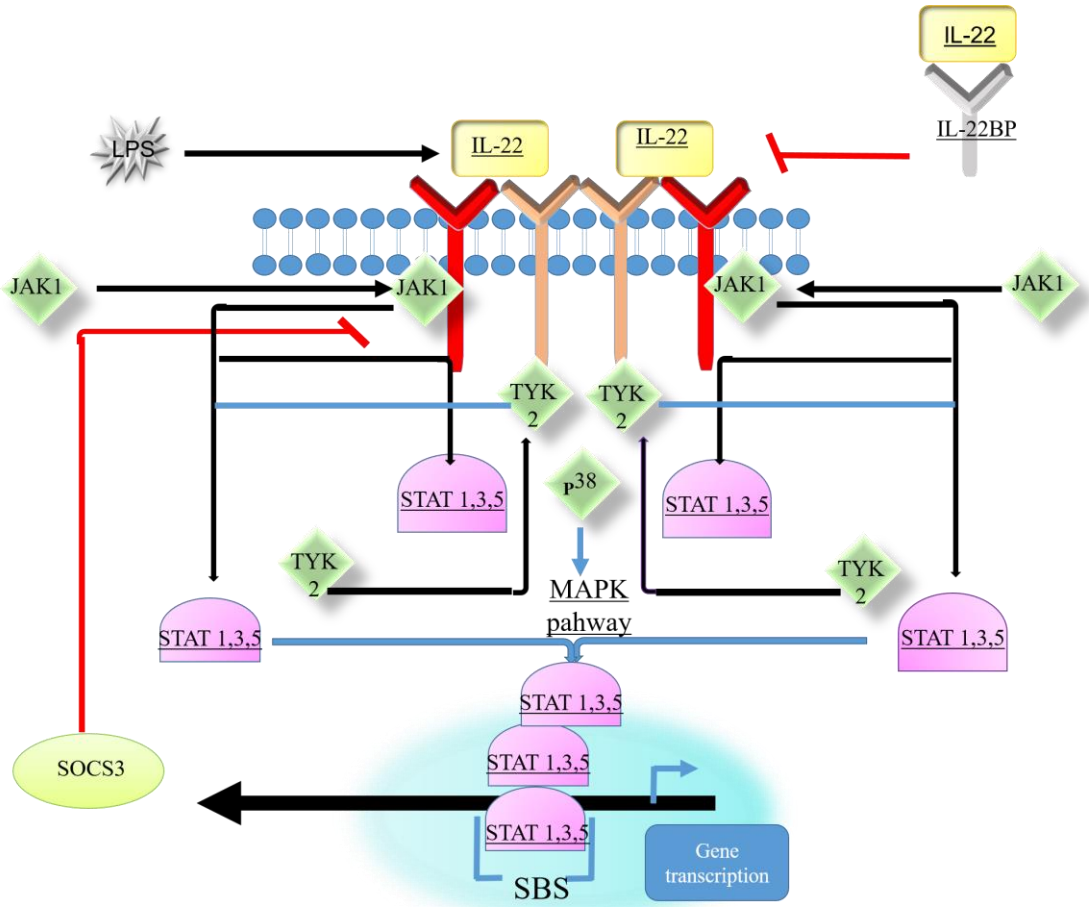

Figure 1. IL-22 signaling pathway. IL-22 binding to IL-22 receptor complex leads to the activation of the receptor-associated Janus kinases JAK1 and Tyk2, followed by activation of transcription factors STAT3, and often STAT1 and/or STAT5. Other signaling pathways that are recruited by this receptor are MAPK (Mitogen Activated Protein Kinase), p90RSK and p38.

Therefore, IL-22 signals non-immune cells, but it is produce by immune cells (13, 14). Binding of IL-22 to its membrane receptor IL22Rα1/IL10Rβ2 is regulated by the competitive binding to the soluble IL-22 binding protein (IL-22Rα2) which has a higher affinity for IL-22 than IL-22Rα1 (17, 18). The source of IL-22 binding protein is mainly $CD11^{+}$ c dendritic cells (DC). IL-22 binding protein is found in many cells and tissues such as the lungs, intestine, skin, pancreas, thymus and spleen (17).

IL-22 function in different tissues

IL-22 receptors are expressed by various cells including keratinocytes, hepatocytes, tracheal and colonic epithelial cells (9-15). There are many documented findings that support a role for IL-22 as a mediator of epithelial barrier function and in maintaining epithelial homeostasis (9-15). IL-22 promotes proliferation and survival by inducing expression of proteins (Muc1, Muc3, Muc10, Muc13) that make up the protective mucus layer, pro-survival genes (Bcl-2, Bcl-XL and Mcl-1), and proliferative factors (c-Myc, cyclinD1, Rb2 and CDK4) (9, 21, 23). In additional, several studies have shown that IL-22 induces mRNA expression of acute phase proteins such as serum amyloid A, α1- antichymotrypsin, and haptoglobin in the HepG2 human hepatoma cell line (13). IL-22 has differential functions in many tissues many of which includes preventing tissue destruction, enhancing wound healing, promoting epithelial cell regeneration, and inhibiting differentiation of keratinocytes (6-30). Below, I will provide a comprehensive review of the role of IL-22 at different barrier tissues.

Very recently, studies have begun to unravel IL-22 role in tissue regeneration in the thymus (7, 8). Van der Brink and colleagues showed that following depletion of CD8+CD4+ T cells by radiation treatment, or by administration of synthetic steroid the

levels of IL-22 in the mouse thymus were elevated. They also demonstrated that IL-22-deficient mice displayed impaired thymic recovery; however, upon treatment with exogenous IL-22, thymic recovery was enhanced. In this study, IL-22 was produced by LTi cells that were induced by IL-23-secreting DC. The authors suggested that IL-22 augments the proliferation and survival of thymic epithelial cells following thymic insult (7). Concurrently, Pan et al. also showed that increased IL-22 expression confers protection following thymic damage induced by high dose of dexamethasone and total body irradiation; both contribute to the depletion of CD8+ CD4+ T cells in the mouse thymus. In addition, these investigators demonstrated that Foxn1 a transcriptional factor that promotes the development of TEC, as well as, genes such as CCL25, and DLL4 were upregulated in TECs following thymic injury. They concluded that thymic insults promote the regenerative program in TECs (8).

It is well documented that IL-22 has protective roles against enteropathogenic bacterial infections such as *Citrobacter rodentium* and *Salmonella enteritidis* (15, 16, 19, 20). The protection occurs when IL-22 acts directly on intestinal epithelial cells, leading to the increase production of antimicrobial proteins such as RegIIIβ and RegIIIγ that promote immune responses against these gut pathogens (15, 16, 19).

Further investigation into the role of IL-22 in the gut and the gut inflammation has led to evidences suggesting that IL-22 has both an inflammatory and a protective role in inflammatory bowel disease (IBD). In one type of IBD (Crohn's disease), studies have shown that upregulated IL-22 levels act on subepithelial myofibolast and promote the expression of other pro-inflammatory cytokines and matrix degrading proteins. Patients

with Crohn's disease have been shown to have an elevated number of CD4+ IL-22-producing cells as well as an increased level of LPS-binding protein (23).

On the other hand, studies have shown that IL-22 confers protection when colitis, another IBD, was induced by acute dextran sodium sulfate (19). In this colitis model, IL-22 acts on the intestinal epithelial cells, resulting in increased expression of pro-inflammatory genes necessary for enhancing the integrity of intestinal barrier through inducing intestinal cell migration and antimicrobial peptides (19).

Yet in another study, in which intestinal inflammation is induced by GVHD, IL-22 was shown to have a protective role. Hanash et al., corroborated this finding in their study by using bone-marrow chimeras as secondary transplant recipients to elegantly illustrate that transplantation of MHC mismatched bone marrow into IL-22-deficient recipients caused more severe pathology in the intestinal tract (21). The findings demonstrated that the recipient-derived hematopoietic cells produce IL-22 which protects epithelial stem cells from GVHD-induced cell death (21).

In addition to the GI tract, IL-22 also has been shown to play a protective role in inflammatory conditions in organs such as the liver, pancreas and lungs. A report by Zenewicz et al. studying the role of IL-22 on hepatocytes during acute liver inflammation demonstrated that IL-22 deficient mice are extremely susceptible to hepatitis, and that adoptive transfer of IL-22+ Th17 cells protected hepatocytes during ConA- mediated hepatitis (22). In another study, it was also shown that IL-22 prevents systemic inflammation though a mechanism that involves expression of lipopolysaccharide-binding protein in hepatocytes (23). Furthermore, IL-22 confers survival of hepatocytes by upregulating anti-apoptotic proteins such as Bcl-xL and Bcl-2 via STAT3 (23).

Data from previous work also suggest that IL-22 mediates direct protection against tissue destruction in a mouse model of hepatitis and acute pancreatitis (24-26). Radaeva et al. reported that there is a significant IL-22 production and T-cell infiltration following Concanavalin A induce hepatitis (24). Colletti et al. expanded on these findings showing that IL-22 plays a direct role in hepatocytes protection which is not limited to T cell-mediated hepatitis. They further demonstrated that there was an increase in IL-22Rα levels at early time point after partial hepatectomy, and that blocking of IL-22 with a neutralizing IL-22 antibody could delayed liver regeneration (25). In the context of the pancreas, Xue, Jing et al., found that IL-22 provides protection against acute pancreatitis by upregulating IL-22Rα1 levels on pancreatic acinar cells following the administration of exogenous IL-22. The effect of administration of IL-22 led to a reduction of acute pancreatitis and associated lung injury, which is mediated through AhR. Mice with blunted AhR activation develop acute pancreatitis and the protective effect of IL-22 is specific because IL-22 blockade abrogate the effect (26).

In the lungs, studies have shown that IL-22 is critical for promoting host protective immunity against bacterial pathogens, reducing inflammation and promoting lung tissue repair (27-30). For example, during *Klebsiella pneumonia* infection IL-22 promotes the production of inflammatory mediators such as IL-6 and G-CSF and chemokines such as CXCL1, CXCL5, and CXCL9 from air way epithelial cells (27). Although IL-22 have no substantial direct role on viral pathogens, Kumar et al. showed that following epithelial cell damage by influenza infection in mice, IL-22 derived from conventional NK cells were crucial for the regeneration of tracheal and epithelial cells after injury (28). In this study, IL-22-deficient mice challenged with influenza virus failed

to regenerate tracheal epithelial cells and displayed a decrease in the proliferation of tracheal epithelial cells. However, adoptive transfer of IL-22 sufficient NK cells into IL-22-deficient mice challenged with influenza virus restored the regeneration of tracheal epithelial cells as well as protect against inflammation (28). In additional, Taube et al. also showed a role for IL-22 produced by innate lymphoid cells in limiting allergenic airway disease, since the administration of exogenous IL-22 to allergen sensitive *Il-22* deficient mice prior to challenge with antigen displayed reduction of allergic asthma mediated cytokines, as well as, a reduction in inflammation and airway constriction (29).

In addition to anti-inflammatory role, IL-22 has been suggested to play a pro-inflammatory role in psoriasis, a chronic autoimmune disease of the skin (14, 16, 31). Investigators focus on characterizing the effect of IL-22 on keratinocytes have so afar shown that IL-22 induces antimicrobial proteins, reduces differentiation-associated protein, and promotes mobility regulating proteins such as matrix metalloproteinase 1 and 3 (13, 14).

The effect of IL-22 on keratinocyte mirrors the phenotype of psoriasis's patients; furthermore, psoriatic plaques and the blood of psoriasis's patients displayed high expression of IL-22 (14, 31). Wolk et al., confirmed the role of IL-22 in psoriasis with the observation that transgenic mice that over-express IL-22 displayed psoriasis-like skin alterations (31). Adding to this finding, Zheng et al., used IL-22-deficient mice to demonstrate that the ablation of IL-22 diminished IL-23-dependent dermal inflammation (32). Overall, these data demonstrate that depending on the cytokine and tissue microenvironments or target cell types, IL-22 display either protective or inflammatory effects (23-32).

Innate lymphoid cells

IL-22 are produce by group three innate lymphoid cells (ILCs). ILCs are a heterogeneous population of cells that are important in innate immunity and lymphoid tissue formation (17, 19, 22-28). ILCs lack lineage markers (LIN-) and in humans are defined as negative for CD1a, CD3, CD11c, CD34, CD123, TCRαβ, TCRγδ, BDCA2, FcεRl, CD19, CD14 and CD16 (22-25). All LIN- ILCs express the lymphoid progenitor marker IL-7 receptor α chain (IL-7Rα; CD127) and the cytokine common gamma (γc) receptor chain (22, 27). ILCs require IL-7 and ID2 (inhibitor of DNA binding-2) for their development and maintenance (23-27).

