Distinct Gene Circuits Control the Differentiation of Innate Versus Adaptive IL-17 Producing T Cells: A Dissertation

DISTINCT GENE CIRCUITS CONTROL THE DIFFERENTIATION OF INNATE VERSUS ADAPTIVE IL-17 PRODUCING T CELLS

A Dissertation Presented

By

Nidhi Malhotra

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A Dissertation Presented By

NIDHI MALHOTRA

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

_____
Joonsoo Kang, Thesis Advisor

_____
Raymond Welsh, Member of Committee

_____
Lawrence Stern, Member of Committee

_____
Eric Huseby, Member of Committee

_____
Julie M. Blander, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Leslie J. Berg, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____
Anthony Carruthers, PhD
Dean of the Graduate School of Biomedical Sciences

Immunology and Virology Program
February 10, 2012

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ABSTRACT

T lymphocytes are distinguished by the expression of αβ TCR or γδ TCR on their cell surface. The kinetic differences in the effector functions classifies γδ T cells as innate-like lymphocytes and αβ T cells as adaptive lymphocytes. Although distinct, αβ and γδ T cell lineages produce a common array of cytokines to mount an effective immune response against a pathogen. The production of cytokine IL-17 is a shared characteristic between the γδ T (Tγδ17) cells and the CD4 T (Th17) cells. γδ T cells develop into Tγδ17 cells in the thymus whereas CD4 T cells differentiate into Th17 cells in response to antigens in the peripheral lymphoid tissues. γδ T cells exported from the thymus, as pre-made effectors, are the early IL-17 producers compared with the late IL-17 producing Th17 cells. In this thesis we describe how TGFβ-SMAD2 dependent pathway selectively regulates Th17 cell differentiation but not Tγδ17 cells generation. We further illustrate the requirement of WNT-HMG box transcription factor (TF) signaling for the thymic programming of Tγδ17 cells.

Cytokine TGFβ in co-operation with IL-6 induces the differentiation of Th17 cells. Conversely, TGFβ signaling also regulates the differentiation and maintenance of $CD4^{+}FOXP3^{+}$ regulatory T cells. The mechanism by which TGFβ signals synergize with IL-6 to generate inflammatory versus immunosuppressive T cell subsets is unclear. TGFβ signaling activates receptor SMADs, SMAD2 and SMAD3, which associate with a variety of nuclear factors to regulate gene transcription. Defining relative contributions of distinct SMAD molecules for CD4 T cell differentiation is critical for mapping the versatile intracellular TGFβ signaling pathways that tailor TGFβ activities to the state of

host interaction with pathogens. We show here that SMAD2 is essential for Th17 cell differentiation and that it acts in part by modulating the expression of IL-6R on T cells. While mice lacking SMAD2 specifically in T cells do not develop spontaneous lymphoproliferative autoimmunity, *Smad2*$^{-/-}$ T cells are impaired in their response to TGFβ in vitro and in vivo and they are more pathogenic than controls when transferred into lymphopenic mice. These results demonstrate that SMAD2 is essential for TGFβ signaling in CD4^{+} T effector cell differentiation and that it possesses functional capabilities distinct from SMAD3.

Although SMAD2 is essential for the differentiation of Th17 cells, TGFβ signaling via SMAD2 is not required for the thymic programming of innate Tγδ17 cells. Among different γδ T cells, Vγ2^{+} (V2) γδ T cells are the major IL-17 producing subsets. We demonstrate that Sry-high mobility group (HMG) box TFs regulate the development of V2 Tγδ17 cells. We show that the HMG box TF, SOX13 functions in a positive loop for the intrathymic generation of V2 Tγδ17 cells. SOX13 regulates the programming of Tγδ17 cells by controlling the expression of B-lymphoid kinase (BLK) in developing immature V2 γδ T cells. BLK is an Src-family kinase expressed by all Tγδ17 cells. Furthermore, we show another HMG box TF, TCF1, the nuclear effector of canonical WNT signaling, is the primary negative regulator of IL-17 production by all γδ T cells. We propose that the antagonism of SOX13 and TCF1 determines the generation of IL-17 producing γδ T cells. We also show that extrinsic cues from αβ T cells do not affect the generation of IL-17 producing γδ T cells. Using OP9-DL1 culture system, we demonstrate that the progenitors of V2 Tγδ17 cells are the c-Kit^{+} early thymic precursors.

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ABBREVIATIONS

DC: Dendritic cell

NK cell: Natural killer cell

NKT cell: Natural-killer like T cell

ILC: Innate lymphoid cell

LTi cell: Lymphoid tissue inducer cell

HSC: Haematopoietic stem cell

CLP: Common lymphoid progenitor

ETP: Early thymic progenitor

V1: $V\gamma1^{+}V\delta6.3^{-}$ γδ T cell

V6: $V\gamma1^{+}V\delta6.3^{+}$ γδ T cell

V2: $V\gamma2^{+}$ γδ T cell

V3: $V\gamma3^{+}$ $V\delta1^{+}$ γδ T cell

V4: $V\gamma4^{+}V\delta1^{+}$ γδ T cell

V5: $V\gamma5^{+}$ γδ T cell

PAMP: Pathogen associated molecular pattern

PRR: Pathogen recognition receptor

TLR: Toll like receptor

NLRP3: NLR family, pyrin domain containing 3

GALT: Gut associated lymphoid tissue

LN: Lymph node

SFB: Segemented filamentous bacterium

SAA: Serum amyloid A

LT: Lymphotoxin

EAE: Experimental autoimmune encephalitis

CIA: Collagen induced arthritis

IBD: Inflammatory bowel disease

CNS: Conserved non-regulatory sequence

CNS: Central nervous system

CTLA4: Cytotoxic lymphocyte associated factor 4

CTLA8: Cytotoxic lymphocyte associated factor 8

SRC: Rous sarcoma oncogene

BLK: B cell lymphoid kinase

ERK: Extracellular signal-regulated kinase 1

ITK: Inducible T cell kinase

PDK1: 3’ Phosphoinositide dependent kinase 1

APC: Adenomatosis polyposis coli

AXIN: Axis inhibition protein

β-TRCP: beta-transducin repeat containing protein

DVL: Dishevelled

SKINT: Selection and upkeep of intra-epithelial T cells 1

IDO: Indoleamine deoxygenase

RA: Retinoic acid

SEFIR: Similar expression to FGF/IL-17R and Toll/IL1R (TIR) domain

TIR: Toll IL-1R inhibitory domain

TF: Transcription factor

AhR: Aryl hydrocarbon receptor

AP1: Activator protein 1

BAX: Bcl2 associated X protein

BCL2: B cell leukemia/lymphoma 2

BCL6: B cell leukemia/lymphoma 6

BCL-XL: Bcl2 like 1

BIM: Bcl-2 interacting mediator of cell death

C/EBP: CCAAT/ enhancer binding protein

EOMES: Eomesdermin

ETV5: Ets variant gene 5

GATA3: GATA binding protein 3

HES1: Hairy and enhancer of split 1

HIF: Hypoxia inducible factor

ID2: Inhibitor of DNA binding 2

ID3: Inhibitor of DNA binding 3

IKbz: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

IRF4: Interferon regulatory factor 4

LEF1: Lymphoid enhancer binding factor 1

MAF: Avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog

NFAT: Nuclear factor of activated T cells

NFkB: Nuclear factor kappa b

PLZF: Promyelocytic leukemia zinc finger

RBPjK: Recombination signal binding protein for immunoglobulin kappa J region

RORα: Retinoic acid orphan receptor alpha

RORγt: Retinoic acid orphan receptor gamma t

RUNX1: Runt related transcription factor 1

SMAD2: Mothers against decapentaplegic homolog 2

SMAD3: Mothers against decapentaplegic homolog 3

SMAD4: Mothers against decapentaplegic homolog 4

SOCS3: Suppressor of cytokine signaling 3

SOX4: SRY (sex determining region Y)-box 4

SOX13: SRY (sex determining region Y)-box 13

STAT3: Signal Transducer and Activator of Transcription 3

STAT5: Signal Transducer and Activator of Transcription 5

T-BET: T-cell-specific T-box transcription factor

TCF1: T Cell Factor1

TIF1γ: Transcriptional intermediary factor 1 gamma

TOX: Thymocyte selection-associated high mobility group box

TRAF6: TNF receptor-associated factor 6

TRIM33: Tripartite motif-containing 33

JNK: c-Jun N-terminal kinase JNK

MAPK: Mitogen activated kinase-like protein

CHAPTER I

INTRODUCTION

INTRODUCTION

The term '*immunity*' was derived from the latin word '*immunis'* that was used to denote individuals exempted from military duty. In biology, it refers to an exemption from the disease state arising from pathogen encounter. The immune system is the collective biological machinery comprising of various organs, cells and effector molecules that provide protection and give rise to immunity.

The Innate and Adaptive immune system

In mammals, the immune system is broadly divided into two functional arms- the innate and the adaptive immune systems, which work sequentially or simultaneously to perform surveillance and protective functions. Cells of the innate immune system act as a first line of defense against foreign assault, and include granulocytic cells such as mast cells, neutrophils, eosinophils, basophils and myeloid cells such as macrophages, dendritic cells (DCs) and lymphoid natural killer (NK) cells (1). These cells recognize pathogen associated molecular patterns (PAMPs) (common antigenic determinants expressed on the surface of microbes) through their germline encoded pattern recognition receptors (PRRs). This recognition is broad and leads to a rapid release of soluble microbicidal and cytotoxic factors, cytokines and chemokines. This unbiased, fast and potent response against invading pathogens acts without the need for extensive cellular expansion and does not generate immunological memory (2).

This innate immune response triggers the development of the slower yet more specific adaptive immune response, which consists of the thymus derived lymphocytes (T cells) and the bone marrow derived lymphocytes (B cells). T and B lymphocytes express a wide array of antigen specific receptors that are generated by random gene rearrangements during their development. The B cell receptor (BCR) is activated by soluble antigens and results in the secretion of antigen specific antibodies. On the other hand, conventional αβ T cell receptors (TCRs) respond to antigenic peptides presented on major histocompatibility complex (MHC) molecules expressed on DCs, macrophages and other nucleated cells. The recognition of antigen by a T cell induces the release of multiple cytotoxic factors and provides signals for clonal expansion, thus amplifying a highly specific immune response to control and eliminate the exponentially growing pathogen. The expansion phase is followed by a contraction phase where most lymphocytic populations undergo apoptosis leaving behind a residual fraction of cells that form the memory cell pool. Memory T and B cells generate a fast and specific response upon secondary challenge by the same pathogen. Memory cells are thus key mediators of immunity and their effective generation is the basic goal for any successful vaccine strategy.