ILCs produce many helper (Th) cell-associated cytokines (Figure 2 and 3) and can be divided into three distinct cell subsets: ILC1s, ILC2s and ILC3s based on their cytokines production and transcription factors expression (17, 2-23-24, 28). Within the group three ILCs (ILC3s) are subtypes that produce IL-22 and depend on the transcriptional factor RORγt (*RORC*- human analogue) for their development and function (16, 21-24, 28). In humans, RORγt+IL-22+ ILC3 cells express the natural cytoxicity triggering receptor 46 (NKP46) as well as NKP30 and NKP44; NKP44 is unique for humans and is not expressed in mice. The ILC3s subtype NCR+IL-22+ is the proposed new nomenclature; previously, these cells have been referred to as NCR22, NKR-LTi, NK22 and ILC22 cells (17, 23-28, 53). NCR+IL-22+ILC3s have been shown to originate from a lineage distinct from the conventional Natural Killer (NK) cells

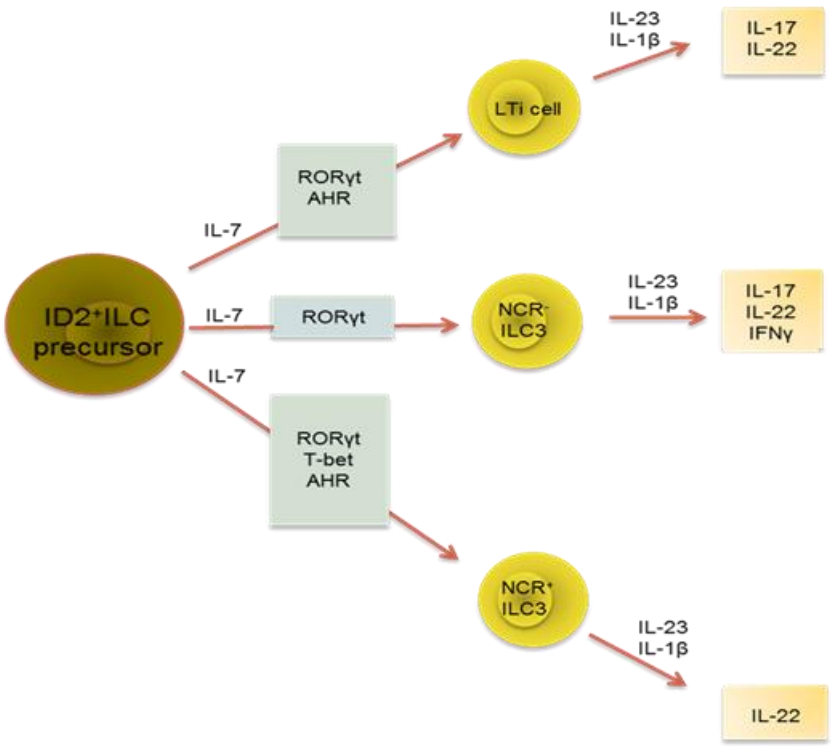

Figure 2. **Group 3 innate lymphoid cells (ILC3s).** Group 3 ILCs are characterized as LIN-cells ($CD3^-CD4^-CD8^-$) that express the transcriptional factor RORγt. There are three subgroups of ILC3s based on the expression of NCR and cytokine production; NCR+ILC3 have been shown to produce only IL-22 while NCR-ILC3 and LTi cell produce IL-17 and IL-22. LTi cells are mostly present during fetal development.

although they both express NK receptor (NKP44) and over 50% of NCR+ILC3s also express CD56 (29, 30). As shown in Figure 2, other subtypes of group 3 ILC3s include NCR-ILC3s and LTi cells. These cells also depend on RORγt and IL-7Rα but have been shown to produce both IL-22 and IL-17. In mice and humans, NCR+IL-22+ILC3s are present in secondary lymphoid organs such as the spleen, lungs, lymph nodes, tonsils and other intestinal lymphoid tissue (17, 23-30). Murine studies have shown that ILC3s development and function depended on several different cytokines, chemokines and

transcriptional factors. ILC3s express the receptors IL-1R1, IL-7R, IL-23R, IL-2Rβγ and c-kit (5, 17, 23, 22-24, 29). Studies have indicated that IL-22–producing ILC3s can be activated by cytokines released by the epithelium (17, 28-31). These cytokines include IL-23, IL-7, IL-21, IL-6 and IL-1β that can stimulate IL-22-producing cells differentiation, function and survival (17, 22-31). In additional, ILC3s express CCR6 the chemokine receptor for CCL20, which influences the localization and expression of IL-22 and may be involved in the migration of ILC3s to different periphery tissues from the bone marrow (17, 22, and 32).

The development of NCR+IL-22+ILC3 and the regulation of IL-22 expression by environmental cues

The transcriptional factors RORγt, AhR and Notch have been shown in murine models to be required for ILC3 development (9, 8, 17, 20, 24, 28, 29, 31). The lack of RORγt in mice impairs the development of ILC3s (17, 28). Several studies have indicated that mouse innate lymphoid cells that are RORγt negative fail to produce IL-22 (7, 8, 18, 24 and 26). *Ahr*-deficient mice showed reduced expression of IL-22 in the small intestine and colon which suggest a role for AhR in the maintenance and functions of ILC3s (17, 39).

There are not many studies addressing the role of Notch signaling in the development of ILC3s. These studies have shown that in mice, AhR induces ILC development in a Notch -1 and Notch -2 dependent manners (33-35). Further evidence supporting a role for Notch signaling includes studies which showed that blocking Notch signaling in hematopoietic cells reduced the levels of NKp46+ ILCs in the intestinal lamina propria (17, 28, 33, 36). Several studies have also link the role Notch signaling in

ILC development and function within a specific microenvironment of ILCs (9, 17, 33-34, 36-38). One study has indicated that prolong Notch signaling in the mouse thymus induced the differentiation of T cells over ILCs (9). Interestingly, another study has also recently shown that higher strength Notch signaling favors ILC2s generation over T cells in the human thymus (38).

IL-22 expression and regulation in T cells population

In humans, IL-22 is produced mainly by activated T cells and NK cells (9, 12, 13). The major αβ T cell subsets that produce IL-22 are T helper 22 (Th22) cells, Th1 cells and Th17 cells. The presence of the different cytokines (Figure 3) and the expression of different transcriptional factors contribute to the differentiation and maintenance of these cell types.

Th17 cells were the first cell subset to be characterize as IL-22 producers. It was later shown that Th22 a subtype of Th17 cells produced IL-22 but not IL-17. It is unclear whether RORγt is necessary for these cells function and maintenance, however like the NCR+IL-22+ILC3s, Th22 cells express CCR6 (12,13). In human peripheral blood, about 35% of all IL-22-producing CD4+ T cells were Th1 cells (10). The cytokines IFN-γ and Tbet are important for IL-22 production by the Th1 cells. Th1 cells have high expression of IL-18R, and studies have shown that IL-12 and IL-18 synergize to enhance IL-22 expression in Th1 cells (10). Other αβ T cell subsets that produce IL-22 include a functional distinct subset of CD8+T cell termed Tc22 cells. Studies have shown that IL-21 can induce human naïve CD8+ T cells to differentiate into Tc22 cells, however, these cells are found mostly in the tissue or blood following infection and cancer (12). These

cells also express the transcriptional factor RORγt. Notch plays an important role in the regulation of peripheral T cell differentiation. Since the Notch

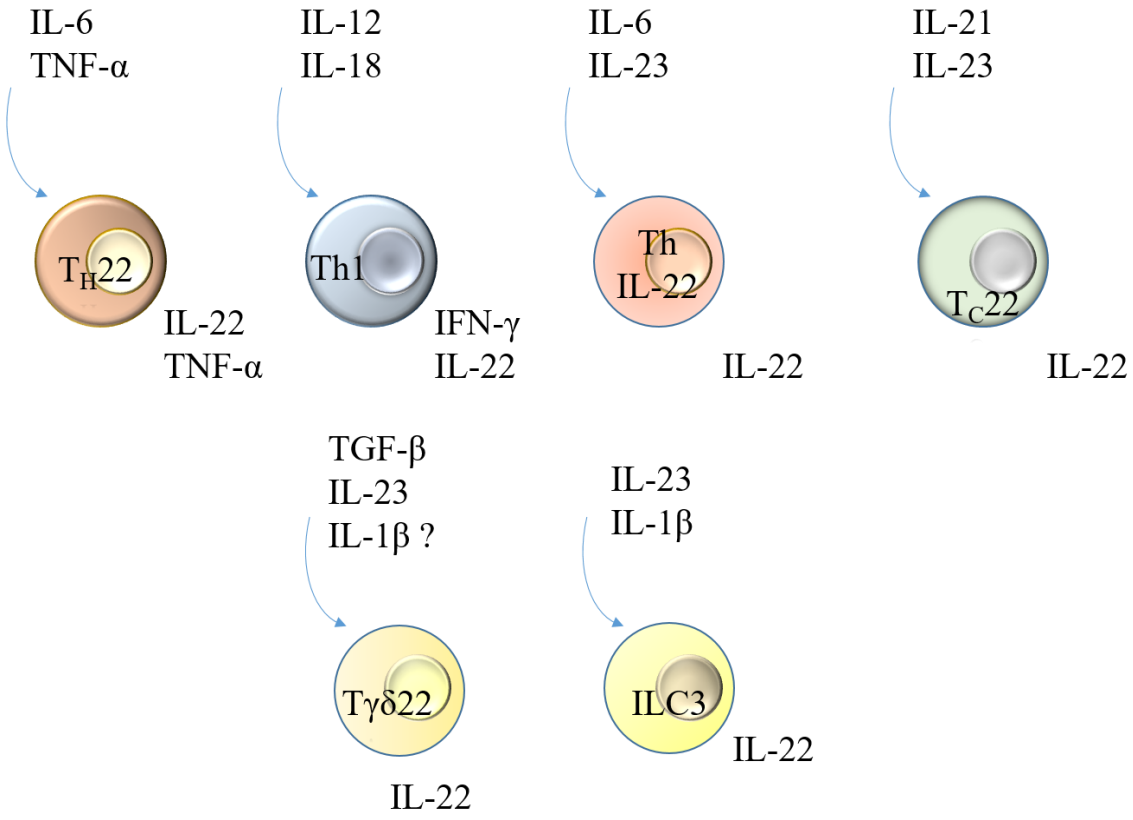

Figure 3. **The different subsets of IL-22-producing cells.** The different cytokines required for the differentiation of cells into the different subsets of IL-22-producing cells.

ligand DL4 promotes the expression of RORγt+ and expansion of CD8+ T cells and CD4+ T cells (40-42). Other innate cell types that produce IL-22 include γδT cells, NK (natural killer) T cells and NK cells. Murine IL-22-producing γδT cells express high level of IL-18R. (8). Studies have shown that in these cells IL-18 together with IL-23 induce

IL-22/IL-17 production, and enhance RORγt+ and IL-23 expression (13). IL-22-producing γδT cells express RORγt+, AHR and CCR6. Studies have also shown that NKT cells isolated from mouse spleen can be stimulated by αCD3/αCD28 to produce IL-22 (13, 28). IL-22-producing NK cells found in the gut mucosa have also been described in both humans and mice.

CHAPTER TWO

SPECIFIC AIMS

Thymic epithelial cells (TEC) are an essential component of the thymic microenvironment and play pivotal roles in the generation of mature T cells expressing T cell receptor αβ chain through a process termed thymopoiesis (1-3). Thymic tissue injuries caused by radiation, chronic inflammation, immunosuppressive therapies and infection lead to alterations in thymic epithelial cell function and survival, resulting in thymic dysfunction leading to compromised immune (1-4). Therefore, the regeneration of thymic epithelial cells following these situations is critical to maintain thymic function. Several murine studies have shown that the production of IL-22 by IL-22- producing cells promote epithelial cells proliferation, differentiation and survival in the gut, lungs, liver and spleen (7-12). Recent studies in mice have shown that following radiation treatment, innate lymphoid cells group 3 (ILC3s) in the thymus produce IL-22 that induces epithelial cell proliferation, leading to the regeneration of thymic function caused by the radiation induced thymic injury (7). Whether IL-22-producing ILC3s are also present in the human thymus has yet to be investigated. I hypothesized that IL-22-producing cells are present in the human thymus. Because, TECs are known to function through cell-cell contacts and the production of cytokines, I further hypothesized that that interaction between TEC and IL-22-producing cells modulates IL-22 expression. Lastly, because TEC express the Notch ligands Delta-like 1 (DL1) and Delta-like 4 (DL4), I investigated whether Notch signaling mediates IL-22 expression in

IL-22-producing cells. To test the above hypotheses, I proposed the following two specific aims:

Specific Aim 1. To determine if IL-22-producing cells are present amongst human thymocytes and the identities of the IL-22-producing cells.

a) Determine whether the LIN- (CD3-CD4-CD8-CD56-) RORγt+ expressing IL-22 cells (ILC3s) are present in pediatric human thymocytes by flow cytometry.
b) Determine whether the sorted LIN- (CD3-CD4-CD8-CD56-) RORγt+ (ILC3s) from pediatric human thymocytes express IL-22 mRNA.

Specific Aim 2: To determine if thymic epithelial cells (TECs) modulate IL-22 expression by thymic and circulating ILC3s and whether the modulation is mediated by Notch signaling via DLL4 ligand.

a) Determine whether co-culturing human thymocytes and TECs increases the frequencies of LIN-RORγt+IL-22-producing cells in a Notch dependent manner.
b) Determine whether TECs also regulate the frequency of circulating peripheral blood LIN-RORγt+IL-22-producing cells in in a Notch dependent manner.

CHAPTER THREE

RATIONALE AND EXPERIMENTAL DESIGN

Specific Aim 1. To determine if IL-22-producing cells are present amongst human thymocytes and the identities of the IL22-producing cells.

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Rationale: A recent study has identified a population of RORγt+ innate lymphoid cells in the mouse thymus. These cells were shown to be critical for thymic tissue repair following radiation damage (21, 22). Literatures have established that RORγt+ IL-22-producing cells are present in tonsil, lung, spleen and intestine in both mice and humans (17, 23-30). Although RORγt+ IL-22-producing cells are found in the mouse thymus, it is unknown whether phenotypically similar populations of RORγt+IL-22-producing cells are also present in the human thymus. The goal of Aim 1 is to determine whether RORγt+ IL-22-producing cells (ILC3s) are present in the human thymus.

Experimental Design:

1A. Determine if the LIN- RORγt+ IL-22-producing cells are present in pediatric human thymocytes. To investigate the presence of IL-22-producing (ILC3s) in the human thymus, we used flow cytometry to determine the phenotype of IL-22-expressing cells within cryopreserved *ex vivo* pediatric human thymocyte samples. For analysis of the percentage of RORγt+ IL-22-producing cells within the lineage negative cells, two different negative control method were used: an isotype control for both RORγt and IL-22, and a RORγt negative control since cells that are RORγt- fail to produce

IL-22. All flow cytometry data were analyzed using fluorescent activated cell sorting (FACS) on a Fortessa and FlowJo software version 7.6.5 (Tree Star, Inc, Ashland, OR). The average percentages of LIN- IL-22+RORγt cells in thymocytes from three different donors were calculated for statistical analysis.

1B. Determine whether sorted lineage negative pediatric human thymocyte sample express IL-22 mRNA. To confirm the presence of IL-22, lineage negative (CD3-CD4-CD8-) thymocytes and the T cells subsets (CD4+ T cells, CD8+Tcells, CD8+CD4+ T cells and CD3-CD4+CD8+ T cells) within the human thymocyte population were sorted from *ex vivo* cryopreserve human thymocytes and IL-22 transcript levels of the sorted populations were measured by RT-PCR. Expression of GAPDH was used to establish relative expression levels.

Specific Aim 2: Determine if thymic epithelial cells (TECs) modulate the expression of IL-22 in RORγt+ IL-22+ cells and whether modulation of IL-22 expression is Notch dependent.

Rationale: TECs have been shown to an essential function in the development of T cells (1, 2, 4, and 14). TECs mediate their functions through bidirectional signaling involving direct contact by ligand–receptor interaction and cytokine production (4, 14, 20 and 21). IL-22-producing cells (ILC3s) can be activated by cytokines produce by epithelium (17, 28). TECs has been shown to express Delta-like 1 and Delta-like 4 ligands that bind to the receptor Notch-1 expressed on other cell types (22, 23, 24).

Several studies have implicated a role for Notch signaling in innate lymphoid cell development and function through the induction of Notch 1 and Notch 2 by AhR (9, 17, 29, 34, 35, 37-39 and 30). Our first goal is to determine whether interaction between thymocytes and TECs can increase the percent and total cell number of IL-22-expressing cells within the thymocyte population; and secondly, to investigate whether IL-22 expression can be increase by TECs in a Notch dependent manner.

Experimental Design:

2A. Determine if interaction between thymocytes and TECs can increase the frequencies of LIN-RORγt+ cells in the human thymocyte population and whether this modulation is Notch dependent. To test whether Notch plays a role in TEC regulation of IL-22 expression by LIN- RORγt+ cells, we used flow cytometry to compare the percent and the total cell numbers of LIN- RORγt+ IL-22-producing cells when thymocytes were co-cultured with two different TEC cell lines: TEC-DL4 which overexpress the Notch ligand DL4 and TEC-84 which expresses low levels of human DL4. Thymocytes were cultured for 2 and 4 days with TEC lines and harvested following 2 hrs of monensin treatment. The cultured thymocytes were analyzed for cytoplasmic IL-22 by flow cytometry. Isotype controls for RORγt and IL-22 were used to define RORγt positive and IL-22 positive population in the LIN- thymocyte subset. The Mann-Whitney U test was used for statistical test and significance was determined at $p \leq 0.05$.

2B. Determine whether TECs also regulate the frequency of circulating peripheral blood LIN-RORγt+IL-22-producing cells in in a Notch dependent manner.

Rationale: The microenvironment of ILCs has been shown to have different influence on their development and their regulation (8, 25, 26, 21, 28 and 31). Since ILC3s have been shown present within the peripheral blood, we next determine whether interaction between PBMCs and TECs could increase d IL-22 expression in ILC3s and whether enhanced IL-22 expression is Notch dependent. To determine if interaction between PBMCs and TECs can increase the percentage and the total cell numbers of RORγt-expressing ILC3s within peripheral blood. Human PBMCs were co-cultured with TEC-DL4 or TEC-84 and then the non-adherent cells were analyzed using flow cytometry. To test this, PBMCs were cultured 4 days with each TEC line, harvested following monensin treatment and IL22-producing cells were assessed by flow cytometry. For analysis, I gated on the LIN- population, then assess fractions that are positive for RORγt and IL-22 as described in previous sections. The Mann-Whitney U test was used for statistical test and the significance was determined at $p \leq 0.05$.

CHAPTER FOUR

RESULTS

Identifying LIN-IL-22+RORγt +cells within the human thymus

IL-22 expressing cells have been identified in mice thymus, however it has yet to be shown that a phenotypically and functionally similar cell subset is present in the human thymus. We have investigated the presence of IL-22-producing cells in the human thymocytes. As shown in Figure 4, LIN- cells were selected for which were further analyzed for the IL-22+RORγt+ cells. The quadrants for positive and negative were based on unstained cells, cells stained with isotype control for IL-22 and RORγt, and by RORγt negative controls gating. Based on our isotype control gating strategy, we observed that an average of 1.08 % of the lineage negative thymocyte population were RORγt+ IL-22+ cell (Table 1). In contrast, gating based on RORγt negative population showed an average 1.49% RORγt+ IL-22+ cells (Table 2). Since these cells express the identification markers associated with group 3 innate lymphoid cells we term them ILC3s and therefore there is an average 1.0-1.5% ILC3s found in the human thymus at steady state.

We next evaluated the presence of other IL-22-producing cells such as γδ T cells, CD8+ and CD4+ T cells which are found in the human thymus that have been indicated to produce IL-22 in other organs (17, 23-30). Figure 5 is a representative flow cytometry profile of the CD3+CD8+CD56- T cells. The CD4- CD8- T cells, CD4+ T cells, CD8+T cells and CD4+CD8+ T cells were defined within the CD4 CD8 lymphocyte gate followed by gating on CD3+ and CD56- cells.

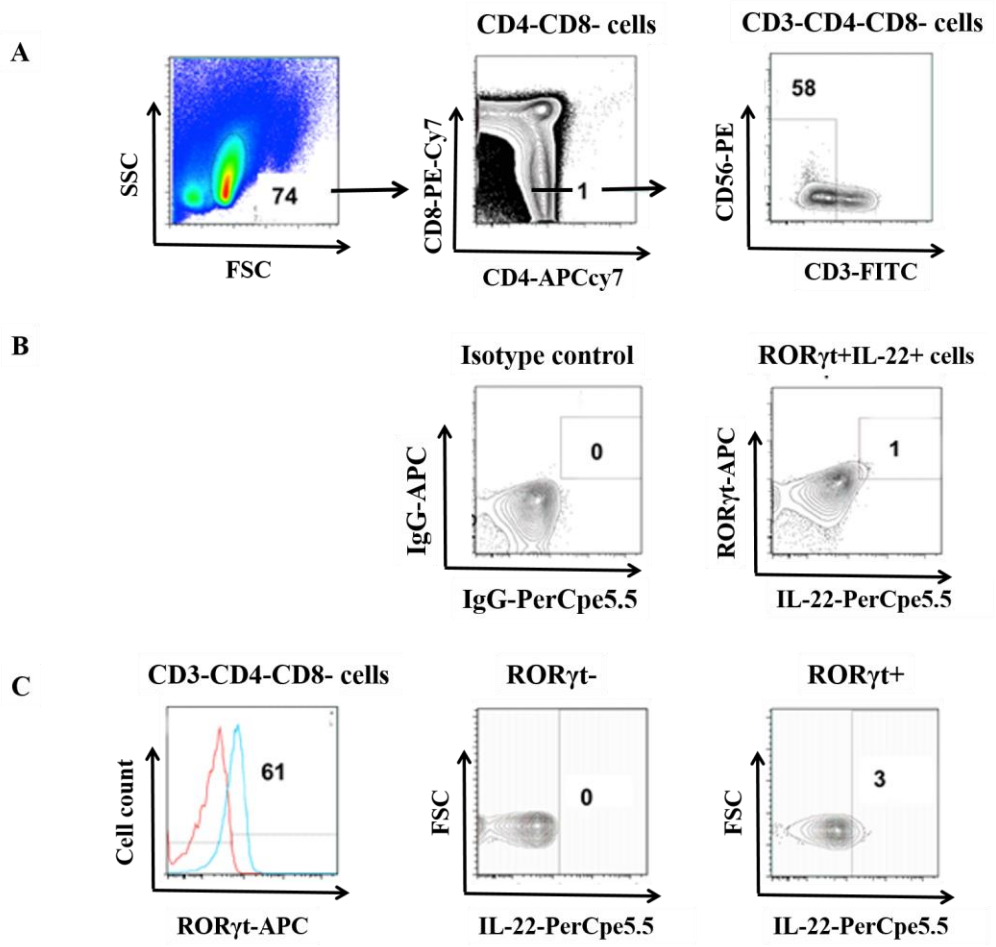

Figure 4. LIN- IL-22+ RORγt+ cells are detected within the human thymus. (*A)* Identification of LIN- cells. 1-2 x 10^6 *ex vivo* thymocytes (sample 64) were stained with surface markers CD3, CD4, CD8, and CD56 and then intracellularly stained for IL-22 and RORγt; middle panel shows the gating for CD4-CD8- cells; right panel shows gating for the CD3 negative and CD56 negative population. (*B and C)* Two strategies to detect RORγt pos IL-22 pos populations: *(B)* Based on isotype control for RORγt; *(C)* Based on RORγt neg and pos populations. Data shown are from a representative analysis of thymocyte sample 64

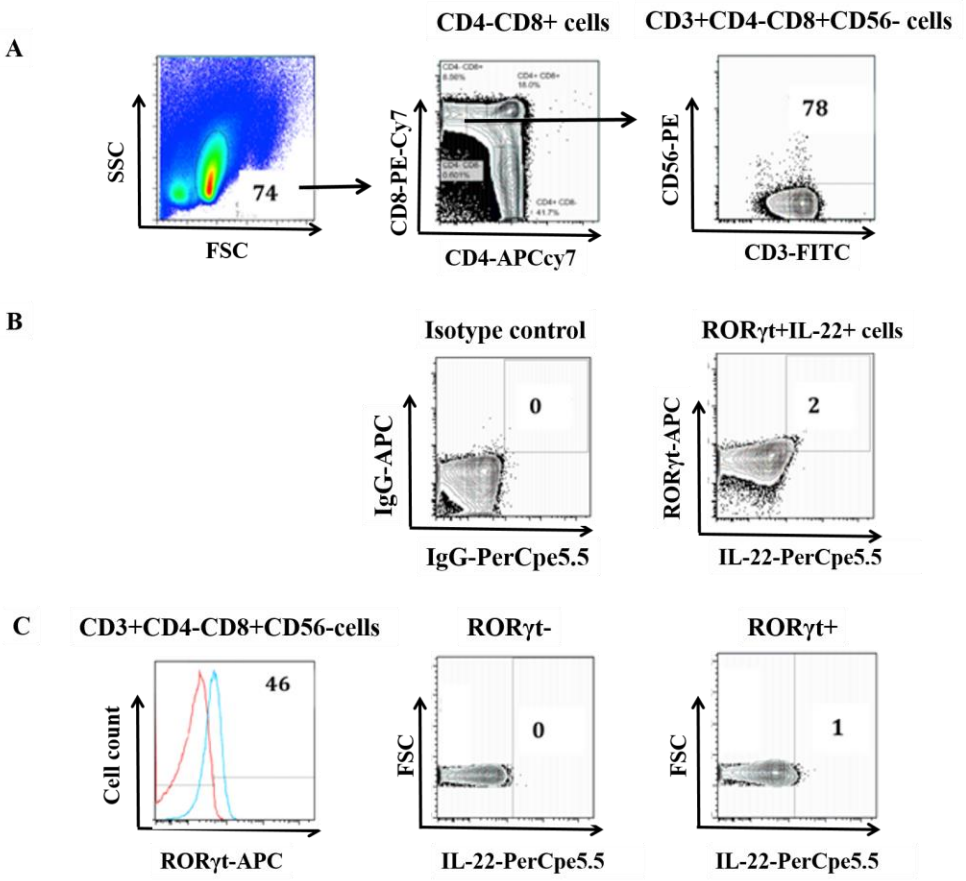

Figure 5. CD3+ IL-22+ RORγt+ cells are detected within the human thymus.
A) Identification of CD3+CD8+CD56- cells. 1-2 x 10^6 *ex vivo* thymocytes (sample 64) were stained with surface markers CD3, CD4, CD8, and CD56 and then intracellularly stained for IL-22 and RORγt. Thymocytes were gated first on CD4-CD8+ then on the CD3+ CD56- population. Data shown are a representative profile of CD3+CD8+CD56- cells using thymocyte sample #64. *B and C)* The CD3+CD8+CD56-RORγt+IL-22+ cells. Data are representative of the gating strategy for isotype control and RORγt negative gating to determine IL-22-expressing cells.

The percentage of IL-22+RORγt+ cells

Thymocyte sample	CD3+ CD4+CD8+ (DP)	CD3+ CD4+ (CD4+ T cells)	CD3+ CD8+ (CD8+ T cells)	CD3+ CD4-CD8- (DN)	CD3- CD4-CD8- (ILC3)
64	0.37	1.01	0.79	0.4	0.32
12	2.17	1.37	2.67	0.27	1.8
14	1.33	0.94	1.97	1.21	1.11
Avg	1.29	1.11	1.81	0.63	1.08

Table 1. Distribution of RORγt+ IL-22+ cells in human thymus Three *ex vivo* cryopreserved pediatric thymocyte samples were evaluated to determine the phenotype of IL-22-expressing cells within the human thymus. 1.0-2.0 x 10^6 thymocytes were stained with surface markers CD3, CD4, CD8, and CD56 then intracellularly stained for IL-22 and RORγt. The percent of CD4, CD8 populations was determined from the viable lymphocyte fraction followed by selecting for CD3 fractions. Quadrants for positive and negative were based on isotype control for IL-22 and RORγt.

For the analysis of the percentage of RORγt+ IL-22-producing cells within the thymocytes population again we used our two-different negative control method: an isotype control for both RORγt and IL-22, and gating on the RORγt negative population. We found cells within the thymocyte population that were CD3+CD8+ (CD8 + T cells), CD3+CD4+CD8+ (double positive (DP) cells), CD3+CD4-CD8- (double negative (DN) cells) and CD3+CD4+CD8-(CD4+ T cells) that express both RORγt and IL-22. Using the isotype control (Table 1), we found that an average percentage of the 1.11% CD3+CD4+ T cells, 1.81% CD3+CD8+T cells and the 0.63% CD3+CD4-CD8- cells express both RORγt and IL-22.

Using the RORγt negative population gating (Table 2) we found that an average of 1.13% CD3+CD4+ T cells, 2.89% CD3+CD8+ T cells and the 3.77% CD3+CD4-CD8- T cells that express both RORγt and IL-22. Taken together the data indicate that 1.2% of IL-22-producing cells in the human thymus are CD4+ T cells, 2.4% are CD8+ T cells, and 2.2% are double negative, or double positive cells. There is a lot of variability between the percentages of each cell population from the different samples. These may be due to biological variability in the human samples.

The percentage of IL-22+RORγt+ cells

Thymocyte sample	CD3+ CD4+CD8+ **(DP)**	CD3+ CD4+ **(CD4+ T cells)**	CD3+ CD8+ **(CD8+ T cells)**	CD3+ CD4-CD8- **(DN)**	CD3- CD4-CD8- **(ILC3)**
64	1.31	1.45	2.16	6.12	0.78
12	6.54	1.35	5.28	2.63	0.41
14	1.66	0.6	1.22	2.55	3.27
Avg	3.17	1.13	2.89	3.77	1.49

Table 2. The frequency of RORγt+ IL-22+ thymocytes in pediatric human thymus determined by using RORyt negative population gating strategy. Three *ex vivo* cryopreserved pediatric thymocyte samples were evaluated to determine the phenotype of IL-22-expressing cells within the human thymus. 1.0-2.0 x 10^6 thymocytes were stained with surface markers CD3, CD4, CD8, and CD56 then intracellularly stained for IL-22 and RORγt. The percent of CD4CD8 populations was determined from the viable lymphocyte fraction followed by selecting for CD3 fractions. The percentage of RORγt+ and IL-22+ positive cells were based gating on

IL-22 mRNA expression in sorted LIN- cells and T cell subsets

To confirm that IL-22 was expressed in subsets of human thymocytes, we next investigate IL-22 mRNA expression in these thymocyte subsets. Cells were sorted based on the expression of CD3, CD4, CD8 and CD56. Five different cell subsets were sorted; these include: CD3+CD4+CD56-(CD4+ T cells), CD3+CD8+CD56- (CD 8+ T cells), CD3+CD4-CD8-CD56- (DN cells), CD3+CD4+CD8+ (DP cells) and CD3-CD4-CD8- cells (ILC3s). The RNA was isolated and IL-22 mRNA expression was evaluated in the five different cell subsets (Table 3).