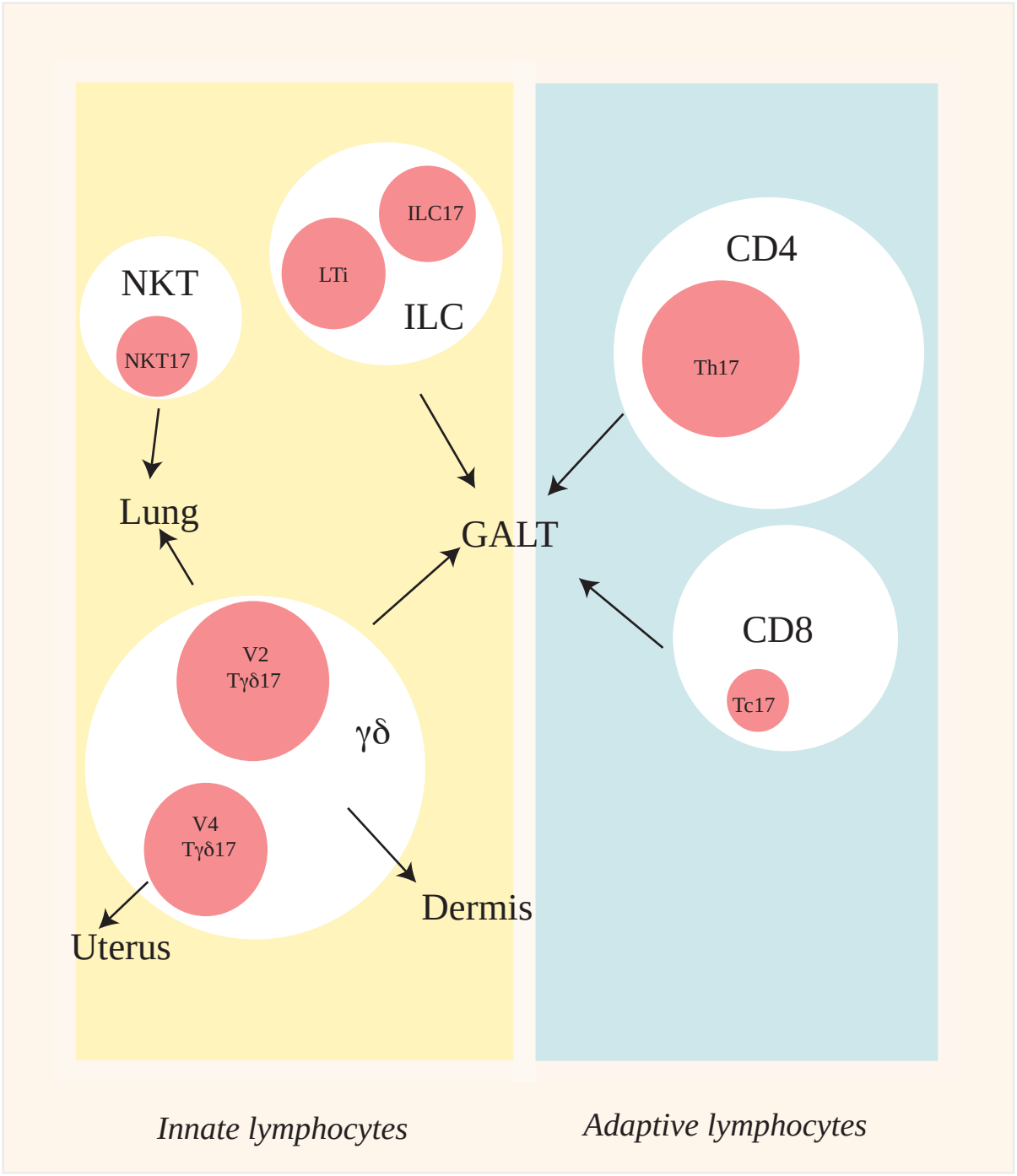
A set of lymphocytes that share overlapping characteristics of the innate and adaptive immune cells are called ‘innate-like lymphocytes’(2). These populations include γδ T cells, intestinal intra-epithelial lymphocytes and NK-like T (NKT) cells. These cells express rearranged antigen specific T cell receptors like conventional αβ T cells. However, similar to innate cells, they can also recognize conserved stress induced

antigens expressed on infected or diseased cells, or even lipid antigens as in the case of NKT cells. Additionally, many of the innate-like lymphocytes such as γδ T cells express toll like receptors (TLRs) that are normally expressed on innate cells, which enable them to recognize and get activated by PAMPs and danger associated molecular patterns (DAMPs) on infected or stressed cells (3). Innate-like lymphocytes exhibit a pre-activated, memory cell-like phenotype and are poised for the rapid mobilization of the effector arsenal. Whether innate-like lymphocytes are capable of generating a memory response is unknown and it appears that most of them respond in a similar fashion upon either a primary or secondary antigenic challenge. As a consequence of these phenotypic and functional properties, these cells are considered to bridge the gap between innate and adaptive immune responses.

A new cluster of cells called innate lymphoid cells (ILCs) has recently been described (4). These cells are of lymphocytic origin, some express NK cell specific markers and have innate properties by virtue of their ability to rapidly secrete cytokines, most notably IL-17 and IL-22, but unlike innate-like and adaptive lymphocytes, they do not express rearranged antigen specific receptors. Most ILCs originate in the gut associated lymphoid tissues (GALT) and in addition to their role in maintaining mucosal tissue homeostasis; they secrete Lymphotoxin that is required for the architectural development of lymph nodes (LNs) and Peyer's patches. The principal cell type critical for the development of lymphoid tissues is called lymphoid tissue inducer (LTi) cells and all GALT ILCs are related to LTi. In other mucosal sites, such as tonsils etc, ILCs producing Th2 cytokines IL-4, IL-5 and IL-13 are found. These ILC2 cells are distinct

Figure 1.1. IL-17 producing innate and adaptive lymphocytes. Among the innate lymphocytes, subsets of γδ T cells form the major IL-17 producing population. These cells reside in the epithelial layer of respiratory tract, reproductive tract, intestinal tract and other tissues. A small subset of NKT cells also produces IL-17. LTi subsets that belong to the ILC lineage, contribute to IL-17 production in the fetal developmental stages while ILC17 subsets are the adult IL-17 producers that reside primarily in the LP. Th17 cells are the major adaptive IL-17 producers and in homeostatic environment, they are found primarily in the LP along with CD8 IL-17 cells.

Figure 1.1


NKT
NKT17
ILC17
LTi
ILC
CD4
Th17
Lung
GALT
CD8
Tc17
V2
Tγδ17
γδ
V4
Tγδ17
Dermis
Uterus
Innate lymphocytes
Adaptive lymphocytes

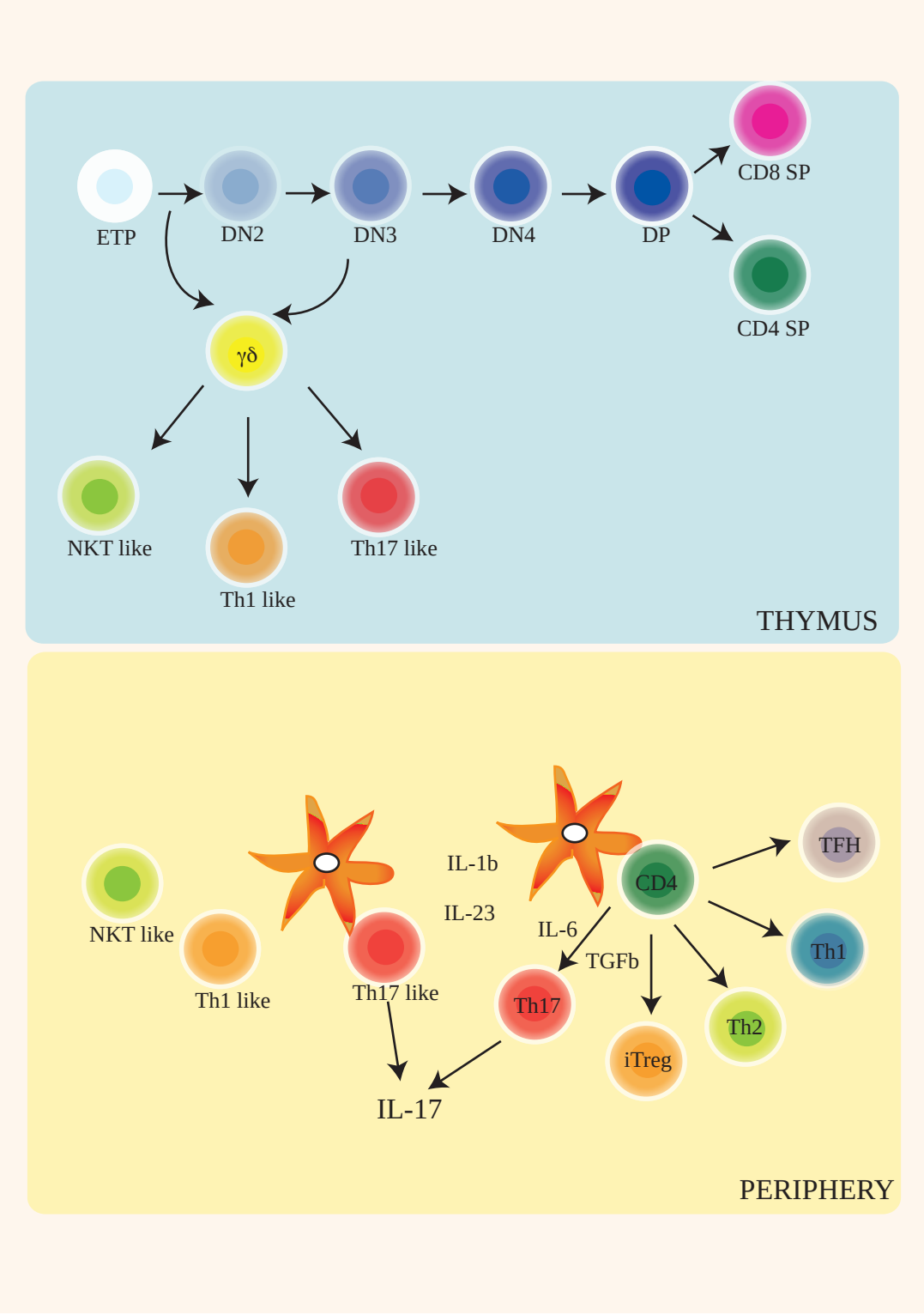
from GALT ILCs and have been shown to play critical roles in clearence of helminth infections.

An important mechanism employed by innate and adaptive immune cells in response to infection and insult is the production of cytokines. Cytokines are small soluble proteins, which act in an autocrine and/or paracrine manner, influencing both the properties of the cell that releases it and the function of other cells that express the receptors for these proteins. Arguably the most immunologically important cytokines released by some ILCs and the innate-like lymphocytes such as γδ T and NKT cells and adaptive αβ T effector subsets is IL-17 (5), (6) (Fig.1.1). While the IL-17 producing innate-like γδ T cells (Tγδ17) originate in the thymus and constitute an early source of IL-17 (5), the adaptive CD4 T cells differentiate into IL-17 producing effectors (Th17) in response to antigenic stimulation in the peripheral lymphoid organs and the inflammatory cytokine milieu (Fig 1.2). Tγδ17 and Th17 cells play critical roles in controlling pathogenic infections. However, the inflammatory IL-17 production also implicates these cell subsets in enhancing pathology in a variety of autoimmune diseases (5), (6). Though both these lymphocytes subsets secrete IL-17, the regulation of IL-17 production pathways in these cell types were predicted to be distinct based on their radically different origins and chronologically segregated participation in immunity to infections using varied modes of cytokine production.

The work in this dissertation focuses on the identification and characterization of the distinct gene networks and key transcription factors (TFs) that program the differentiation of adaptive Th17 cells versus innate-like Tγδ17 cells. This Introduction is

Figure 1.2. Thymic versus peripheral effector differentiation of γδ and CD4 T cells respectively. γδ T cells differentiate into effector cell subsets in the thymus. These cells are exported into the peripheral tissues as pre-made memory like cells, thus they rapidly secrete their effector cytokines upon brief stimulation of their cytokine or toll like receptors. On the contrary, CD4 T cells differentiate into functional Th1, Th2, Th17, iTreg or TFH cell subsets upon antigen encounter and in the presence of a specific cytokine milieu in the periphery.