Phenotype of sorted cell subsets

	(ILC3)	(CD4+ T cells)	(CD8+ T cells)	(DN)	(DP)
Thymocyte sample	CD3- CD4-CD8-	CD3+ CD4+CD56-	CD3+ CD8+CD56-	CD3+ CD4-CD8-CD56-	CD3+ CD8+CD4+
64	+	*NP	+	+	+
12	+	+	-	-	-
21	-	+	-	-	-
Final result	+	+	±	±	±

Table 3. The mRNA expression of IL-22 in sorted cell subsets. $40x10^6$ cells were sorted based on the expression of CD3, CD4, CD8 and CD56. The RNA was isolated for each of the sorted subsets and used to perform RT-PCR for IL-22 expression. The **+** sign indicates positive IL-22 expression, *NP indicates that the IL-22 was not evaluated while **–** sign indicates no IL-22 expression. Data are representative of three different patient thymocyte samples.

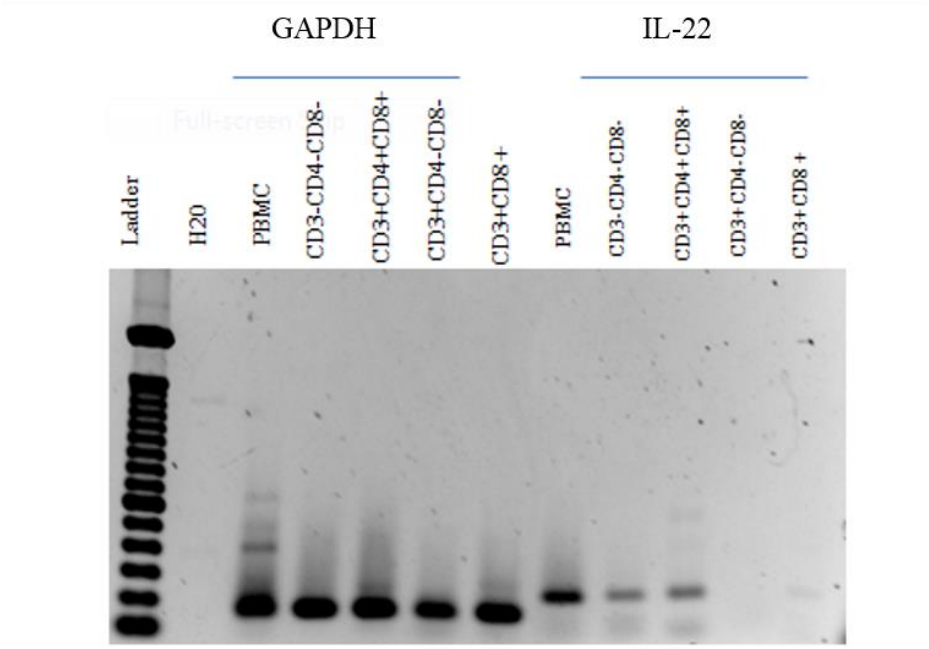

Figure 6. IL-22 mRNA expression in sorted LIN- and T cell subsets. IL-22 relative to GAPDH in LIN- cells sorted from ex vivo cryopreserved thymocyte samples as determined by RT- PCR. PBMC was used as a positive control since it has been shown that PBMC have IL-22 expression. Data are representative of 3 independent experiments using thymocyte 64.

We observed differential expression of IL-22 in the cell subsets for the 3 different thymocyte samples assessed. Thymocyte sample 64 (Figure 6) displayed high IL-22 mRNA expression by all cell subsets except CD4 + T cells that were not process due to low quality RNA. For thymocyte sample 12 we observed IL-22 mRNA expression for only ILC3s and CD4+ cells. While thymocyte 21 showed detectable level of IL-22 mRNA by only CD4+ cells. Overall we found that there was expression of IL-22 mRNA

in the CD3-CD4-CD8- cells and CD3+CD4+CD56- cells with variable expression of IL-22 mRNA in the CD3+CD8+CD56- cells, and CD3+CD4+CD8+ cells (Table 3).

The expression of IL-22 by IL-22+RORγt+ cells following TEC/thymocyte co-culture

We analyzed thymocytes cultured with TEC for the expression of IL-22. Figure 6 is a representative flow panel showing IL-22 and RORγt expression following thymocyte co-culture with TEC; the negative and positive quadrants were established by using both isotype control and RORγt negative control. The data suggest that TECs have some effect on the expression of IL-22 by LIN-RORγt+ cells. However, these differences are not statistically significant (Figure 7). We also found that the percentage and total cell number of the LIN- RORγt+ IL-22+ cells, and the total cell number of LIN- RORγt+ cells within the thymocyte population co-cultured with TEC did not statistically increased in comparison to cells cultured without TEC (Figure 8).

Similarly, we compared the percent and the total cell numbers of the CD3+ RORγt+ IL-22+ T cell subsets within the thymocyte population of each treatment conditions, and found no significant changes in the percent and the total cell number of CD3+ RORγt+ IL-22+ cells, or in the total cell number of CD3+ RORγt+ cells subsets following thymocyte-TEC co-culture (data not shown).

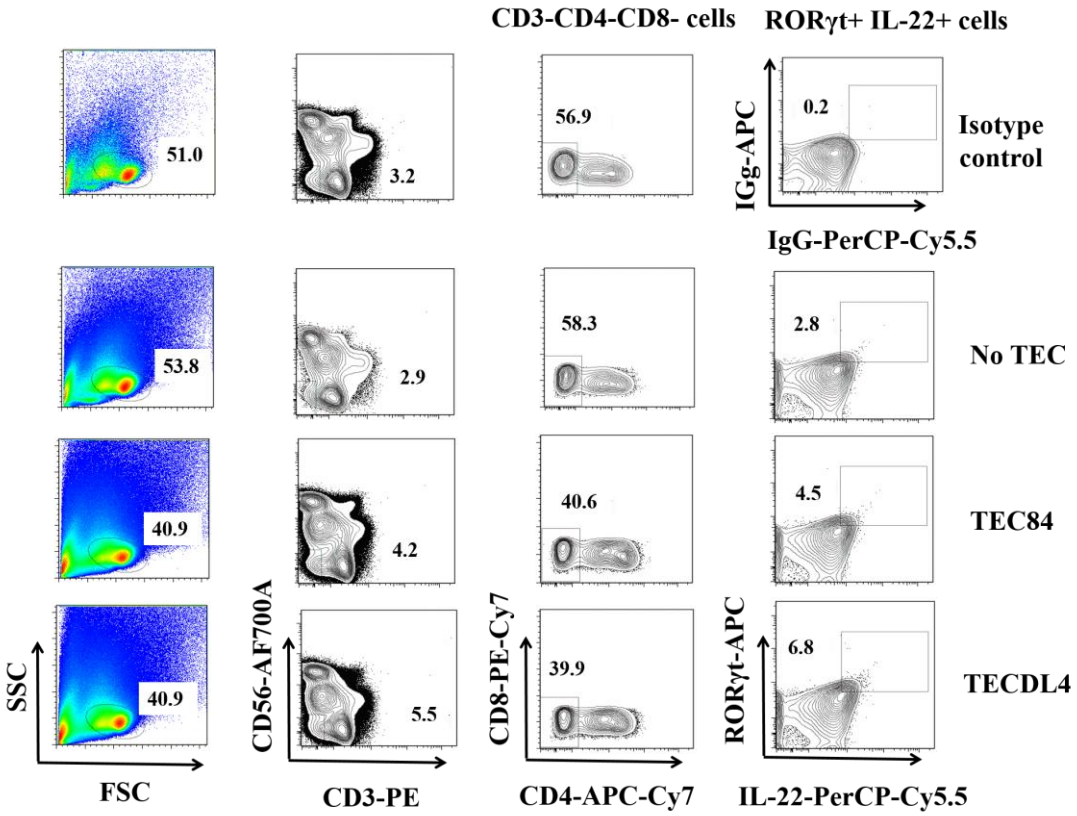

Figure 7. IL-22 and RORγt expression of thymocytes co-cultured with thymic epithelial cells (TECs). Following TEC84 or TECDL4 co-culture with thymocytes (sample 64), thymocytes were harvested, treated with monensin for 2 hrs and analyzed for the expression of CD3, CD4, CD8, IL-22 and RORγt. The positive and negative quadrants were determined using isotype control. The data shown are representative of four independent experiments.IL-22 and RORγt expression in the lineage negative cells within thymocyte population.

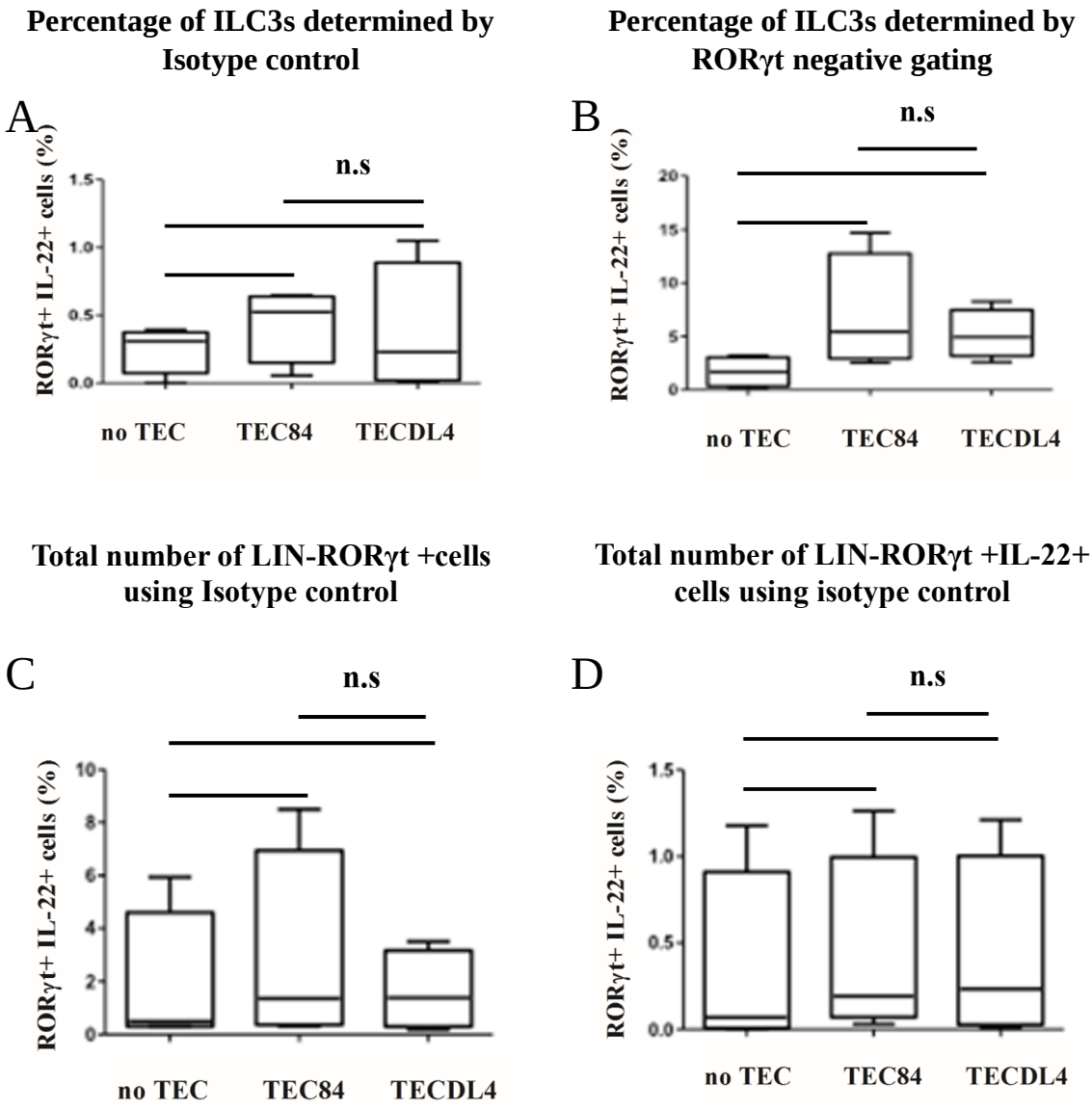

Figure 8. The frequency of IL-22+RORγt+ cells among thymocytes does not significantly increase following TEC/thymocytes co-culture. *(A and B)* Boxplot of RORγt+ IL-22+ cells percent determined by isotype control and RORγt+ negative gating respectively. *(C and D)* LIN-RORγt+ cells and LIN-RORγt+ IL-22+ cells. Data are presented as the total cell number of LIN-RORγt+ cells and LIN-RORγt+ IL-22+ cells within the thymocyte population of co-culture cells. Data represent one of four independent experiments. Boxplot depicts mean, ± SEM and statistics (n.s: not significant, $p>0.05$) represent Mann-Whitney comparisons.

The expression of IL-22 by IL-22+RORγt+ cells following TEC/PBMC co-culture

Upon the examination of IL-22 expression following TEC/PBMC co-culture we found that the frequencies of the LIN-RORγt+ IL-22+ cells within the PBMC/TEC co-culture increased in comparison to the frequencies of LIN-RORγt+ IL-22+ cells cultured alone (Figure 9 and 10). Statistical analysis using data from both controls confirmed that the LIN-RORγt+ IL-22+ cells within the PBMC-TECDL4 co-culture significantly increased in comparison to LIN-RORγt+ IL-22 cells cultured alone (Figure 11A and 11B). Cell co-culture with TEC84 showed significant increased when evaluated with negative gating but not isotype control gating. Thus, we observed no significant difference in the expression of IL-22 by ILC3s when co-cultured with TEC expressing a low level of Notch ligand or a high level of Notch ligand based on negative control gating strategy. However, we did observe a significant difference between ILC3s culture with TEC with low Notch ligand expression or high Notch ligand expression based on our isotype control. While the percentages changed significantly, the total number of LIN-RORγt+ IL-22+ cells and LIN-RORγt+ cells within PBMC-TEC co-culture showed no significant difference as compared to the PBMC cultured alone (Figure 11C and 11D).

We also found that the frequency of IL-22+RORγt+ cells within the CD3+CD4+ (Figure 12) and CD3+CD8+ (Figure 13 and 14) population significantly increased following PBMC/TEC co-culture using both gating strategy. However, there is no statistically significant difference between cells that were culture with TEC that have low Notch ligand expression or high Notch ligand expression.

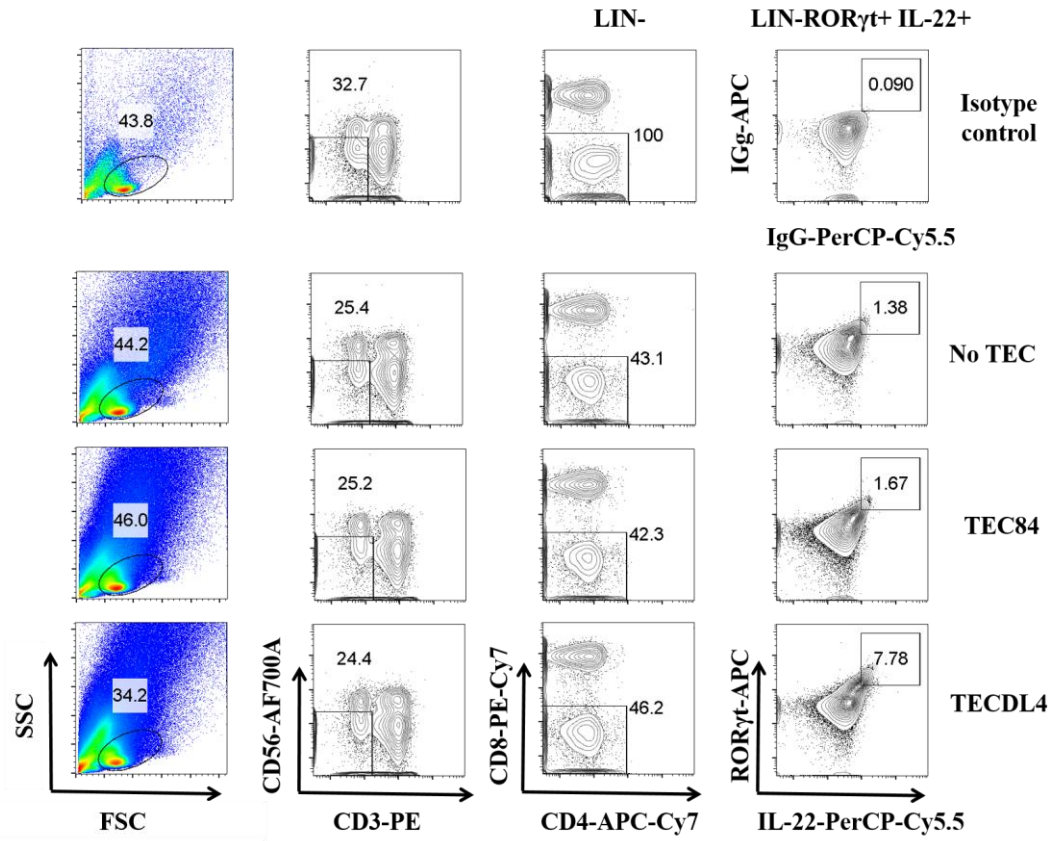

Figure 9. IL-22 and RORγt expression following PBMC/TEC co-culture. Following 72hr co-culture with TEC84 or TECDL4 PBMCs were treated with monensin for 2 hrs and analyzed for the expression of CD3, CD4, CD8, IL-22 and RORγt. The positive and negative quadrants were determined using isotype control. The data shown are a representative of four independent experiments.

There were no differences in the total number of IL-22+RORγt+ cells or RORγt+ cells within the CD3+CD4+ (data no shown) and the CD3+CD8+ (Figure 14C and 14D) populations in the PBMC cultured alone, or co-cultured with low Notch ligand expressing or high Notch ligand expressing TECs.

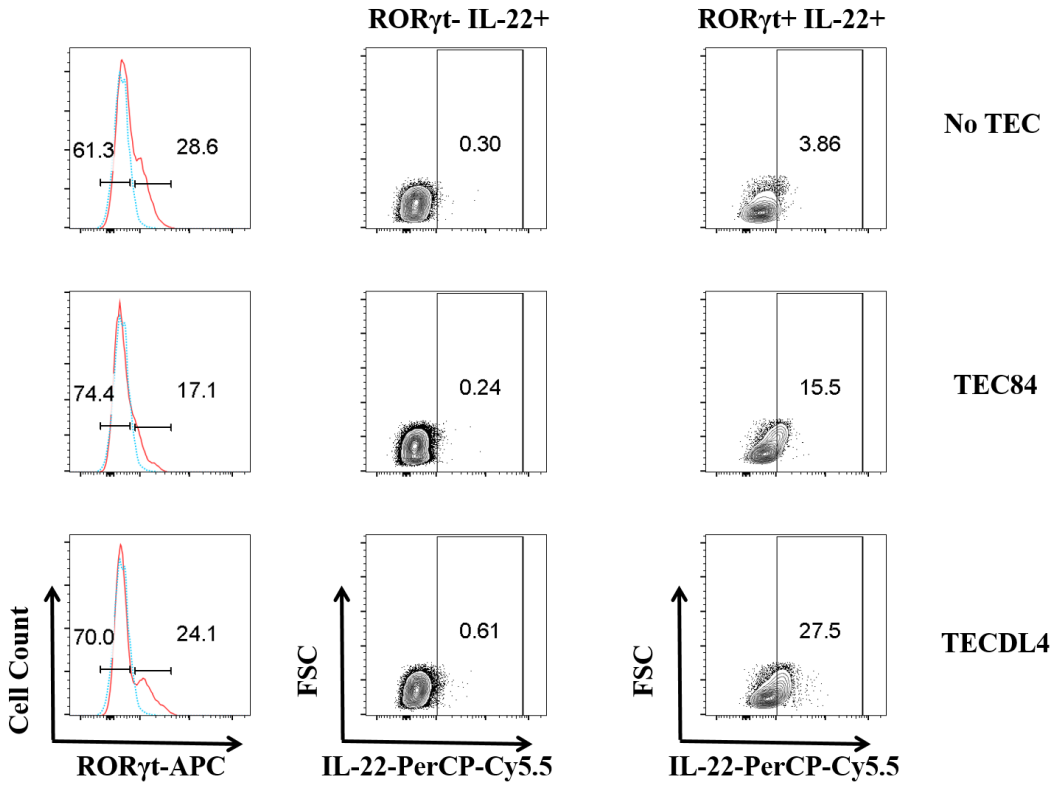

Figure 10. The percentage of LIN- RORγt+ cells following PBMC/TEC co-culture. LIN- RORγt+ IL22+ cells were analyzed using RORγt negative gating to determine positive and negative staining for IL-22. Numbers shown are percentages within each fraction. Data are a representative of 4 different PBMC-TEC co-cultures.

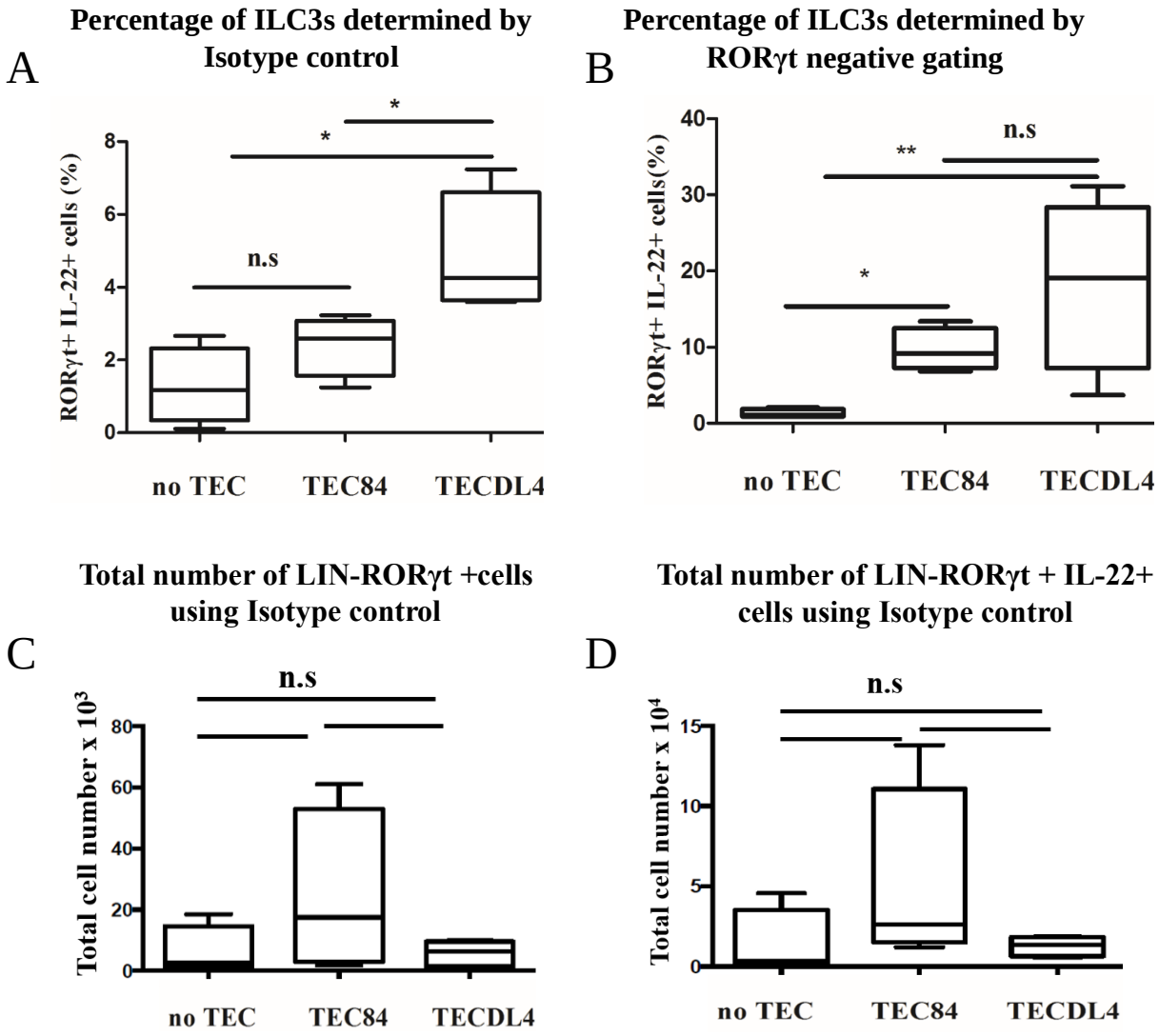

Figure 11. The frequency of LIN- RORγt +IL-22+ cells increased when PBMC were cultured with TEC. (A) Boxplot showing the percentages of RORγt+ IL-22+ cells as determined by isotype control. (B) Boxplot showing the percentage of RORγt+ IL-22+ cells determined by RORγt negative gating. (C and D) The total number of LIN- RORγt+ and LIN- RORγt +IL-22+ cells did not increase when cultured with TEC. Data were obtained using isotype control. Similar data were obtained using RORγt negative control. Boxplots depict mean, ± SEM and the statistics (*$p<0.05$) are represented by Mann-Whitney comparisons. Data are representative of 4 independent experiments.

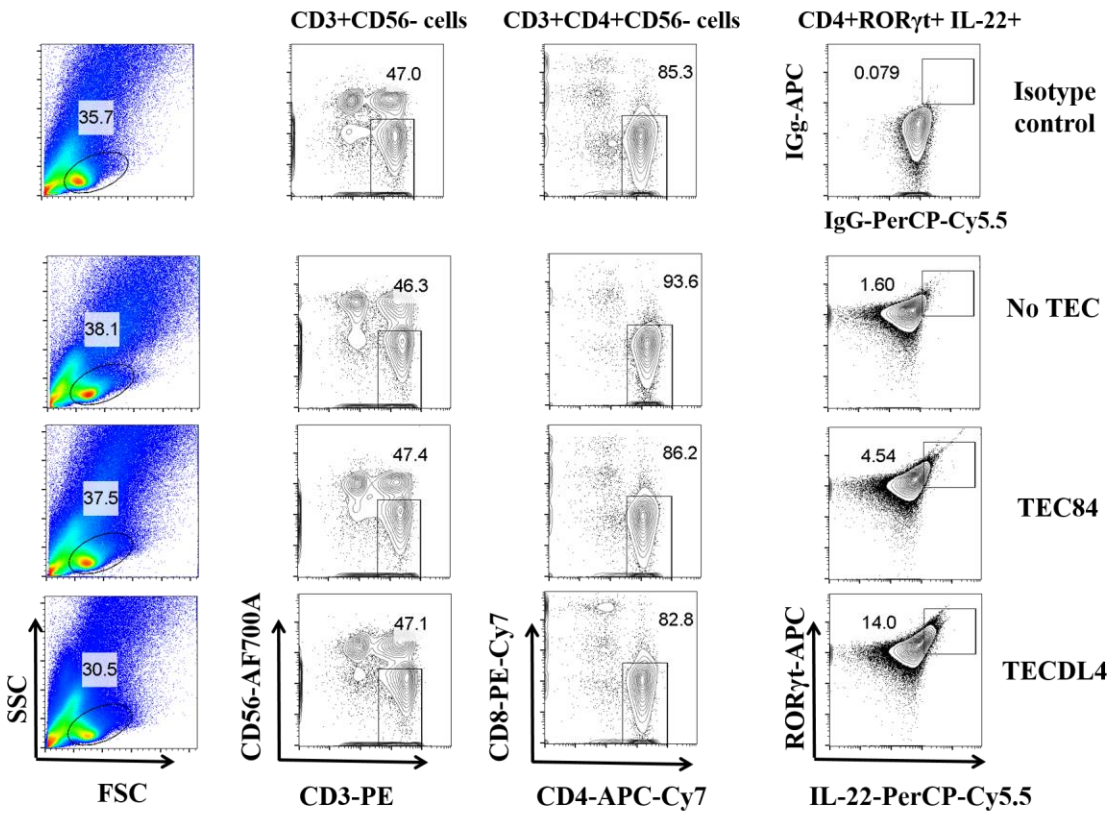

Figure 12. The frequency of CD3+CD4+IL-22+RORγt+ cells significantly increases when PBMC were cultured with TEC-DL4. Following 72hr co-culture with TEC84 or TECDL4 PBMCs were treated with monensin for 2 hrs and analyzed for the expression of CD3, CD4, CD8, IL-22 and RORγt. The positive and negative quadrants were determined using isotype control. The data shown are representative of IL-22 and RORγt expression in the CD3CD8 cells within lymphocyte population of cultured cells. Data are representative of 4 independent experiments.