Figure 1.2


ETP
DN2
DN3
DN4
DP
CD8 SP
CD4 SP
γδ
NKT like
Th1 like
Th17 like
THYMUS
NKT like
Th1 like
Th17 like
IL-1b
IL-23
IL-6
CD4
TFH
Th1
TGFb
Th17
Th2
iTreg
IL-17
PERIPHERY

divided into three major sections. **First part of the introduction focuses on the cytokines of the IL-17 family, second part describes the IL-17 producing innate and adaptive cells, and the last section reviews the current understanding of the TF networks that control the IL-17 production by these different cell subsets.**

1. IL-17 and IL-22: Signature cytokines produced by Tγδ17 and Th17 cells

1.1. IL-17 in immunity

Cytotoxic T lymphocyte antigen 8 (CTLA-8), more commonly known as IL-17A, was identified in a subtractive hybridization screen of a rodent T cell library (7). Since its discovery in 1993, several structural homologues of IL-17A have been identified and the IL-17 family now consists of 6 members (A-F) (8). IL-17A, often termed, as IL-17, and IL-17F are the most well characterized members of the IL-17 family. These cytokines are produced by GALT ILCs, γδ T cells and NKT cells, and adaptive lymphocytes such as Th17 and $CD8^+$ Tc17 cells. Among the fast-acting lymphocytes upon pathogen encounter, γδ T cells are the dominant producers of these cytokines while Th17 cells are the major late IL-17 producers (5).

The *Il17a* and *Il17f* genes are located in close proximity on chromosome 1 of the mouse genome, and their respective proteins share high amino acid sequence homology, especially in the conserved Cysteine amino acid residues (8). These cytokines mostly form covalent homodimers and rarely exist in heterodimers (9), (10). The signaling from IL-17A and IL-17F induces the expression of genes encoding proinflammatory cytokines (TNFα, IL-1β, G-CSF, GM-CSF), antimicrobial peptides (S1008, βdefensin1, RegIIIγ),

chemokines (CXCL1, CXCL5, CCL2 and CCL7) and matrix metalloproteases (MMP1, MMP3, MMP13) in epithelial cells, endothelial cells, chondrocytes and adipocytes. IL-17A also synergizes with other inflammatory cytokines such as TNFα and IL-1β to promote the activation of tissue infiltrating neutrophils (11).

With their above-mentioned inflammatory actions, IL-17 cytokines help in the rapid elimination of extracellular pathogens such as *Klebsiella pneumoniae* (lung infection), *Citrobacter rodentium* (gut infection) and *Candida albicans* (muco-cutaneous infection) (12), (13), (14). However, contrary to its protective role, the IL-17 mediated release of multiple inflammatory mediators from different tissue cells, and the recruitment of other inflammatory cells, exacerbate the inflammation in several autoimmune disorders like collagen induced arthritis (CIA) and experimental autoimmune encephalitis (EAE) in mice and in corresponding human diseases (6), (13) (rheumatoid arthritis and multiple sclerosis).

Both IL-17A and IL-17F bind to the same receptor, which is a heterodimer of IL-17RA and IL-17RC, which is expressed on lymphocytes, epithelial and endothelial cells of various tissues (8). However, the binding affinity of IL-17A to the receptor complex is much stronger as compared to IL-17F (15), (16). In contrast with the large body of knowledge that has accumulated on the generation of Th17 cells and the function of IL-17 family members, relatively little is known about the signal transduction events downstream of IL-17. The IL-17 receptor heterodimeric complex contains extracellular fibronectin III like domains and intracellular- SEFIR (Similar Expression to FGF, IL-17R and Toll/IL-1R (TIR)) domains (17). The SEFIR domain containing adaptor protein Act1

directly associates with IL-17RA and IL-17RC to mediate downstream signaling events (18). The Act1 adaptor recruits TNF receptor associated factor 6 (TRAF6) and other proteins to initiate the canonical NFκb signaling pathway (19), (20). This activates the NFκb subunits p50 and p65 and induces the expression of other NFκB family member, the TF Iκbζ. Other signal transduction programs influenced by IL-17 signaling are the MAPK and AP1 pathways. MAPK signaling downstream of IL-17 helps stabilize the mRNA transcripts of chemokines such as *Cxcl1.* IL-17A also induces the transcription of the genes that encode the TF CCAAT/enhancer binding protein (C/EBP) (21). This TF further promotes the induction of proinflammatory cytokines, IL-6 and Lipocalin2. Overall, IL-17 cytokine signaling amplifies a positive loop where factors that will help in the secretion of more IL-17 as well as augment the inflammatory reaction are released in the milieu. Magnification of this response, thus mediates the pathology observed in the autoimmune disorders.

The distinct and redundant functions of IL-17A and IL-17F, which are often co-transcribed and co-expressed in the same cell types, have been elucidated using *I17a*$^{-/-}$, *I17f*$^{-/-}$ and *I17a*$^{-/-}$*I17f*$^{-/-}$ mice (13). Mice with genetic ablation of *Il17a* or *Il17f* genes remain healthy; however, deficiency of both *Il17a* and *Il17f* genes increases susceptibility to opportunistic infection by *Staphylococcus aureus* that forms mucocutaneous abscesses around the mouth and nose of these mutant mice.

The *Citrobacter rodentium* infection in mice results in colonic hyperplasia and increased villi length. Normal mice clear the bacterial burden and colitis within two to three weeks of infection. However, deficiency of *Il17a* and *Il17f* impacts the control of

intestinal *Citrobacter* bacterial load in these mice. The uncontrolled bacterial numbers results in the dissemination of bacterial antigens to the spleens causing splenomegaly and increased lymphocyte activation. Interestingly, *Il17f* deficiency results in a more severe *Citrobacter* infection characterized by splenic hypertrophy and acute inflammatory changes in the colon compared to *Il17a* deficiency, suggesting differential requirements of IL-17F and IL-17A in the maintenance of mucosal homoeostasis. Both these cytokines regulate *Citrobacter* and other bacterial infections by inducing secretion of antimicrobial peptides and chemokines from the intestinal epithelial cells (13).

As opposed to the dominant role of IL-17F in clearing *Citrobacter* infection in mice, IL-17A is the primary driver of increased inflammatory responses in autoimmune diseases such as EAE, CIA and neutrophilic airway inflammation (13). The possible explanations for the functional difference between these two cytokines despite their structural homology are: Firstly, a signal of greater magnitude ensues downstream of IL-17A binding to its receptor, which may induce the expression of more inflammatory mediators than induced by IL-17F signaling alone (15), (16). Secondly, IL-17RA and IL-17RC complexes are differentially distributed in different tissue cell types (13). Thirdly, certain cell types preferentially secrete IL-17F over IL-17A and vice-versa. For instance, colonic epithelial cells have also been shown to specifically express *Il17f* transcripts (13), suggesting an increased requirement of IL-17F in regulating intestinal homeostasis. Therefore, it is thought that the quality of IL-17 signaling varies in different tissues depending on the relative abundance of these cytokines and differential expression of the receptors.

Another member of IL-17 family that functions similar to IL-17A and IL-17F is IL-17C. IL-17C is secreted by Th17 cells under inflammatory responses (22). Under homeostatic conditions, however, it is primarily produced by epithelial cells (23). Unlike IL-17A and IL-17F that signal by binding to IL-17RA/IL-17RC heterodimers, IL-17C signals by binding to IL-17RA/IL-17RE heterodimers (23). IL-17RE is predominantly expressed on epithelial cells with the highest expression on colonic epithelial cells. Similar to its receptor, the cytokine IL-17C is mainly produced by the epithelial cells upon activation with TLR ligands such as flagellin and peptidoglycan and other inflammatory stimuli like IL-1β and TNFα. IL-17C participates strongly in regulating homeostasis in the GALT by inducing the expression of a plethora of anti-microbial peptides, defensins and chemokines. The expression of these compounds is severely affected in *Il17c*$^{-/-}$ or *Il17re*$^{-/-}$ colons. IL-17C synergizes with IL-17A and IL-17F in augmenting inflammation in autoimmune diseases like EAE (22). In comparison to *Il17a* or *Il17f* deficient mice, *Citrobacter* infections in *Il17re*$^{-/-}$ mice result in much more severe gut pathology associated with high mortality rates (23). This indicates that while these cytokines have similar functions that are synergistic, they also perform distinct functions.

In sum, IL-17 family cytokines are critical regulators of inflammatory responses. These cytokines are essential for clearing the pathogenic infections. However, they can also cause severe unwanted damage to the host either due to an exaggerated response to the pathogens that can damage bystander cells or by their direct participation in exacerbation of autoimmune reactions to the self. For the latter to occur, multiple triggers are required and the first step is the generation of the self-reactive lymphocytes. Once the

reaction is triggered the path to self-destruction is led by the synergistic actions of the IL-17 cytokine family released by the innate and adaptive lymphocytes.

1.2. IL-22 maintains the mucosal epithelial integrity

Importantly, along with IL-17 family cytokines, other cytokines are released by subsets of innate and adaptive IL-17 producing cells that synergize with IL-17A and IL-17C to strongly upregulate the expression of anti-microbial peptides, defensins and chemokines. The most prominent of these co-factors is IL-22 (22),(3). Similar to IL-17 family cytokines, especially IL-17C, IL-22 is required for controlling and clearing *Citrobacter* bacterial infections in the gut (24). Although IL-22 has an additive effect in enhancing inflammation in IL-17 dependent autoimmune infections, IL-22 production in the absence of IL-17 cytokines leads to protective responses. For example, IL-22 aids in the proliferation of epithelial tissues of several tissues including the intestine thereby accelerating the recovery of mice after chemical induced colitis (25).

IL-22 is secreted by Th17, Tγδ17, γδ intestinal IELs and ILC ($NKp46^+$ or NK22 and ILC22) (4). As discussed in more detail in subsequent sections the regulation of *Il17* and *Il22* transcription is distinct. In particular, *Il22* transcription is highly dependent on aryl hydrocarbon receptor activated by halogenated aromatic hydrocarbons (e.g. dioxin) and by-products of dietary vegetables (e.g. tryptophan-linked phytochemical compounds), making it a rheostat linking environmental toxins and diet to epithelial homeostasis, with both detrimental and beneficial impacts to host (26),(27). IL-22 receptor complex is predominantly expressed in epithelial cells and it consists of IL-22R

and IL-10Rβ heterodimer (25). IL-22 receptor activates STAT3 and induces the anti-apoptotic proteins (BCL-2 and BCL-XL), cell cycle regulators (Cyclin D1 and CDK4) and mucus secretion promoting molecules. These factors aid in the survival, repair and proliferation of epithelial cells in the large intestine, skin, liver and lung (25).

The presence of multiple members of the IL-17 cytokine family and other Th17-related cytokines such as IL-22 released in a temporally coordinated manner by the innate and adaptive immune cells in the gut and other tissues most exposed to the external universe best illustrates a complex and intricate network of cells and factors required for mucosal epithelia homeostasis. It is not known how the differential temporal and spatial distribution of these cytokines and their receptors during homeostasis and infection establishes the healthy balance between inflammation and epithelial integrity. Understanding how the heterogeneous populations of effectors that produce IL-17 and IL-22 are distinctly made, maintained and functionally engaged is a key to defining the elements that constitute optimal immune responses at the mucosal epithelia.

2. IL-17 producing lymphocytes

2.1 Adaptive $CD4^+$ T effector lineages

Conventional αβ T cells fall into two main classes that are distinguished by the expression of cell-surface proteins, CD4 and CD8, which mark helper and cytotoxic T cells, respectively. CD4 and CD8 T cells originate as separate branches of αβ T cells in the thymus. The TCR on a CD4 T cell recognizes antigenic peptides derived from extracellular or intracellular proteins presented by the MHC Class II proteins that are

expressed on professional antigen presenting cells (APC) such as macrophages, dendritic cells and B cells. On the other hand, the TCR of a CD8 T cell, recognizes mainly intracellular antigens that are bound to the MHC class I molecules, which are expressed on all nucleated cells. CD4 and CD8 entities on these two T cell types are co-receptor chains that assist the respective TCR's engagement to APCs, by binding to the invariant regions of MHC molecules and thus promoting T cell activation.

$CD4^+$ T helper effector cells (Th) promote B cells to secrete antibodies, enhance CD8 T cell responses and $CD8^+$ T memory cell generation, potentate the microbicidal activities of macrophages, and recruit eosinophils, basophils and neutrophils to the site of infections. CD4 T cells also secrete different cytokines and chemokines to orchestrate immune responses by both hematopoietic and parenchymal cells. Immune responses controlled by Th subsets are countered by a specialized suppressor $CD4^+$ T cell subset termed regulatory T cells (Tregs). "Natural" Tregs (nTregs) differentiate in the thymus, arising from DP thymocytes, post thymic selection events that also govern conventional T cell development. nTregs were identified by their distinct expression of IL-2 receptor α chain (CD25) in the thymus (28). They express TCRs that recognize self-antigens with intermediate affinity to escape negative selection in the thymus. Subsequent to their identification, it was shown that they express forkhead family TF FOXP3 (29), (30) whose deficiencies or dysfunction underlies the devastating multi-organ autoimmunity in *scurfy* mice and results in immunedysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX) syndrome in humans (31), (32). FOXP3 acts as a central

transcriptional activator and suppressor of several genes in Tregs and thus specifies a significant part of their transcriptional signature. For example, FOXP3 regulates the expression of genes that are considered hallmarks of Treg cells such as *Il2ra, Ctla4, Tnfrsf18, Itgae, Gpr83* and *Nrp1* (33). It also suppresses the transcription of *Il2*. The lack of IL-2 production and the constitutive expression of CD25 enables Tregs to consume the IL-2 available to effector T cells and thereby competitively restrict effector T cell proliferation. Tregs also suppress proliferation and activation of conventional αβ T cells through other mechanisms such as direct cell-cell interactions, by release of suppressive cytokines like TGFβ and IL-10 (34), (35) and by rendering DCs relatively quiescent via CTLA-4 engagement of its ligand B7 molecules(36). The ligation of CTLA4 with B7 on DCs induces trans-endocytosis of B7 molecules (37) and subsequently limits the CD28-B7 interactions, to inhibit effector T cell activation. Furthermore, this interaction has also been proposed to result in an inside out signaling, where suppressive factors such as IDO (indoleamine 2-3 deoxygenase) are induced in T cells that breakdown tryptophan into products, which inhibit T cell proliferation (38).

Apart from nTregs that arise in the thymus, CD4 T cells can differentiate into multiple effector subsets in the peripheral lymphoid organs. Before the discovery of different CD4 T cell effectors, it was thought that a CD4 T cell participates in two kinds of immune responses: antibody mediated (humoral) and cell mediated. However, the humoral and cell mediated responses did not always work in parallel. In 1986, the work of Tim Mossman and Bob Coffman showed for the first time that long term CD4 T cell lines could be subdivided into two groups based on their cytokine secretions, those that

produced IFNγ (Th1) and those that produced IL-4 (Th2) (39). This led to the realization that CD4 T cells represent a pool of heterogeneous populations, which possess different effector functions. How these effectors are generated and what TFs regulate their differentiation have been two dominant themes in immunological research for over 20 years. In the presence of different cytokine microenvironments, a naïve CD4 T cell can differentiate into at least five distinct functional lineages: Th1, Th2, Th17, induced $FOXP3^+$ Tregs and T follicular helper (TFH) cells. The in vitro T helper cell cultures, often used to study these functional lineages, utilize a specific Th lineage-promoting cocktail of recombinant cytokines and Abs that block cytokines of alternate lineage. However, in vivo, several of these effectors co-exist with each other, indicating that the rules of Th subset differentiation are much more complex than those mimicked in vitro. Overall, it is the relative dominance of a specific effector arm or the co-functioning of different effector lineages that determines the clearance of a pathogenic infection with limited immunopathologies or susceptibility to autoimmune inflammation.

2.1.1. $IFN\gamma^+$ Th1 cells

Th1 cells secrete IFNγ as their signature cytokine and TNFα and Lymphotoxin β as their secondary cytokines. Their primary role is to help in clearance of intracellular viral, protozoan and bacterial infections (40). Outside their roles in clearing infections, Th1 cells are also implicated in the pathogenesis of autoimmune disorders such as Inflammatory Bowel Disease (IBD) and EAE. The key cytokine and the TF that are necessary for Th1 cell differentiation are IL-12 and T-BET respectively (41). T-BET

induces chromatin remodeling at the IFNγ locus, promotes upregulation of the IL-12 Receptor β2 (IL-12Rβ2) subunit to amplify IL-12 signaling while inhibiting T cell differentiation to other functional lineages. *In vitro*, a naïve CD4 T cell can be polarized towards the Th1 lineage by the engagement of TCR in the presence of IL-12. During in vivo infections, activated innate cells such as NK cells and innate-like γδ T cells act as an early source of IFNγ. Early IFNγ synergizes with TCR stimulation to induce T-BET expression in naïve CD4 T cells. Subsequently, IL-12 released from pathogen-activated macrophages and DCs augments T-BET expression to polarize CD4 T cells toward Th1 lineage (42). IFNγ signaling maintains multiple aspects of inflammatory processes: it augments antigen processing and presentation by APCs, stimulates IgG2α antibody production from B cells, and enhances phagocytotic and nitric oxide releasing activities of macrophages. Further, IFNγ augments the recruitment of granulocytes and myeloid cells such as neutrophils and macrophages to the site of infections. These functions of IFNγ capture most activities associated with Th1 cells and IFNγ-deficiency results in the susceptibility to an array of intracellular pathogens in mice (40). Although IFNγ is the central cytokine of a Th1 response, Th17 cells that predominantly produce cytokine IL-17 can also convert to Th1 lineage-like cells by producing IFNγ. The reverse conversion, Th1 to Th17 cells, is not observed. In vivo, Th17 cells that co-express IL-17 and IFNγ have been suggested to be the primary pathogenic effector cells in autoimmune diseases (43).

2.1.2. IL-4$^+$ Th2 cells

Initially characterized as helper cells for B cell activation and class switching, Th2 cells are important in protecting the host against extracellular infections of worms (Helminths), parasites, and are also implicated in aggravating allergic disorders such as asthma and atopic dermatitis. The signature cytokines released by Th2 cells are IL-4, IL-5 and IL-13 (42). The differentiation of CD4 T cells to the Th2 lineage requires the presence of IL-4 along with T cell activation signals. The exact source of IL-4 in vivo for initial priming of Th2 differentiation remains unclear. Memory CD4$^+$ T cells, basophils, NKT cells and γδ T cells are some of the early producers of IL-4 that can program naïve CD4$^+$ T cell differentiation towards the Th2 lineage. IL-4R signaling on T cells activates the TF STAT6 that in turn induces the expression of the TF GATA3, which docks onto the *Il4* gene loci, respectively (44). GATA3 can program Th2 cell development when overexpressed in T cells (45). Moreover, it can direct cells towards the Th2 lineage even in the absence of the TF STAT6 (46). One of the TFs that can upregulate GATA3 expression in CD4 T cells is IRF4 (47). Interestingly, IRF4 is also critical for Th17 cell differentiation and Treg cell function (48), (49). The exact underlying differences in the function of IRF4 in these T cell lineages are not fully defined. However, it is likely that many TFs critical for effector cell differentiation and function have multiple co-factors and depending on the cell type-specific TF network, they regulate discrete set of genes. Throughout T cell development, major TFs are recursively used at different stages of maturation, with constantly changing repertoire of target genes under their regulatory umbrella. This reutilization is economical, conserves cellular energy and confers onto

cellular systems rapidity of response to changing environmental cues by limiting the number of effectors needed to alter global genome activity. The differentiation of CD4 T cells is an excellent case study where the same cytokines and TFs can have cell type specific functions by interacting with a few effector lineage specific factors.

2.1.3 Follicular helper T cells (TFH cells)

CD4 T cells, whose primary function is to provide help to B cells, have been recently grouped as a distinct lineage called as TFH cells. These cells are identified by their expression of chemokine receptor CXCR5, cytokine IL-21 and the TF BCL6 (50), (51), (52). In the absence of BCL6, TFH cell differentiation does not occur (52), while its constitutive expression drives this differentiation (53). BCL6 represses the differentiation of Th1, Th2 and Th17 cell lineages while specifically maintaining the TFH cells. These results suggested BCL6 as the master regulator for TFH cell differentiation and placed TFH cells as a distinct lineage. Multiple factors such as cytokines IL-6 and IL-21 (52), (54), (55) and B cells have been proposed to be required for TFH cell differentiation but the detailed process for their in vivo differentiation is not known yet. TFH cells are located in the Germinal Centers (GC) of lymphoid organs where they help B cells for Class Switch Recombination, memory B cell formation and plasma cell differentiation among other functions (56). B cells and TFH cells engage in mutual interactions and help in the development of each other. Like other T helper subsets, TFH cells also exhibit plasticity and in infectious settings, can contribute to IFNγ and IL-4 production. The production of cytokine IL-21 and the requirement of TFs STAT3 and IRF4 are shared

features between TFH cells and Th17 cells (56). Moreover, similar to IL-21, IL-17 has also been shown to induce GC formation in the autoimmune BDX2 mice (57). Whether TFH cells co-operate with Th17 cells for GC formation under homeostatic conditions is not known.

2.1.4. TGFβ-dependent iTreg cells

$CD4^+$ T cells can adopt regulatory fates in the peripheral lymphoid organs in the presence of antigenic stimulations and the cytokine TGFβ. TGFβ can turn on *Foxp3* expression in $CD4^+$ T cells and generate induced Tregs (iTregs) (58). FOXP3 provides these cells with immunosuppressive properties similar to nTregs. The gene expression profiling iTregs showed that they express a subset of the nTreg gene signature, such as *Nrp1, Itgae* and *Gpr83* genes, but lack the expression of other central nTreg-associated transcripts such as *Il2ra, Tnfrsf18* and *Ctla4* (33). In vitro and in vivo Treg functional assays have shown that iTregs possess limited suppressive activity. The reasons for their limited activity include: a) Partial epigenetic modifications at certain regulatory Conserved Non-coding Sequence (CNS) elements at *Foxp3* gene locus that render FOXP3 expression unstable in the absence of continuous TGFβ availability, thereby permitting a reversion to conventional CD4 T effector cells (59), (60) and (b) The inability of induced FOXP3 to activate all of its target genes found in nTregs (33).

Although, it can be said that iTegs do not completely mimic nTregs, the fact that TGFβ can induce these cells is relevant in vivo especially under non-homeostatic conditions when a locally present iTreg cell could help in suppressing the inflammatory

responses induced by CD8 T cells, Th1 or Th17 cell types during infections or autoimmune disorders. Also, TGFβ is central to the differentiation of not only iTregs but also for Th17 cells. Therefore, the understanding of Th17 cell generation is tightly connected to the differentiation of iTregs.