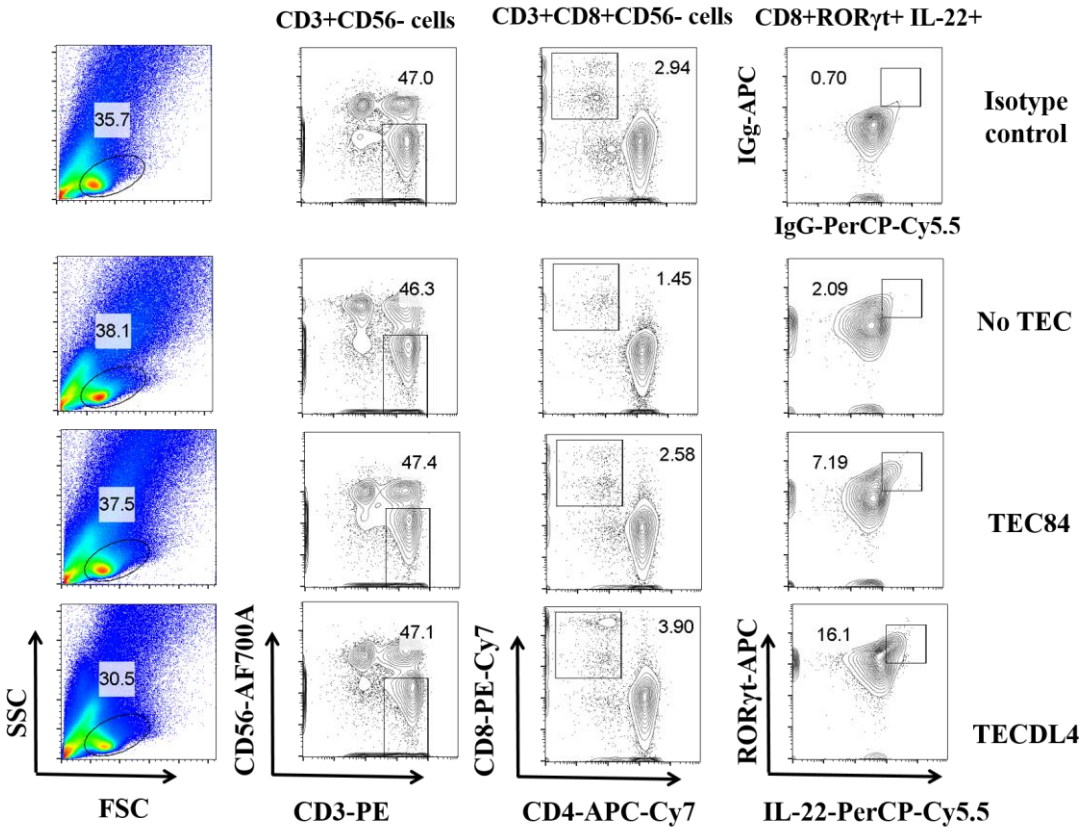

Figure 13. The frequency of CD3+CD8+ IL-22+RORγt+ cells increases when PBMC were cultured with TEC-DL4. Following 72hr co-culture with TEC84 or TECDL4 PBMCs were treated with monensin for 2 hrs and analyzed for the expression of CD3, CD4, CD8, IL-22 and RORγt. The positive and negative quadrants were determined using isotype control. The data shown are representative of IL-22 and RORγt expression in the CD3CD8 cells within lymphocyte population of cultured cells.

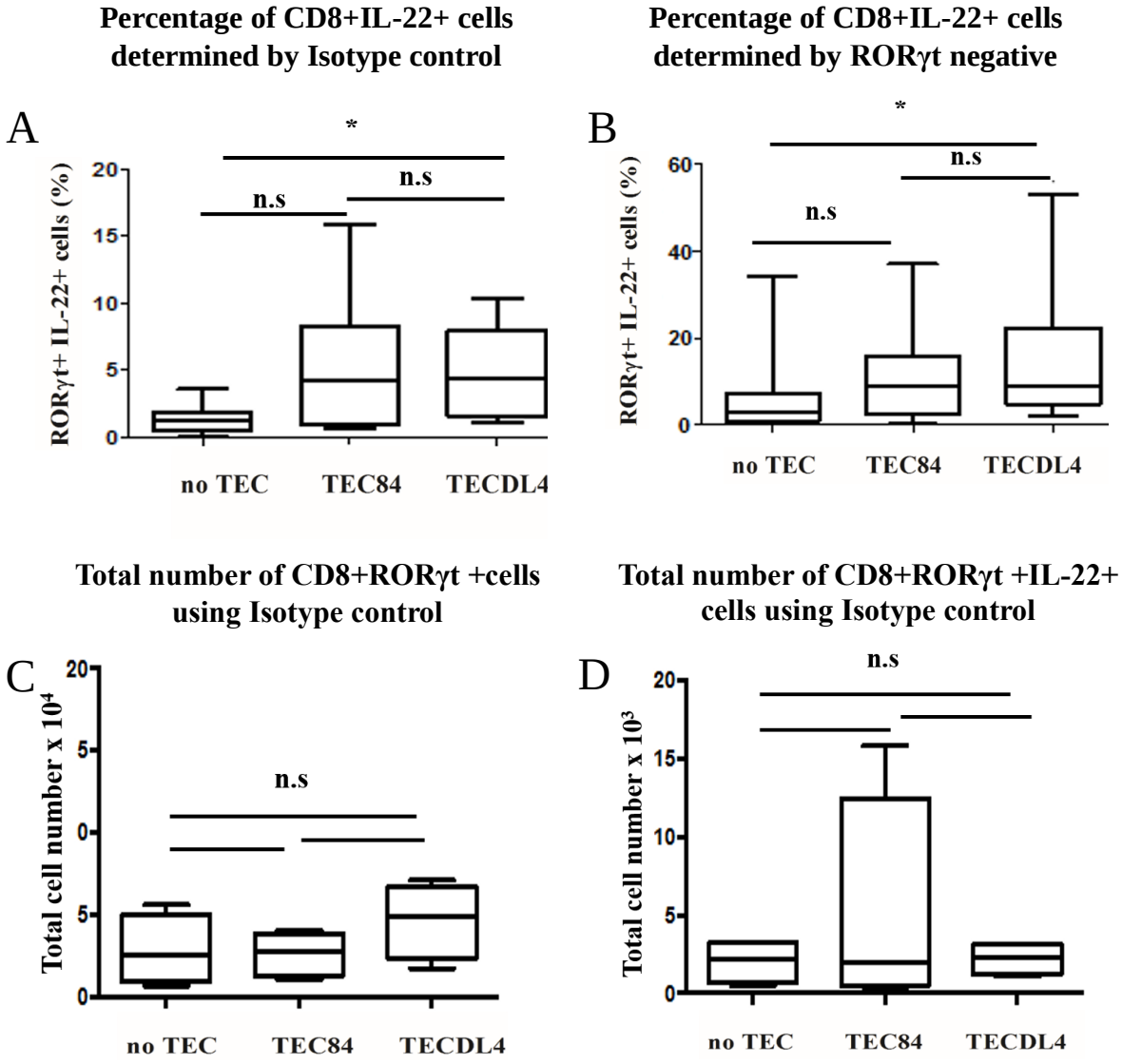

Figure 14. The frequency, but not the total cells number of CD3+CD8+ cells increases when PBMC were cultured with TEC-DL4. *(A)*. Boxplot showing the percentage of CD8+ RORγt+ IL-22+ cells as determined by isotype control. *(B)* Boxplot showing the percentage of CD8+RORγt+ IL-22+ cells determined by RORγt negative gating. (*C and D)* Boxplot of the total cell number of CD8+RORγt+ and CD8+RORγt+ IL-22+ cells did not increase when cultured with TEC. Data were obtained using isotype control. Similar data were obtained using RORγt negative control. Boxplots depict mean, ± SEM and the statistics (*$p<0.05$) are represented by Mann-Whitney comparisons. Data are representative of 4 independent experiments.

CHAPTER FIVE

DISCUSSION

IL-22-producing cells are present in the human thymus

It was recently reported that IL-22-producing ILCs are present in the mouse thymus, and play a critical role in the proliferation and regeneration of thymic epithelial cells following thymic insults (6). IL-22-producing cells, particularly the ILC3s have been shown to be present in most secondary lymphoid tissues in humans and mice (9-13, 53). In fact, IL-22 is constitutively produced by ILC3s in the small intestine. Studies have demonstrated that the ILC3s in the gut produce IL-22 under steady states through ILR1-MyD88 signaling (18). Furthermore, in the gut, IL-22 protects intestinal epithelial cells from bacterial infections through the production of antimicrobial peptides and promotes tissue repair through the induction of epithelial cell proliferation (11).

However, it is still unknown whether ILC3s are present in the human thymus. Here we have investigated the presence of IL-22-producing cells in the human thymus. Our analysis of *ex vivo* thymocytes from pediatric thymus demonstrated that there are LIN- RORγt+ IL-22+ cells present in the human thymus at the protein and mRNA levels. We did see a variability in the percentage of LIN- RORγt+ IL-22+ cells; these variations may simply be due to the biological variability between the patients.

Alternatively, it is possible that the health condition of the patients at the time of their surgery when their thymus was removed, as well as, the medications they were administered before or during the surgery could contribute to the variability observed. At this point these are only mere speculations; however, the present or absence of other health conditions that may have affected thymic function should be examined in

future studies. In addition to ILC3s in the thymus, we reported that at steady state there are CD4+ cells, CD8+ cells, CD3+CD4-CD8- (possible γδ T) cells, and CD4+CD8+ (DP) cells which express IL-22 at the protein level.

Although we observed low to undetectable levels of IL-22 mRNA expression in CD4+ cells, CD8+ cells, CD3+CD4-CD8- cells, and CD4+CD8+ cells within the thymocyte population this is somewhat not surprising. Wolk et al., and others have previously reported that the highest expression of IL-22 is observed following infection or damage, when T cells such as CD8+ T cells, CD4+ T cells and γδ T cells are activated (8). Therefore, it is likely that in the absent of tissue damage or inflammation in the thymus, DP T cells, and γδ T cells IL-22 mRNA levels are lower than ILC3s, CD8+ T cells, and CD4+ T cells and these cells may require activation following injury or inflammation to produce a higher level of IL-22 mRNA expression. It is also possible that some population of cells that produce IL-22 at the protein levels in the thymus at steady state are so low that it is more difficult to detect their IL-22 mRNA expression. Therefore, more sophisticated technology may be need to be employed to answer these questions.

Furthermore, most studies evaluated IL-22 mRNA expression in murine stimulated- T cells lines and stimulated -human peripheral blood, have reported that IL-22 can only be found in activated T cells (13). On the other hand, other studies have demonstrated that T cells can produce IL-22 under steady state conditions (12). These T cells include human CD4+ T cells and CD8+ T cells that can differentiate into IL-22-producing cells known as T_H22 and T_C22, respectively. The T_H22 and T_C22 cells are

normally found present in the skin of normal and inflamed individuals (12). Furthermore, naïve T cells can secrete IL-22 without the need for T effector cells polarizing cytokines (10).

Studies have indicated that IL-22 involved in homeostasis at barrier organs in the individuals healthy as well as in individuals with inflamed states (9-13). Therefore, it is likely IL-22 release is governed by factors such as internal micro-environmental stimuli, the cytokine milieu and the tissue in which IL-22 is expressed. Therefore, it is highly possible that in the thymus, direct interaction between thymocytes and different type of thymic stromal cells could induce the necessary stimuli for IL-22 expression. The fact is that the mechanism underling IL-22-expressing cells differentiation, development and regulation in the human thymus is yet to be studied.

Thymic epithelial cells do not modulate IL-22 expression *ex vivo*

It is well established that direct contact between developing thymocytes within the thymus and thymic epithelial cells can induce the expression of different cytokines by the thymic epithelial cells (12, 13 and 15). These cytokines in turn are known to induce expression of other cytokines; certain the cytokine profile can induce differentiation of ILC3s and T cell subsets (Figure 2). We next assess the effects of thymic epithelial cells on LIN- RORγt+ cells in terms of IL-22 expression. We co-cultured thymocytes with thymic epithelial cells and evaluated the percentage of IL-22- producing cells using flow cytometry. Although we observed a trend suggesting an increase in ILC3 RORγt+ expressing IL-22 (Figure 7) following thymocyte/TEC co-culture, there were no statistical significant differences between cell cultured with or without TEC. The finding

indicates that TEC alone may not be sufficient to modulate IL-22 expression in ILC3s in the human thymus.

IL-22 production by most cell subsets requires cytokine induction such as, IL-7, IL-1β, IL-23 and IL-6 (33-46). Studies have shown that ILC3 development and proliferation require a milieu of cytokines particularly in an infectious state or during repair. These includes IL-23 and IL-1β which are required by ILC3s for effector function both *in vitro* and *in vivo* (41, 45). IL-1β in combination with IL-17 and IL-2/IL-15 can induce the proliferation, accumulation and activation of ILC3s (41). Other important cytokines include IL-7, TSLP, and SCF these cytokines are necessary for ILC3 maintenance (33, 41, and 42). These cytokines are produce by cells such as fibroblast, epithelial cells and different stromal cells and should be produce in our co-culture system. However, the production of other cytokines such as IL-23 may require the present of other cell types that is a normal part of the thymic stroma such as CD103+ DCs. Studies have shown that DCs regulated ILC3s activities (17). ILCs can be activated directly by dendritic cells (DCs) cytokines such as IL-23 (6, 17). Therefore, it is also likely that DCs as well as thymic epithelial cells may be required for IL-22 production by LIN- RORγt+ IL-22+ cells.

Another alternative is that the cytokines that are produce in our co-culture system could be blunting IL-22 expression. In fact, the TECs in our co-culture system express TGF-*β*, and studies have shown that TGF-*β* can drive IL-17 production and inhibit IL-22 expression in Tc22 and Th22 cells depending on the context of the tissue (11, 15, and 33). Studies have shown that ILC3s can switch from producing of IL-22 to produce TNF and IL-2 (33). Furthermore, it has been previously demonstrated that the loss of RORγt

stability due to the absence of IL-7 and its ability to enhance LTα1β1 expression can result in the conversion from IL-22-producing ILC3s (LTi) into IFN-γ-producing ILC3s (19,33, 34). Studies have shown that ILC3s generated in the presence of IL-2, IL-15 and IL-12 cytokines can result in the conversion from IL-22-producing ILC3s (LTi) into IFN-γ-producing ILC3s (33-35). Whether this is true for other ILC3s in the human thymus is unknown. To analyze these possibilities in the future, it is necessary to measure the supernatant in the co-culture assay using ELISA to determine whether cytokines such as IL-7, IL-1β, IL-23, IFN-γ, IL-2, TNF and IL-6 are produced in our culture system. Furthermore, flow cytometry analysis of thymocytes co-cultured with TEC with the addition of cytokines combination (IL-7, IL-1β, IL-23 and IL-6) should be performed to elucidate the mechanism of IL-22 regulation by TEC.