2.1.5. IL-17/22^{+} Th17 cells

After the discovery of Th1 and Th2 cells, there were a number of observations that remained unexplained. For instance, though Th1 cells were implicated in potentiating autoimmune disorders, mice deficient in IFNγ or IFNγ receptor were found to have an increased susceptibility towards CIA and EAE (61). Similarly, EAE was exacerbated in the mice deficient for the Th1 inducing cytokine IL-12 (62). Subsequently, it was discovered that IL-12 cytokine shares its p40 subunit with IL-23 cytokine and mice deficient specifically in IL-23 were found to be resistant to EAE (63). Though IL-23 did not promote IL-17 production from CD4 T cells, it was shown to promote the expansion and survival of cells that produce IL-17, which were later termed as Th17 cells. IL-23 expanded Th17 effector T cells were able to transfer EAE more effectively than EAE induced by the transfer of Th1 cells alone (64). These results challenged the importance of Th1 cells and IL-12 in autoimmune diseases and shifted attention toward IL-23 and Th17 cells.

Th17 cells were identified as an independent effector T cell lineage in 2005 by Casey Weaver's laboratory (65). Subsequent studies showed that in vitro stimulation of naïve CD4 T cells with TCR engagement in the presence of immunoregulatory cytokine

TGFβ and proinflammatory cytokine IL-6 results in their polarization towards the Th17 cell lineage (66),(67), (68). Th17 cells produce IL-17A and IL-17F as their hallmark cytokines and IL-17C, IL-23, IL-21 and IL-22 as secondary cytokines. Th17 cells, as described previously, have dual roles in inducing inflammation in many autoimmune disorders while also providing protection against extracellular microbes (*Candida albicans*, *Citrobacterium rodentium* and *Klebsiella pneumoniae)* and some intracellular pathogens (*Francesella tularensis)* (14), (69), (70). In vivo, a very small percentage of CD4 T cells in the peripheral lymphoid organs secrete IL-17. A relatively significant proportion (around 10%) of CD4 T cells present in the lamina propria (LP) of the small intestine and colon of mice produce IL-17. However, the cellularity of LP lymphocytes is very low under homeostatic conditions (71). Infections by pathogens such as *Citrobacter rodentium* (72) or induction of autoimmune diseases (EAE) (22) increase the development of Th17 cells. Therefore, these models are often used for studying the cytokines and transcription factors required for Th17 cellular differentiation.

The generation of Th17 cells requires three simultaneous signals, TCR activation, co-stimulation and cytokine receptor activation. Distinct components of the TCR signal transduction pathway convey the quality or quantity of signals to integrate the existing activities of TFs or for the de novo induction of new TFs involved in T cell effector differentiation. One such example is the Tec family kinase ITK, which acts downstream of the TCR to relay the signals for activation of the TF Nuclear Factor of Activated T cell (NFAT). Mutations affecting *Itk* do not completely eliminate TCR signals but result in a decreased T cell response. *Itk* deficient CD4 T cells are impaired in the expression of

Il17a, but they can express other Th17 cell associated cytokines normally, including the closely linked *Il17f* (73). The defect in IL-17A expression arising from *Itk* deficiency is caused by a decreased binding of NFATc1 specifically to the *Il17a* promoter. These results suggested that expression of *Il17a,* but not *Il17f,* is controlled in part by the strength of TCR signaling and that proper Th17 differentiation requires "strong" signals, if the loss of ITK is viewed primarily as a setting for increased signal threshold required for T cell activation. However, there are caveats to this interpretation. ITK-mediated Ca^{2+}/NFAT signaling is likely to be influenced by CD28 costimulatory signaling that also activates ITK (74). CD28 has been implicated as both a positive and negative regulator of Th17 differentiation (75), (76), greatly complicating the issue. In humans, it has been reported that relatively weak, but not strong, T cell activation signals promote Th17 differentiation (77), suggesting that signals in addition to those generated by TCR have major modulatory effects on *Il17* transcription.

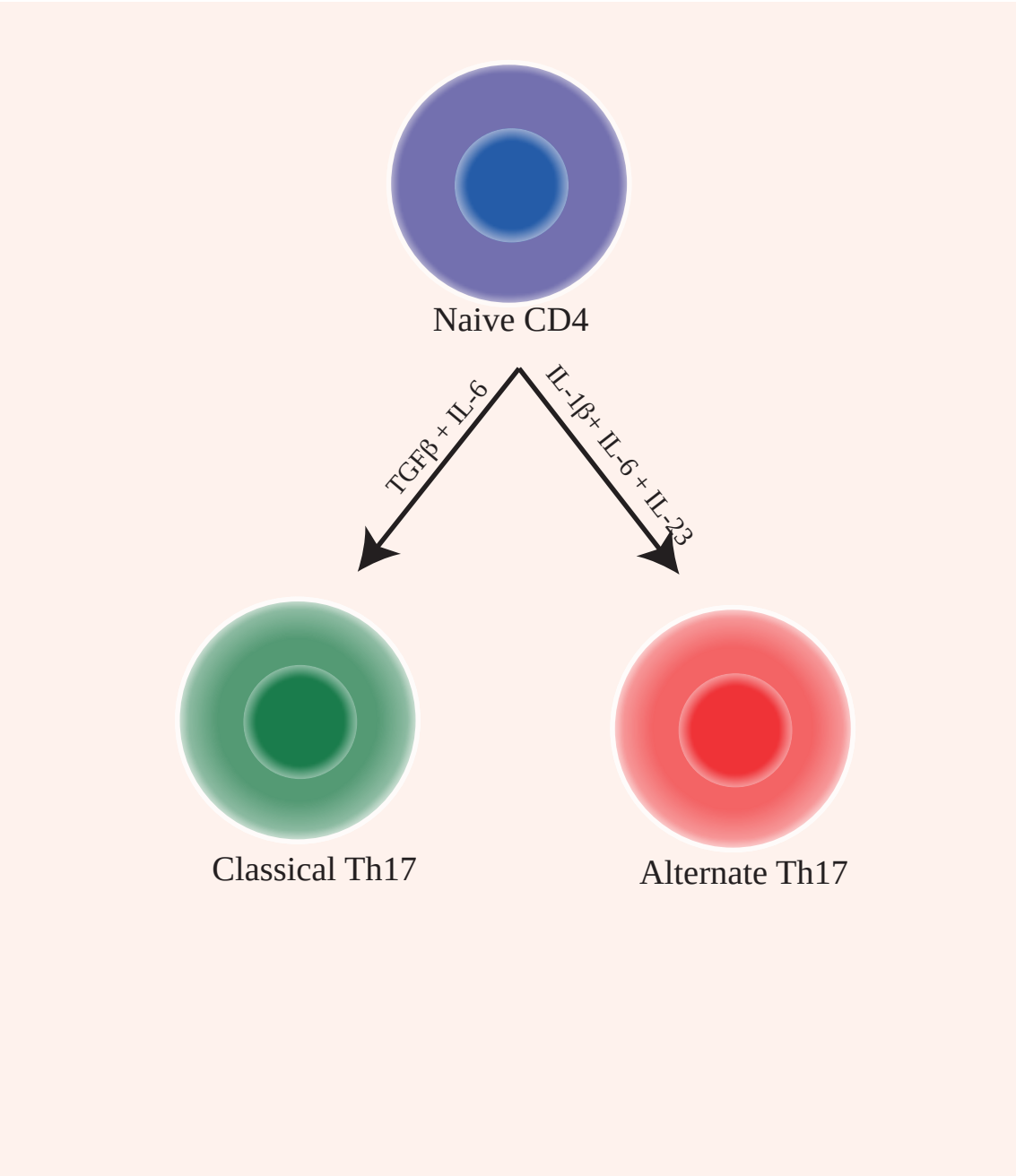
Aside from TCR signaling the driving force for the Th17 cell generation is the presence of a specific cytokine milieu. Initial studies of murine Th17 cells implicated TGFβ and IL-6 as the Th17 differentiation factors. The dual role of TGFβ in inducing the suppressive iTregs and inflammatory Th17 cells strongly reinforced TGFβ as the kingpin determining the fine balance of immune homeostasis and inflammation, with the concentration of TGFβ put forth as a major deterministic parameter of the balancing act (78). However, Th17 cell differentiation is not entirely controlled by TGFβ, as other inflammatory cytokines IL-1β and IL-23 can also program Th17 differentiation during T cell activation (79), (80). Therefore, Th17 cells are now sub-divided into two main

categories (80), (81) based on their generation in the presence of two distinct cytokine environments: Th17 cells generated in the presence of TGFβ and IL-6 are called Classical Th17 cells or Th17(β) cells; and Th17 cells generated in the presence of IL-1β with IL-6 and IL-23 have been named Alternate Th17 cells or Th17(23) cells (Fig.1.3). The comparison of gene expression profiles of Th17(β) and Th17(23) cells showed increased expressions of *Il18r, Tbx21 (Tbet), Cxcr3* and *Il2* in the latter. The induction of these Th17 genes is associated with the development of IL-17^{+}IFNγ^{+} CD4 T cells that develop during EAE and IBD and are thought to contribute to the inflammation and pathology in these disease settings (80), (43).Thus, it has been suggested that classical Th17 cells are not pathogenic, but that inflammatory cytokine generated alternate Th17 cells are. The cytokines that are involved in Th17 cell differentiation are described in more detail below.

Transforming Growth Factor β (TGFβ) is a morphogen in the Bone Morphogenic Protein (BMP) family and a cytokine that is critical for angiogenesis, epithelial to mesenchymal transition, cell growth and division, carcinogenesis, and development and homeostasis of the immune system (82). In the immune system, it affects most, if not all, cell types: It regulates the proliferation and responses of T cells and NK cells, mediates class switching of B cells for IgA production, regulates NKT cell development and inhibits the maturation and expansion of multiple cell types, including APCs (83) (84). Most critically, TGFβ is the central cytokine required for the maintenance of peripheral tolerance by controlling the nTreg development and imposing quiescence on most

Figure 1.3. Distinct sub-types of Th17 cells. CD4 T cells can be differentiated into Th17 cells in the presence of TGFβ and IL-6 or with IL-1β, IL-6 and IL-23 cytokines. The Th17 cells generated in the presence of TGFβ are called Classical Th17 cells, whereas IL-1β induced Th17 cells are Alternate Th17 cells. These sub-types secrete IL-17 but they express many distinct markers that could categorize Alternate Th17 cells as more inflammatory than Classical Th17 cells.

Figure 1.3


Naive CD4
TGFβ + IL-6
IL-1β+ IL-6 + IL-23
Classical Th17
Alternate Th17

hematopoietic cell types. The absence of TGFβ in mice leads to deregulated immune cell activation resulting in an early onset fatal multi-organ autoimmunity (85).

TGFβ has three isoforms, of which TGFβ1 is predominantly expressed in the immune system. TGFβ engages a receptor comprising of two subunits, TGFβRII and TGFβRI, which are serine threonine kinases and transduce signals through phosphorylation of downstream transcription factors called SMAD proteins. SMAD2 and SMAD3 (receptor SMADs) present in the cytoplasm undergo phosphorylation upon the receptor engagement and are imported to the nucleus with or without SMAD4, where they affect a large number of genes primarily involved in cell cycle and differentiation (84). The details of the TGFβ signal transduction cascade will be described in depth in Chapter II, while the functions of TGFβ in different cell types, particularly Th17 cells, remains the focus here.

Dissection of the role of TGFβ has been performed by using multiple models that alter TGFβ signaling globally (*Tgfb*$^{-/-}$ mice) and in a T cell specific fashion (*Cd4cre-TgfbrII*$^{fl/fl}$ mice) and transgenic mice expressing a dominant negative form of TGFβRII, (DNR), specifically in T cells (85), (86), (83). Similar to *Tgfb*$^{-/-}$ mice, *Cd4cre-TgfbrII*$^{fl/fl}$ mice and DNR mice suffer from spontaneous T cell mediated autoimmune disorders. One of the reasons for the autoimmune manifestations in the absence of TGFβ signaling is the drastic reduction in the cellularity of nTreg cells. A number of studies were subsequently conducted to investigate whether TGFβ affects thymic differentiation of nTregs or their survival in peripheral lymphoid organs. Analysis of DNR mice showed normal nTreg frequencies in the thymus, but a stark reduction of nTregs in peripheral

lymphoid organs (83). However, analysis of *TgfbrI* CKO neonatal mice (3-5 days old) showed an early requirement of TGFβ in nTreg development in thymus (87). Further investigations have shown a requirement of TGFβ in protecting nTregs from apoptosis by maintaining a balance of pro-apoptotic proteins (BIM and BAX) and the anti-apoptotic TF BCL2 (88). Apart from maintenance of nTregs, TGFβ as mentioned before, is necessary for the differentiation of iTregs, which require the action of SMAD TFs to induce FOXP3 expression (89).

TGFβ signaling was also shown to play a key role in mouse Th17 cell differentiation (67). It was shown that T cells from *Tgfb* CKO and DNR mice did not efficiently differentiate into Th17 lineage cells and these mutant mice were protected from EAE upon MOG peptide immunization (90). Conversely, transgenic overexpression of *Tgfb* in mice increased the generation of Th17 cells and correspondingly increased the severity of EAE. It was also shown that T cell derived TGFβ functions in an autocrine or paracrine fashion to induce and maintain Th17 cell differentiation (90).

For Th17 cell differentiation, TGFβ by itself can upregulate the expression of RORγt, the master TF of all IL-17 and IL-22 producing lymphocytes (78). IL-6 signaling activates STAT3, which further amplifies RORγt expression and IL-17 transcription. Various mechanisms by which TGFβ can regulate Th17 cell differentiation have been proposed. It has been shown that TGFβ mediated suppression of Th1 and Th2 cell differentiation pathways is one of the fundamental parameters in which TGFβ promotes IL-6 dependent IL-17 production (91). Further, TGFβ can directly enhance IL-6 signaling by increasing the expression of the *Il6ra* subunit of IL-6R and by inhibiting the

expression of STAT3 inhibitory SOCS3 proteins (92), (93). Thus, TGFβ enhances and maintains IL-6 activated STAT3 phosphorylation to promote sustained IL-17 production (93). Importantly, the concentration gradient of TGFβ has been suggested to determine the fate of effector T cells. It has been shown that lower concentrations of TGFβ drive Th17 cell differentiation while higher concentrations are inhibitory, instead promoting the iTreg differentiation (78). TGFβ is one of the most ubiquitously expressed cytokines in the immune system, with nearly all hematopoietic cells capable of expressing it. However, complex processing is necessary for its full function (94) and the active form is extremely difficult to detect in vivo. Given TGFβ's pleiotropic and potent effects, the in vivo availability of paracrine TGFβ is likely to be tightly controlled and full understanding of TGFβ function will require more in depth understanding of anatomical distributions of TGFβ producing micro niches in homeostatic and inflammatory settings.

IL-6 as the essential cytokine for Th17 cell differentiation was discovered when naïve CD4 T cells cultured in the presence TGFβ and supernatant from LPS stimulated DCs produced IL-17. This IL-17 production was completely abolished by the blockade of IL-6 signaling (66). Further, it was shown that addition of recombinant IL-6 with TGFβ induced Th17 cell differentiation, while addition of TGFβ alone resulted in FOXP3 induction (95). IL-6 is an inflammatory cytokine that functions in acute phase response during infections. It was initially cloned as a growth factor for B cells which induced differentiation of plasma cells (96). IL-6 also acts as a co-stimulatory factor for T cells helping in their activation, proliferation and memory cell generation (97). The production

of IL-6 by the DCs, monocytes, macrophages and B cells is stimulated by external inflammatory stimuli such as LPS, IL-1 and TNFα. IL-6 binds to a receptor complex consisting of IL-6Rα and the common signal transducer of IL-6 cytokine family gp130 subunit. The T cell stimulation of IL-6R activates Janus kinases 1 and 2 (JAK1, JAK2) and Tyk2, which in turn recruit and phosphorylate STAT3 (97).

For Th17 cell differentiation, IL-6 activated STAT3 induces the transcription of several key TFs (*Rora, Rorc* and *Batf*) and cytokines (*Il17a* and *Il17f)* (98). Although IL-6 has been considered the most critical cytokine for Th17 cell induction, it has now been shown that Th17 cells can be generated in an IL-6 independent manner. Cytokine IL-21 has been shown to partially compensate for the absence of IL-6 and it can participate in Th17 cell differentiation in *Il6* deficient mice (99). Although IL-21 can contribute towards Th17 cell differentiation, its major action is to maintain these cells by inducing the expression of *Il23r* on developing Th17 cells (100). Interestingly, a recent study showed that normal proportions of Th17 cells are observed in the spleens of the $Il6^{-/-}$ mice (101). Th17 cells form a small proportion of the memory T cell pool compared with their relatively larger proportions in the LP of the intestine. Despite the normal Th17 cell proportions in the spleen, the loss of IL-6 impaired IL-17 production from LP CD4 T cells under homeostatic as well as inflammatory environments. This defect was shown to be due to distinct populations of DCs that were localized in the spleen and LP in mice. It was shown that the DCs in the LP secreted TGFβ and the Vitamin A metabolite Retinoic Acid (RA) to preferentially induce Treg differentiation (101). In this setting, IL-6 was proposed to interfere with the inhibitory effects of RA on Th17 cell differentiation.

However, Vitamin A-deficient mice (102) or RA Receptor A (*Rara*)-deficient mice exhibit a striking decrease in Th17 cell proportions in the GALT (103), ruling out the previous conclusion that RA favors Treg cell development at the expense of Th17 differentiation (104) and raising doubts regarding the IL-6 and RA interaction.

IL-23 cytokine is composed of p19 and p40 subunits, the latter being a common subunit of IL-12 and IL-23. The generation of *p19* deficient mice led to the distinction of the signaling between these two cytokines (63). Similar to the cytokines, IL-23R and IL-12R also share a common subunit. However, while signaling through IL-12 predominantly activates STAT4, IL-23 signaling instead leads to a strong activation of STAT3 (105). IL-23 (*p19*) deficient mice failed to clear *Citrobacter* infection (67). Furthermore, *p19* deficient mice were also resistant to the development of EAE (63) implicating a central role for IL-23 in Th17 cell differentiation. However, the lack of IL-23R on naïve $CD4^+$ T cells and the normal IL-17 production by the IL-23R deficient T cells (43) suggested that IL-23 is specifically required for the expansion and pathogenicity of already differentiated Th17 cells (106), (107). Consistent with this IL-23 alone cannot generate Th17 cells in vitro nor can it replace IL-6. IL-23 is required for generating pathogenic IL-17^+IFNγ^+ dual cytokine producer cells, as these cells do not develop in *Il23ra* deficient mice, which correlates with increased resistance to EAE induction (107). In Th17 cells, IL-6 has been shown to be the major driver of *Il23r* expression (100), which is further amplified by IL-23 itself. IL-23R expression, however, is not restricted to CD4 T cells. It is also expressed on T$\gamma\delta$17 and IL-17 and IL-22 producing ILCs (108), (3), (109). IL-23R

expression on innate effectors does not depend on IL-6 (110) and an alternate pathway for innate IL-17 production exists (Narayan et al., in press), as discussed in more detail in Chapter III. The activation of IL-23R signaling induces *Il22* expression and consequently IL-23 participates indirectly in IL-22 mediated protective and pathological responses (111). AHR, which is expressed in all innate and adaptive IL-17 producing cells, also increases *Il22* transcription (26), (3). Which of these pathways is dominant in IL-22 production is not known. Interestingly, a recent study showed that IL-23R expression and IL-23 mediated IL-22 production is reduced in the absence of AhR in the ILCs (27). This suggests that AhR and IL-23R pathways converge for IL-23 production; the detailed links that would connect the AhR- IL-23R pathway are not yet understood.

IL-1β is one of the 11 members that comprise the IL-1 family of cytokines. IL-1β is primarily known for its function in innate responses. Its signaling through the IL-1 receptor leads to NFκB activation, which potentiates inflammatory responses (112). IL-1R1 is highly expressed on all innate and adaptive IL-17 producing lymphocytes. During Th17 cell differentiation, IL-1β synergizes with TGFβ to enhance IL-17 production (113), (79). Interestingly, an association of IL-1β with hyper IL-17 production was observed upon a gain of function mutation in the NLRP3 inflammasome (114). An inflammasome is a molecular platform that senses endogenous danger signals (uric acid, cholesterol and others) or exogenous pathogen signals (through PAMPs) and induces a signaling cascade to drive the secretion of inflammatory cytokines (115). In mice, a point mutation in the *Nlrp3* gene lowers the threshold of NLRP3 activation. This induces

strong expression of IL-17 inducing cytokines such as IL-1β in DCs resulting in the development of an autoinflammatory disease characterized by increased Th17 cell responses.

Previously published results showed that IL-1β and IL-6 can induce Th17 cell differentiation, but this inductive condition was thought to be dependent on the endogenous TGFβ secretion by the activated T cells. However, subsequent results demonstrated that IL-17 could be induced in the presence of IL-1β with IL-6 and IL-23 even when TGFβ signaling was blocked (80). Moreover, in the absence of IL-1R1 on CD4 T cells, in vivo and in vitro Th17 cell differentiation is dramatically compromised. IL-1β mediated IL-17 induction involves the induction of IRF4 and RORγt and overexpression of both TFs has been shown to be required to restore IL-17 production in *Il1r1* deficient T cells (79).

In addition to specific cytokine environments, another unique factor driving in vivo Th17 cell differentiation is the gut microbiota. The release of IL-17 cytokines, as mentioned before, is necessary for the maintenance of homeostasis in the dynamic gut environment. Apart from Th17 cells, the LP also contains other IL-17 producing subsets that include LTi-related ILCs, γδ T, $CD8^+$ T, and iNKT cells. The generation of Th17 cells requires the presence of gut flora and they are completely absent in the germ free mice (116). While the development of Tγδ17 cells is independent of microbial flora, their expansion in the LP is limited in the germ free mice (117). Further, the development of $ROR\gamma t^+$ ILCs is also not dependent on the gut microbiota (118). One of the mechanisms by which

gut flora induces Th17 cell differentiation is by releasing the ATP in the gut lumen (116). ATP then activates a subset of $CD70^{hi}$ $CD11c^{low}$ DCs present in the LP. This leads to the transcription of the genes for Th17 inducing cytokines such as *Il6, Il23a, Tnf,* and for integrin genes αV and $\beta 8$ that converts latent TGFβ to its functionally active state. The mechanistic link between the activated ATP receptors expressed on a subset of DCs and the altered gene expression that favors the induction of Th17 gene expression program in the engaged naïve T cells is not well defined. How bacteria and ATP release in the lumen influence iTreg cell differentiation in the gut is also not known.

The gut flora in the intestinal lumen belongs to several genera. Not all the bacteria have the same ability to induce Th17 cell responses. A pioneering work from the Littman lab showed that C57BL/6 (B6) mice bred at Taconic contained more Th17 cells than those from the Jackson Laboratory (119). Detailed studies led to the identification of a family of bacteria termed segmented filamentous bacteria (SFB) as the causative agent of the expanded Th17 cells in Taconic B6 mice (120). It was shown that the colonization with SFB alone could confer high Th17 state in Jackson B6 mice. SFB induces genes that encode for Serum Amyloid A (SAA) proteins in the LP DCs. These SAA proteins have the capacity to induce the secretion of proinflammatory cytokines from LP DCs to promote Th17 cell differentiation. Whether this is the sole mechanism responsible for the unique ability of this bacteria to promote Th17 differentiation remains to be determined.