Circulating LIN- RORγt + cells can produce IL-22 independent of thymic epithelial cells *in vitro*

IL-22- producing cells are present in peripheral blood mononuclear cells (17, 15, and 43). We have also shown that the total PBMC have higher expression levels of IL-22 mRNA (Figure 5). Studies have indicated that the microenvironment of ILC3s play a role in their development and function (47-53). This led us to look at the ability of thymic epithelial cells to modulate expression of IL-22 in circulating ILC3s.We observed a trend towards increase in IL-22 secretion (Figure 7) by RORγt-expressing ILC3s following PBMC /TEC84 co-culture. However, statistical analysis of our data showed conflicting results. There was no statistical significant difference (Figure 11A) between cell cultures with or without TEC84 using isotype control gating, but there was statistical significant difference (Figure 11B) between cell cultured with or without TEC84 using

RORγt negative control gating. These discrepancies may be due to more variance generated using isotype control gating. We may be able to see a statistically significant increase by increasing the number of experiments.

However, if we go by the isotype control gating and assume that thymic epithelial cells alone may not be sufficient to modulate peripheral ILC3-derived IL-22 *in vitro* then as mentioned above these results may be due to the nature of the cytokine production profile in the PBMC that can act as stimulators that leads to IL-22 production in culture. In supporting this notion, there are studies showing that T_H22 cells in the peripheral blood can be stimulated by dendritic cells promoting their differentiation from naïve to IL-22- producing cells (12). Furthermore, T_H22 are polyclonal TCRαβ+CD4+ T cells that are autoreactive to CD1a, a MHC Class1 like molecules (12). Whether T_C22 have a similar population of cells is yet to be shown. Over all, in our co-culture sytem of PBMC there may be factors increasing IL-22 expression by the IL-22-producing cells which could explain why there is no difference between PBMC culture alone, and PBMC-TEC84 co-culture.

Alternately, there are other factors that regulate the development, proliferation, and maintenance of ILC3s and IL-22-producing T cells and can influence IL-22 production. These includes AHR, RORγt, STAT3 and Notch signaling (45-59). Possot C et al., have shown that ILCs progenitors can develop independently of the thymic environment under the influence of Notch 2 signaling (48). Therefore, it is of importance to examine the role of Notch in the ability of ILC3s and T cells to produce IL-22.

The role of Notch signaling in TEC modulation of IL-22 expression by LIN-IL-RORγt + cells

Notch signaling is involved in several cellular processes such cellular proliferation, border formation and program cell death (50). In mammalians, there are four Notch receptors (Notch 1-4) that bind to five different Notch ligands (*Delta-like 1, 3, 4 and Jagged 1 and 2*). The role of Notch in the crosstalk between developing thymocytes and thymic epithelial cells are well documented (50, 57). Thymocytes express Notch receptors while Notch ligands genes such as the *Delta like1, 3, 4* and *Jagged 1, 2* have been shown to be expressed by thymic epithelial cells (57). Studies in mice have also shown that Notch signaling can upregulate IL-22 production from CD4+ T cells through the regulation of AhR expression (49). Studies have also shown that Notch is involved in the activation and differentiation of CD8 + T cells (54, 58). However, whether Notch can also induce IL-22 production by CD8 + T cells is unclear. Mounting evidence has implicated a role for Notch signaling in innate lymphoid cell development and function through the induction of Notch -1 and Notch 2 expression by AhR (47, 48, 51, and 52).

Therefore, we next investigated the possibility that thymic epithelial cells mediate IL-22 expression in ILC3s through Notch signaling. When PBMCs co-cultured with TECDL4, there was a significant increase in the frequency of IL-22-producing cells (ILC3s, RORγt+ CD4+ and RORγt+ CD8) when compared to PBMC cultured without TEC. Whether the increase was mediated by Notch signaling remains to be determined. Overall our data suggest that Notch signaling in particularly through DL4 ligand, at least partly mediates the increase in the frequencies of IL-22 positive cells within the ILC3s,

RORγt+CD8 + and RORγt+CD4 + T cells. However, we cannot exclude the possibility that Notch mediates IL-22 expression indirectly thought Notch mediated effector functions. Therefore, it is possible that Notch signaling contributed to the differentiation of effectors cells (Th1, Th17, and Th22) which causes the induction of cytokines such as IL-1β, IL-6 and IL-23 which can up-regulate IL-22 expression (58).

It is also important not to dismiss the potential effect of alloreactivity in the induction of IL-22 because TECs and PBMCs do not come from the same donor and are HLA-missed match. The fact is that the significant increase in the frequency of IL-22 expression by RORγt+CD8+ T cells and RORγt+CD4 + T cells could be a result of activated allogeneic T cells responding to allogeneic TECs as in our co-culture system. The allogeneic activated T cells may in turn cause increase production of cytokines that can up-regulate the expression of IL-22 in the RORγt –expressing CD8+ and CD4+ T cells and ILC3s independent of Notch signaling. We can rule out a role for Th17 effector cells in the production of IL-22, since we did not detect IL-17 expression among the PBMC when co-cultured with TEC (data not shown). With these data one may want to infer that TEC, Notch signaling and allogeneic T cell activation is involved in modulating effector functions leading to the upregulation of IL-22 by ILC3s and other IL-22-expressing T cells in our co-culture system.

Taking all of this into consideration, we expected to see a difference between cells that were co-culture with TEC84 and TECDL4, since TECDL4 has a higher expression of DL4 ligand. However, we observed no significant difference in the expression of IL-22 by ILC3s and T cells within the PBMC-TEC84 and PBMC–TECDL4 co-culture based

on negative control gating strategy with RORγt, but we did observe a significant difference based on our isotype control. I am unsure of the reason behind this difference other than there been more variable using my negative control gating (may also be due to technical differences in my culture system from experiment to experiment). These variables may be also solved through performing experiments and adding more samples.

Another consideration is that although TEC84 cells express a low level of DL4, it is possible that they have other ligands such as Jagged1 that could come into play. In this scenario, the differential effects on IL-22 production could be in part due to differential binding and signaling induced by various Notch ligands. It is also possible that within our co-culture system different triggers may contribute to the cytokine–production profile that induce IL-22-producing cells. These signals could be induced through a combination of the TEC, Notch signaling and allogenic reactivity.

For now, more experiments are necessary to further elucidate the role of Notch signaling in the regulation of IL-22 expression. Therefore, to clarify whether Notch signaling through DL4 plays a role in TEC modulation of IL-22 expression by ILC3s, CD8+ T cells and CD4+ T cells it is necessary to blocking Notch receptors via soluble DLL4 to prevent Notch signaling and then examine IL-22, RORγt and cytokines expression after PBMC-TEC co-culture. Furthermore, since other ligands such as Jagged 1 and 2 that are expressed by thymic epithelial cells are likely involved in the Notch signaling, it is important to establish that only DL4 contribute to the increase frequency

of IL-22 expression by RORγt–expressing CD8+ T cells, therefore, co-culture assays should be perform using γ-secretase inhibitor to block Notch signaling.

Another experiment to answer these questions is to isolate LIN- cells from PBMC and carry out co-culture assays to determine whether similar observation is seen when LIN- cells are cultured alone without other lymphoid cells. I have attempted this experiment, however we failed to detect any IL-22 expression. One of the problems I think was due to the absence of the necessary cytokines to facilitate ILC3 maintenance and proliferation. I did add in IL-7, however as mentioned in my introduction there are many additional cytokines necessary for ILC3 survival and proliferation. Therefore, it is essential to repeat these studies using these necessary cytokines.

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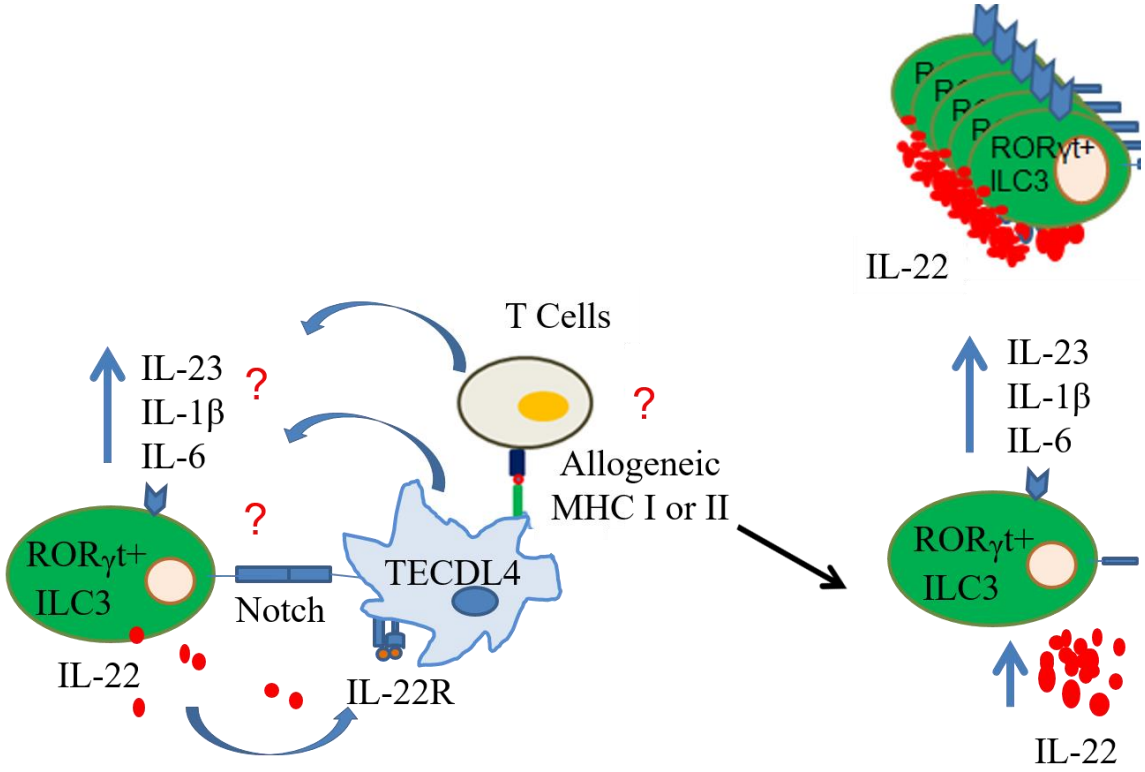

Figure 15. Model for the regulation of IL-22 production of circulating ILC3s. Notch signaling mediates thymic epithelial cells up-regulation of IL-22 through nonspecific regulation of notch on T cell effector functions. Allogeneic activated T cells generate effector cells that produce cytokines which in turn induce IL-22 production. Direct contact between thymic epithelial cells and circulating cells induce increase production of cytokines that up-regulate IL-22 production in a notch independent or dependent notch manner.

Significance of this study

Due to the importance of the thymus in establish peripheral tolerance and educating T cells understanding how the thymus recover from damage should as radiation and infection is relevant. IL-22 role in regenerating thymic epithelial cells is of importance for the development future treatments and therapies as well as reestablishing the thymic microenvironment following damages from chemotherapies and chronic infections such as HIV, or even the effects cause by aging within the thymus.

CHAPTER SIX

MATERIALS AND METHODS

Cells and cell lines

Human Thymocytes: Cryogenically preserved *ex vivo* human thymocytes that were previously isolated from human pediatric thymus tissue were quickly thawed and incubated with thaw media (RPMI 1640 supplemented with 30% FCS, 100 µg/ml DNase I, and 10 µg/ml gentamicin) for 1hr at 37ºC. The dead cells were separated by Ficoll-Hypaque centrifugation using lymphocyte media (Corning, Cell gro). Cells were resuspend in RPMI-1640 media with L-glutamine supplemented 5% fetal bovine serum, 100U/mL penicillin-streptomycin and 5% human AB serum then counted using hemocytometer and used for co-culture assays and flow cytometry.

Peripheral mononuclear blood cells: Cryogenically preserved human peripheral blood mononuclear cells were previously obtained from healthy patients' blood and separated were quickly thawed and incubated with RPMI 1640 supplemented with 5% FCS, 100 µg/ml DNase I, and 10 µg/ml gentamicin for 1hr at 37ºC. The dead cells were separated by Ficoll-Hypaque centrifugation using lymphocyte media (Corning, Cell gro). Cells were then resuspend in RPMI-1640 media with L-glutamine supplemented 5% fetal bovine serum, 100U/mL penicillin-streptomycin and 5% human AB serum and counted using hemocytometer and used for co-culture assays and flow cytometry

Thymic epithelial cells: Previously, a primary TEC culture from pediatric thymus tissue was generated and described (4). TEC cell lines TEC84 express low levels of the human Notch ligands, DL1 (<1000 transcript copies/µg RNA) and DL4 (3000 transcript copies/µg RNA). The TEC-DL4 cell line was also previously generated by infecting the TEC-84 cell line with retrovirus containing Migr1-DL4-GFP to enhance the potential of the TEC-84 cell line. The generated cell line was termed TEC-DL4 cell line which homogenously express GFP and express high levels of the human Notch ligand, DL4. (4). TEC-84 and TEC-Dl4 were used in cell co-culture assays.

Flow cytometry and data analysis

For phenotyping experiments, 1-2x10^6 thymocytes were treated for 2 hours with monensin (Biolegend). Then incubated with Rat anti-human antibodies: anti-CD3 FTTC (eBioscience; UCRHT) or anti-CD3 PE (eBioscience; UCRHT), anti-CD4 AF750 (eBioscience; RPAT4), anti-IL-17eF450 (eBioscience; RPATH), anti-CD8 PE-Cy7 (eBioscience; RPAT8), anti-CD56 PE (eBioscience; HCD56), or anti-CD56 AF700 (bioscience; HCD56) for 30 minute on ice in the dark. To characterize RORγ+ IL-22+ cells, following the above surface staining, the cells were fixed and permeabilized at RT for 20 minutes with IC fixation buffer 1X Permeabilization buffer (eBioscience). Then incubated with Rat antihuman IL-22 PerCpeF710 and RORγ+ APC, and for the isotype control the cells were incubated with IL-22 Rat IgG2a PerCpeF710 (EBR2α) and RORγ+ Rat IgG2a APC (EBR2α) for 30 min an ice in the dark. The samples were analyzed using BD LSRFortessa with tree star Flow Jo Software version 7.5.6.