The release of ATP and SAA for enhancing Th17 cell differentiation is a feature of commensal bacteria that reside under homeostatic conditions in the gut. Interestingly, other mechanisms are at play to increase Th17 cell differentiation upon pathogenic gut

infections in mice. Pathogenic bacteria such as *Citrobacter rodentium* attach to the intestinal wall and cause the loss of epithelial integrity by inducing massive apoptosis of the epithelial cells. The infected apoptotic vesicles that contain self-antigens and TLR ligands like LPS are phagocytosed, causing activation of TLRs expressed on the phagocyting cells (72). The activated phagocytes secrete cytokines TGFβ and IL-6 and prime the gut environment to generate Th17 cells. In a reciprocal condition, when uninfected apoptotic vesicles are released and phagocytosed, it results in the secretion of only TGFβ by phagocytes, which helps in the generation of FOXP3 expressing iTregs in the LP. Interestingly, while blocking apoptosis by using mutant *Citrobacter* strains or by inhibiting caspase mediated apoptosis blocks overt IL-17 induction, the basal Th17 cell numbers were maintained. This emphasizes that the sensory cells in the LP are triggered by various stimuli released by the gut flora. While strong signals like pathogenic assault potentially induce a stronger inflammatory response dependent on TLR activation, constant, tonic stimuli ensure continuous generation of Th17 cells.

Although CD4 T cells release most of the IL-17 in the adaptive T cell compartment, CD8 T cells can also contribute towards IL-17 production. Cytotoxic T cells capable of IL-17 synthesis (Tc17) can be found in the LP (71). This suggests that to maintain intestinal homeostasis, there is a strong requirement for IL-17 produced by multiple cell types in the gut, which is contributed by nearly all of the major subsets of the adaptive and innate lymphocytes.

2.2. γδ T cells: innate sources of IL-17

Despite the increased attention received by Th17 cells in past few years, much of the IL-17 released during an inflammatory response is actually derived from ILCs and the innate-like γδ T cells (5). Unlike Th17 cells that differentiate in a foreign antigen specific manner in the context of an established inflammatory milieu, ILCs and γδ T cells are pre programmed effectors that can secrete cytokines immediately upon pathogen detection. Given that Tγδ17 cells can be activated by TLR signaling plus IL-23 (3), (121) to secrete effector molecules, without an absolute need for the engagement of clonal TCR, the size of the responding population at the initial phase of pathogen detection is very large compared to adaptive T cells ruled by the clonal selection. For instance, during *Mycobacterium tuberculosis* infections in mice, γδ T cells constitute the dominant IL-17 producing population present in the lungs soon after infection (122). Similarly, during EAE development, a major fraction of γδ T cells make IL-17 in the draining lymph nodes at early time-points compared to a minor involvement of Th17 cells (123). Furthermore, very few Th17 cells are found in healthy animals and they are primarily localized to the gut. However, Tγδ17 cells reside in multiple organs such as the epithelium of respiratory tract, reproductive tract, lung, dermis, as well as in blood, peritoneum and lymphoid tissues (5).

γδ TCRs are proposed to recognize antigens of limited complexity that are upregulated on stressed, damaged, infected or transformed cells. One class of such antigens are non-classical MHC Class I molecules such as T10/22 and Qa, which are upregulated upon stress (124). However, as emphasized earlier, Tγδ17 cells have the

ability to secrete large amounts of cytokines without the need of TCR engagement, by the mere activation of their innate toll-like receptors, or inflammatory cytokine receptors. This innate sensing and activation are programmed during differentiation processes in the thymus.

2.2.1. Thymic development of γδ T cells: ***Developmental stages***

Both γδ T cells and αβ T cells develop from multi-potent precursors in the thymus. Thymic T cell development begins with the settlement of uncommitted early thymic progenitors (ETPs) that still possess limited myeloid development potential, which gets restrained by the thymic environment. The loss of multi-potency and the progressive commitment to T cell lineage is a gradual process. The sequential progression of thymocyte precursors initiates from the DN1 stage ($CD4^-CD8^-ckit^+CD44^+CD25^-$) to DN2 stage ($CD4^-CD8^-ckit^-CD44^-CD25^+$) followed by DN3 stage ($CD4^-CD8^-c\text{-}Kit^-CD44^+ CD25^+$), which contain mostly αβ T cell lineage committed cells, and finally completed by DN4 stage ($CD4^-CD8^-c\text{-}Kit^-CD44^-CD25^-$) (125). The gene rearrangements at δ, γ and β loci occur primarily as cells transit from DN2 to DN3 stages (126), (127). The productively rearranged *Tcrg* and *Tcrd* genes are necessary for γδ T cell differentiation whereas successful *Tcrb* gene rearrangement (β-selection) commits DN3 cells to the αβ T cell lineage (128). Most γδ T cells arise from the DN2 stage but some γδ T cells can arise from DN3 cells (129), however in DN3 cells *Tcrg/d* expression is inhibited and very little intracellular TCRγ or TCRδ chains are detectable (130). The β chain on DN3 cells forms a complex with an invariant pre-TCRα chain forming a pre-TCR. The cells

expressing pre-TCR quickly transition from DN4 stage to the double positive (DP) stage characterized by the co-expression of CD4 and CD8 co-receptors. At this stage, cells rearrange *Tcra* gene and start expressing TCRαβ on their cell surface. This is followed by the positive and negative selection events and eventual downregulation of CD4 or CD8 co-receptors giving rise to mature CD4 SP or CD8 SP T cells. With the elucidation of the steps leading to development of these T cell types, it became necessary to understand how the precursor cells undergo this binary (αβ/γδ) cell-fate decision process.

2.2.2. Thymic development of γδ T cells: *αβ/γδ T cell lineage commitment*

Two evolving models have been advanced to account for αβ/γδ T cell lineage commitment. The TCR instructive model proposes that the strength of TCR signaling specifies the fate of T cells to develop into γδ or αβ T cells (131). This model proposes that γδ T cells develop from relatively strong signaling in DN3/DN4 precursors while signaling of lower duration or intensity directs the fate of T cells towards immature αβ lineage DP thymocytes. In contrast, the stochastic model states that there is heterogeneity among the DN1/DN2 cells (variations in gene expressions), which are predestined towards γδ or αβ T cell fate (132). It further proposes that the acquisition of a functional TCR primarily enables the survival and maturation of the lineage-biased cells.

The initial evidence for stochastic model was provided by the data showing that DN2 cells can be separated by their expression of high or low levels of the IL-7 receptor (IL-7R). Intrathymic injection experiments showed that IL-7R^{hi} DN2 cells were more likely to differentiate into γδ T cells whereas IL-7R^{lo} cells differentiated more toward αβ

T cell lineage (133). Subsequently, TF SOX13 had been identified as the first γδ lineage restricted marker as it was selectively expressed in γδ T cells and not in any αβ T cells (134). The expression of SOX13 is higher in IL-7R^{hi} DN2 cells that are biased to give rise to γδ T cells. The overexpression of SOX13 promoted the γδ T cell development at the expense of the αβ T cell lineage. In fact, the presence of SOX13 in the DP cells induced the expression of γδ T cell genes such as *Tcrg* and *Blk,* in the αβ T cells. Complementary experiments showed a loss of γδ thymocytes in fetal *Sox13*$^{-/-}$ mice. These results have placed SOX13 as a candidate for γδ T cell lineage fate determination. However, the loss of SOX13 did not result in a complete loss of γδ T cells nor did the ectopic *Sox13* expression convert all precursors towards γδ T cell lineage, leaving open the possibility that other factors and events, including TCR-mediated selection, are necessary for full γδ T cell lineage commitment.

Recently, it has become apparent that γδ thymocytes segregated based on the germline encoded TCR repertoire have distinct developmental requirements (Narayan et al., in press). Given that these separable γδ thymocyte subsets are endowed with unique effector capacities it became important to determine how these γδ subsets are different from each other developmentally and whether γδ T cell development is in reality a mix of distinct processes, not under one deterministic checkpoint. Systematic gene expression profile analysis of all emergent γδ thymocyte subsets distinguished by the germline encoded TCR repertoire indicates that there are three distinct subtypes in γδ T cell lineage and that they are as different from each other as they are from αβ T cells (Narayan et al., in press). While more detailed discussion on this subset heterogeneity

will follow, this result will require reassessments of most studies on γδ T cell development that have treated γδTCR$^+$ thymocytes as one uniform population.

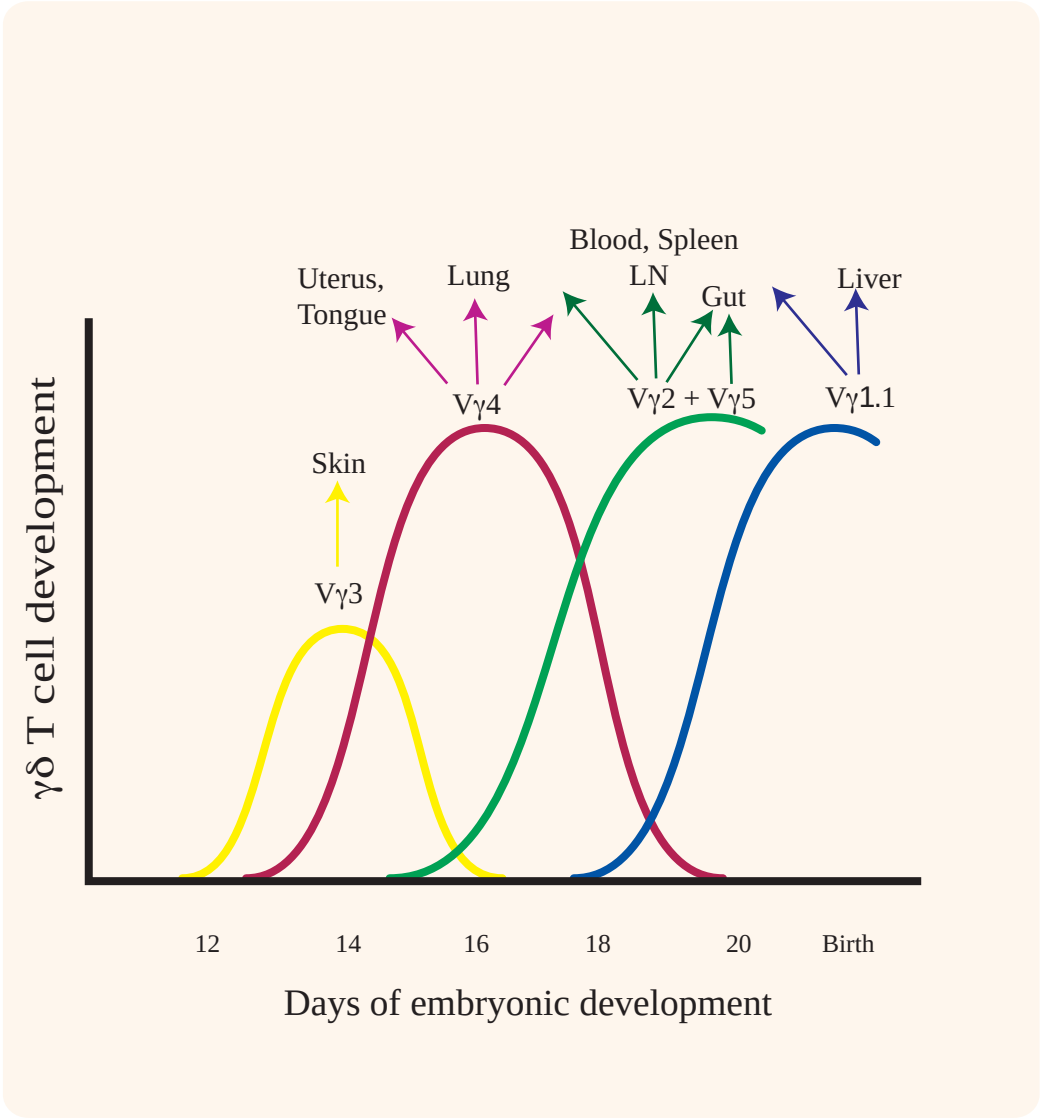
2.2.3. Thymic development of γδ T cells: ***Repertoire of γδ T cells***