Cell Co-culture

Co-culture of thymocyte with TEC-84 or TEC-DL4, and co-culture of PBMC with TEC-84 or TEC-DL4: $3x10^4$ TEC-84 or TEC-DL4 were cultured at $3x10^4$/mL in a flat bottom 24- well culture plate with TE media (3; Dulbeecco's modified Eagle's medium: F12 medium with 5% fetal calf serum, 5.5µg/ mL bovine insulin, 0.4 µg/ml Hydrocortisone, 9ng/mL, cholera toxin, 0.3% adenine hydrochloride, 1mM sodium pyruvate, 10ng/mL epidermal growth factor, 2.5 µg/ mL, amphotericin B, and 55 ng/ mL gentamicin sulfate) until 50% confluent. $1\text{-}2X10^6$ thymocytes/well or PBMC/well were co-cultured with 50% confluent TEC-84 or TEC-Dl4in RPMI-1640 media with L-glutamine supplemented 5% fetal bovine serum, 100U/mL penicillin-streptomycin and 5% human AB serum. Cells were cultured for 2, 4 and 8 days then treated with monensin for 2hrs before harvest. The total number of live cells harvested was counted on a hemocytometer using Trypan blue (Life technologies) to exclude the dead cells. Harvested cells were analyzed using flow cytometry to compare IL-22 and RORγt expression of cells culture with or without TEC-84 or TEC-DL4.

Cell Sorting

For cell sorting of LIN- cells and T cell subsets, 40 $x10^6$ thymocytes were surface stained with anti-CD3 FTTC (eBioscience; UCRHT, anti-CD4 AF750 (eBioscience; RPAT4), anti-CD8 PE-Cy7 (eBioscience; RPAT8), anti-CD56 PE (eBioscience; HCD56). FACS sort was performed with a FACSAria cell sorter (BD Biosciences). Purified LIN- cells and CD8+ T cells, CD4 + T cells, CD8+CD4+T cells, CD3+CD8+CD4+ T cells were used for RNA isolation described below.

RNA isolation and Reverse Transcriptase-PCR

RNA from sorted cell subsets and total RNA from PBMC was isolated using Trizol reagent (Sigma-Aldrich) according to the manufacturer's protocol. Isolated RNA was then treated with DNase using Ambion DNA-free kit and 0.5 to 1 μg of RNA was used for cDNA synthesis using the Invitrogen's SuperScript II synthesis kit with Oligo-dT primers, per the manufacture's instruction. The resulting cDNA was used for PCR with primers as follow IL-22: forward 5'- TCTCCTTCCCAGTCACCAGTT and reverse 5'TCATGATGGAGTTTGGCTTCC, GAPDH; forward 5'-GCACCGTCAAGGCTGAGAAC and reverse 5' GCCTTCTCCATGGTGAA. PCR thermal cycler profile was run as follows: 95C 4min, 95^0C 15min, 55^0C 20 min, 72^0C 40 min, 72^0C 40 min, 72^0C 4 min 10^0C 5min.The PCR products were run on a 1.8% gel, then stained with ethidium bromide and image using BioRad ChemiDoc XRS + w/image lab software.

Statistical Analysis

Statistical analysis was performed using Prism 5.0 (Graph Pad software). Comparison between two groups was performed with the unpaired Man- Whitney U test, values of $p<0.05$ are considered statistically significant.

REFERENCES

1. Legrand, Nicolas, et al. "Human Thymus Regeneration and T Cell Reconstitution." *Seminars in Immunology* 19.5 (2007): 280-8.

2. Nunes-Cabaco H. and Sousa AE., et al. “Repairing thymic function”. *Current opinion in Organ Transplantation* 18.3 (2013): 363-368.

3. Jason Gill, Mark Malin, Jayne Sutherland et al. “Thymic generation and regeneration”. *Immunological Reviews* 195 (2003): 28-50

4. Ioannis Politikos and Vassiliki A. Boussiotis. “The role of the thymus in T-cell immune reconstitution after umbilical cord blood transplantation”. Blood November 20: 124(22)3201-3211.

5. Werner Krenger, Bruce R. Blazer and Georg. A Hollander “Thymic T- cell development in allogeneic stem cell transplantation” Blood.2011.117(25): 6768-6776

6. Kumar P., Malarkannan S., et al. “IL-22: An Evolutionary Missing- Link Authenticating the Role of the Immune System in Tissue Regeneration”. *Journal of Cancer* 4.1(2013): 57-65.

7. Dudakov, Jarrod A., et al. Interleukin-22 Drives Endogenous Thymic Regeneration in Mice. *Science* 336 Vol., 2012.

8. Bin Pan, Kailin Xu, et al. Acute ablation of DP thymocytes induces up-regulation of IL-22 and Foxn1 in TECs. *Clinical Immunology*, Volume 2014,150:101-108

9. Enayat Nikoopour et al.IL-22, cell regeneration and autoimmunity. *Cytokine* (2014):2-7

10. Eyerich, Stefanie, et al. "IL-17 and IL-22: Siblings, Not Twins." *Trends in Immunology* 31.9 (2010): 354-61.

11. Rutz S., Céline E., and Wenjun O., "IL-22, Not Simply a Th17 Cytokine." *Immunological Reviews* 252.1 (2013): 116-32

12. Gregory F. Sonnenberg, and David Artis *et al. "*Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surface by IL22”. *Nature Immunology*.2011. 5(12): 383-390

13. Wolk K., Witte E., and Witte K., et al. “Biology of Interleukin-22.” *Semin.in Immunopathology.*2010. 32: 17-31

14. Wolk, K., et al. "IL-22 Increases the Innate Immunity of Tissues." *Immunity* 21.2 (2004): 241-54.

15. Zenewicz, LA., and Richard A. F., “Recent Advances in IL-22 Biology. *International Immunology* 23 Vol., 2011.

16. Kreymbory K., and Burkhard B., “IL22 vs IL22: The tissue matters” *Open Autoimmunity Journal* 2 (2010):181-186.

17. Martin, JCJ et al. “Interleukin-22 Binding Protein (IL-22BP) Is Constitutively Expressed by a Subset of Conventional Dendritic Cells and Is Strongly Induced by Retinoic Acid.” *Mucosal immunology* 7.1 (2014): 101–113.

18. Monica Romera-Hernandez, Patricia Aparicio-Domingo, Tom Cupedo. “Damage control: Rorγt+ innate lymphoid cells in tissue regeneration”. *Current Opinion in Immunology,* 25.2 (2013)156-160.

19. Zenewicz LA., et al. ‘Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease’. *Immunity* 29.6 (2008): 947-57.

20. Basu, Rajatava, et al. "Th22 Cells are an Important Source of IL-22 for Host Protection against Enteropathogenic Bacteria." *Immunity* 37.6 (2012): 1061-75.

21. Hanash, Alan M, et al. "Interleukin-22 Protects Intestinal Stem Cells from Immune-Mediated Tissue Damage and Regulates Sensitivity to Graft Versus Host Disease." *Immunity* 37.2 (2012): 339-50.

22. Zenewicz, Lauren A. et al. “IL-22 but Not IL-17 Provides Protection to Hepatocytes during Acute Liver Inflammation.” *Immunity* 27.4 (2007): 647–659.

23. Wolks, K. et al. IL-22 induces lipopolysaccharide-binding protein in hepatocytes: potential systemic role in Crohn’s disease. *J. Immunol.* 178.9 (2007) 5973-5981

24. Radaeva S., et al. "Interleukin 22 (IL-22) Plays a Protective Role in T Cell-Mediated Murine Hepatitis: IL-22 is a Survival Factor for Hepatocytes via STAT3 Activation." *Hepatology* 39.5 (2004): 1332-42.

25. Ren, Xiaodan, Bin Hu, and Lisa M. Colletti. "IL-22 Is Involved in Liver Regeneration after Hepatectomy." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 298.1 (2010): G74–G80.

26. Xue, Jing et al. **"**Aryl Hydrocarbon Receptor Regulates Pancreatic IL-22 Production and Protects Mice from Acute Pancreatitis". *Gastroenterology* 143.6 (2012) 1670 - 1680

27. Aujila S.et al, "IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia". *Nat Med* 14 (2008) 275-281.

28. Kumar, Pawan et al. "IL-22 from Conventional NK Cells Is Epithelial Regenerative and Inflammation Protective during Influenza Infection." *Mucosal immunology* 6.1 (2013)

29. Taube, C et al. "IL-22 Is Produced by Innate Lymphoid Cells and Limits Inflammation in Allergic Airway Disease." *PLoS ONE* 6.7 (2011): e21799.

30. Monticelli, Laurel A., Gregory F. Sonnenberg, and David Artis. "Innate Lymphoid Cells: Critical Regulators of Allergic Inflammation and Tissue Repair in the Lung." *Current opinion in Immunology* 24.3 (2012): 284-9.

31. Wolk, K., et al. "IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis". *Eur. J. Immunol*.36.5 (2006)1309-1323

32. Zheng, Y., Danilenko, D.M., Valdez, P.et al. "Interleukin-22, a T (H) 17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis". *Nature* 455:648 (2007).

33. Kilig M., et al. "Recognition strategies of group 3 innate lymphoid cells". *Frontiers in Immunology* 2014.142 (4): 1-8

34. Vonarbourg, Cedric et al. "Progressive Loss of RORγt Expression Confers Distinct Functional Fates to Natural Killer Cell Receptor-Expressing RORγt^{+} Innate Lymphocytes." *Immunity* 33.5 (2010): 736–751

35. Spits, H., and Tom C., "Innate Lymphoid Cells: Emerging Insights in Development, Lineage Relationships, and Function." *Annual Review of Immunology* 30.1 (2012): 647-75.

36. McKenzie A.N. J., et al. “Innate lymphoid cell-how did we miss them?” *Nature Reviews Immunology* 2013.13:75-87

37. Liying Guo, Iikka S. Junttila and William E. P., et al. “Cytokine-induced cytokine production by conventional and innate lymphoid cells”. *Trends in Immunology* 2012 972 1-9

38. Spits, H., and Tom C., "Innate Lymphoid Cells: Emerging Insights in Development, Lineage Relationships, and Function." *Annual Review of Immunology* 30.1 (2012): 647-75.

39. Caroline E. Sutton, Lisa A. Mielke and Kingston H.G. Mills. “IL-17-producing γδ T cells and innate lymphoid cells.” *Eur. J. Immunol*. 2012. 42: 2221-2231

40. Hergen Spits and James P. Di Santo. “The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling.” *Nature Immunology*. 2011.12: 21-27

41. Cedric Vonarboung and Andras Diefenbach. “Multifaceted roles of interleukin-7 signing for the development and function of innate lymphoid cells.” *Seminars in Immunology*. 2012. 24: 165-174

42. Cording. S, et al. "Development and regulation of RORγt+ innate lymphoid cells. *FEBS Lett.* (2014).

43. Hazenberg M. D., and Spits H., “Human Innate Lymphoid Cells”. *Blood*. 2014. 1-43

44. Ahn Y-O., Vernieris R.M., Blazar R. B., et al. “Lineage relationships of Human IL-22 producing CD56+ RORγt+ Innate Lymphoid cells and Conventional NK cells”. *Blood*. 2013. 1-26.

45. Lee, Youngae, et al. "Intestinal Lin-c-Kit+NKp46-CD4- Population Strongly Produces IL-22 upon IL-1ß Stimulation." *The Journal of Immunology* 190.10 (2013): 5296-305.

46. Schinichiro Sawa et al. “Lineage Relationship Analysis of RORγt Innate Lymphoid cells.” *Science*. 2010 October; 330: 665-669

47. Lee J S., et al. “AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch”**.** *Nat Immunol.* 2011.13 (2) :144-51

48. Possot C, et al. ‘Notch signaling is necessary for adult, but not fetal, development of RORγt (+) innate lymphoid cells’**.** *Nat Immunol*. 2011 12(10):949-58

49. M. S. Alam, Y. Maekawa et al. “Notch signaling drives IL-22 secretion in CD4+Tcells by stimulating the aryl hydrocarbon receptor”. *PNAS*. 2010 March; 107 (13) 5943-5948

50. Radke F., et al. “Regulation of innate and adaptive immunity by Notch”. *Nature Reviews Immunology*.2013. 13:427-437

51. Belz GT., et al. ‘The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. *” Nature Reviews Immunology.* 2013. 14(4): 389-395

52. Gentek R., et al. “Modulation of Signal Strength Switches Notch from an Inducer of T cells to an Inducer of ILC2”. *Front Immunol*. 2013 4:334

53. Tait Wojno, and Elia D. Artis, David “Emerging concepts and future challenges in innate lymphoid cell biology”. *J. Expt. Med.*2016

54. Kuijk et al., “Notch controls generation and function of human effector CD8+ T cells”. *Blood*.2013;121(14)2638-2646

55. Felix N.J., and Allen M. P. “Specificity of T cell alloreactivity” *Nature Reviews Immunology*. 2007. 7: 942-95

56. Felix N.J. and Allen M. P., et al. “Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes”. *Nature Immunology*. 2007. 8(4): 388-397

57. Kyoko Masuda, Wilfred T.V. Germeraad, et al. “Notch activation in thymic epithelial cells induces development of thymic microenvironments” Molecular Immunology.2009. 46 (8–9) 1756-1767

58. Barbara A, Osborne and Lisa M Minter. “Notch signaling during peripheral T-cell activation and differentiation”

59. Sanos S.L, Vononbourg C., et al. ‘Control of epithelial cell function by interleukin-22-producing RORγt^{+} innate lymphoid cells’. *Immunology Review* 132 (2011): 453-465.

VITA

Nadine Morgan was born in Manchester, Jamaica. After completing her high school education in Jamaica, she relocated to California where she began to pursue an education in science. After graduating from California State University Los Angeles with a Bachelor of Science degree in Biology, she obtained her post-baccalaureate training at Wright State University in Dayton Ohio. In 2012, she began her graduate work in the Infectious Disease and Immunology program at Loyola University Chicago in pursuit of a Master of Science degree. In 2013, Nadine joined the research lab of Dr. Phong Le where she investigated IL-22- producing cells presence in the human thymus and the regulation of these cell types by thymic epithelial cells. Following the completion of her master training, Nadine plans to continue her research training at the University of Alabama at Birmingham in the Microbiology and Immunology department.