γδ T cells are the first T cells to develop in most organisms. *Tcrg* and *Tcrd* gene rearrangements have been detected in the murine thymus at embryonic day 13 (E13) (135). In theory, the clonal diversity among γδ TCRs is greater than that of αβ TCRs or BCRs, but whether this diversity is relevant for antigen recognition or γδ T cell function has not been established. Instead, γδ T cell function correlates strongly with the germline encoded *Tcrg* and *Tcrd* genes. For example, Vγ3:Vδ1 (V3) T cells in the murine skin (Dendritic Epidermal T cells, DETCs) and Vγ4:Vδ1 (V4) T cells in the reproductive tracts express canonical γδTCRs, with no diversity in junctional sequences (136), (137). It is believed that the highly restricted TCRs expressed by γδ T cell subsets enable them to recognize ligands that are specifically expressed in infected, diseased or stressed cells. In addition, IL-4 producing NKT-like Vγ1.1:Vδ6.3 (V6) T cells in the liver also lack clonal TCR diversity (138). Combined with Vγ2$^+$ (V2) and Vγ5$^+$ (V5) T cells constituting the bulk of adult Tγδ17 cells and intestinal IELs (iIELs), respectively (139), there is an overwhelming evidence that γδ T cell function is linked to germine encoded elements of γδTCR, not the RAG-mediated clonal TCR diversity that underpins adaptive αβ T cell selection and function.

Activation of *Tcrg* V gene segments for recombination is not random, being tightly regulated based on the proximity to J gene segments and cis acting promoter

sequences (140). The early fetal thymus (E13-E15) contains large proportions of V3 cells, which mainly home to the skin. The *Vg3* gene at the *Cg1* locus rearranges exclusively at this fetal stage and the development of these cells specifically requires the fetal stroma (141). Following V3 cells, a wave of V4 cells originate at E15-E17 in the fetal thymus. These cells home to the reproductive organs (uterus and vagina), oral-respiratory tract (tongue, lungs), and can be found in circulation in the blood and the lymph nodes in the adults. Similar to DETC cells, V4 cells also arise mainly in the fetal thymus as *Vg4* gene rearrangements wane after birth. Adult thymus derived V5 subset of γδ T cells makes up a major population of iIELs. These cells were initially suggested to undergo extrathymic development as they could develop in athymic mice. However, subsequent cell fate mapping studies revealed that committed IEL precursors are exported from the thymus to cryptopatches. (139). The main γδ T cell subsets originating in the adult thymus are $V\gamma1.1^+$ and $V\gamma2^+$ (V2) cells. $V\gamma1.1^+$ cells can be divided into $V\delta6.3^+$ cells (V6) cells and the rest of $V\gamma1.1^+V\delta6.3^-$ (V1) γδ T cells. V6 cells produce IL-4 while V1 cells are the primary IFNγ producers of γδ T cell lineage. **Hence, V1, V2 and V6** γδ **T cell subsets are the main circulating and tissue resident populations of** γδ **T cells that are found in the adult blood, spleen, LNs and in non-lymphoid tissues.** V1 and V6 cells are particularly prevalent in the liver, whereas V2 cells form a major subset of lymphocytes present in the lungs and the dermis (Fig.1.4).

Figure 1.4. γδ T cells arise in sequential waves in the fetal thymus that home to specific tissues. V3 and V4 cells form the early waves of γδ T cell development, and are generated at ~E13 and ~E15 respectively. V2 cells start arising at ~E17 but they continue to develop in the thymus through adulthood. V5 cells form the major proportion of Intra-epithelial lymphocytes (IELs) in the intestine. Vγ1.1+ cells predominantly arise post-natal and are further segregated into V1 and V6 cell types.

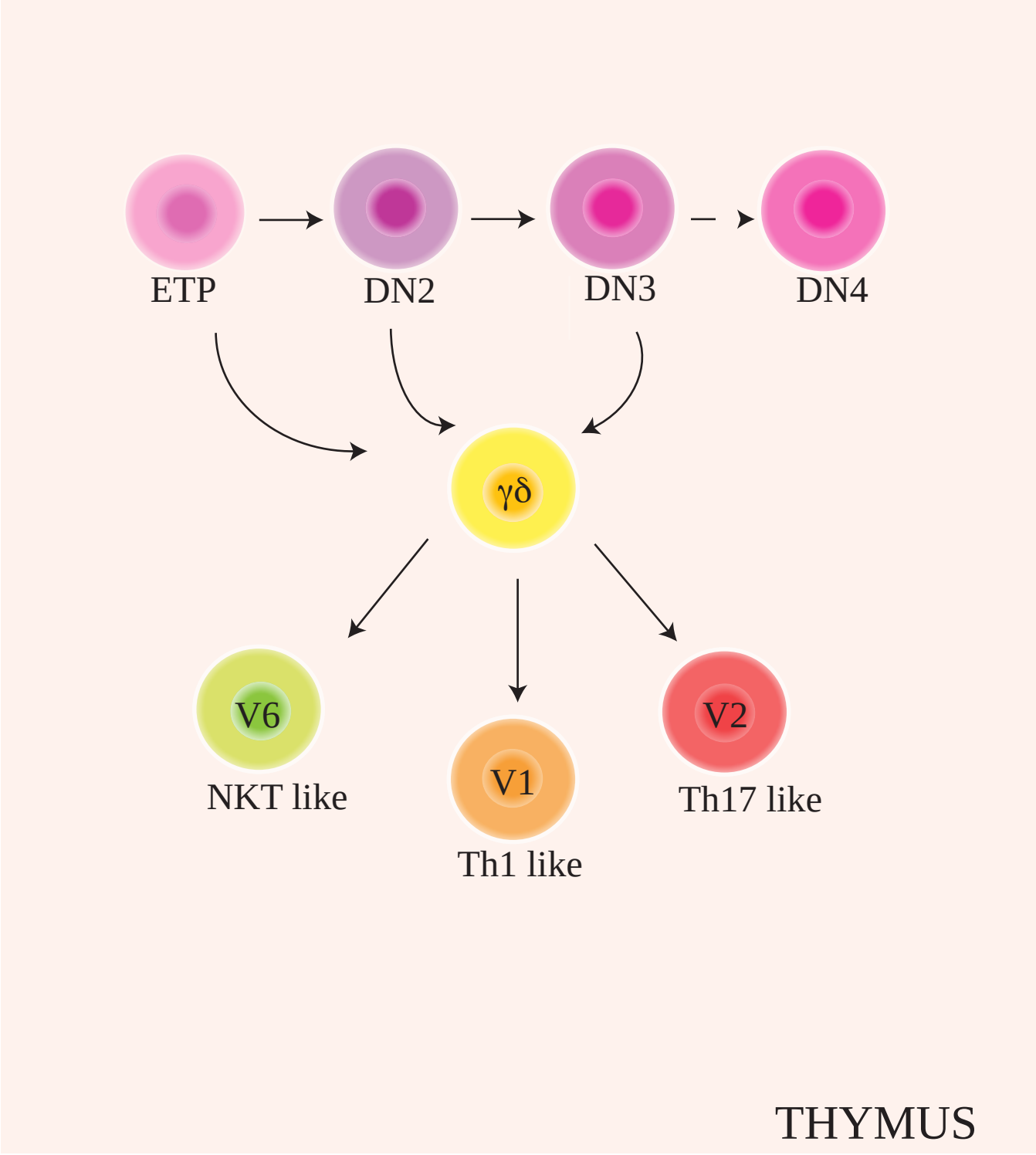

Blood, Spleen
LN
Uterus,
Tongue
Lung
Gut
Liver
Vγ4
Vγ2 + Vγ5
Vγ1.1
Skin
Vγ3
γδ T cell development
12
14
16
18
20
Birth
Days of embryonic development

2.2.4. Thymic development of γδ T cells: *γδ T cell subset function*

Similar to CD4 T cells that are divided into Th1, Th2, Th17 and iTreg lineages based on their functions, γδ T cells also possess distinct innate effector lineages that secrete a discrete cluster of effector cytokines. CD4 αβ T cell helper/effector lineages develop in the peripheral lymphoid tissues in response to antigenic stimulation of the TCR and specific cytokines (section 2.1). But, γδ T cell effector subsets are programmed in the thymus and are exported as pre-made memory cells in the peripheral lymphoid and non-lymphoid organs (3). Unlike αβ T cells, γδ T cell effector subsets is segregated on the basis of specific Vγ and Vδ gene usage. Thus, γδ T cell functional subsets that originate in the murine thymus are composed of Tγδ17 cells made up of adult-thymus derived V2 and fetal-thymus derived V4 cells, Th1-like IFNγ producing V1 cells and NKT-like IL-4 and IFNγ dual producing V6 cells. The gene expression analysis of different γδ subsets during their development in the thymus showed that the acquisition of these effector functions occurs in a step-wise manner (Narayan et al., in press) (Fig.1.5). γδ cell subsets emerge as immature cells that can be identified by their high expression of Heat Stable Antigen (HSA, CD24). At this earliest identifiable developmental stage V2 cells have been shown to be molecularly distinct from V1 and V6 cells, while V1 and V6 cells are nearly identical. Upon transit to mature ($CD24^{lo}$) state, V1 and V6 cells diverge molecularly to become distinct effectors. Critically, the intrathymic differentiation of V2 Tγδ17, V1 (Th1-like) and V6 ($IL\text{-}4^{+}$ NKT-like) subsets are uniquely guided by the expression of TFs RORγt, EOMES and PLZF, respectively (Narayan et al., in press). The

Figure 1.5. γδ **T cells effector lineages.** γδ T cells in an adult thymus are comprised of Th1 like-V1 cells, NKT like-V6 cells and Th17 like-V2 cells

Figure 1.5



same TFs program αβ T cell effector function with RORγt, EOMES and PLZF controlling IL-17, IFNγ and IL-4 expression, respectively (71), (142), (143).

γδ T cell subsets serve unique functions in different tissues. For example, V3 DETCs are the only resident lymphocyte populations in the murine skin. They play an important role in the wound repair processes by releasing KGF and EGF necessary for the wound closure (144). V5 iIELs are located primarily in the small intestine where they are required to maintain intestinal homeostasis by promoting the repair of damaged epithelium (145) as well as for limiting bacterial penetration into the host tissue by their release of antimicrobial lectins (146). V6 cells help in IgE antibody production by B cells by their capacity to secrete IL-4. In *Itk*$^{-/-}$ mice the expanded V6 cells have been shown to be responsible for hyper IgE syndrome (147).

A major function of all γδ subsets is to protect against pathogenic infections. γδ T cells impact immune responses to diverse bacterial, protozoan and viral pathogenic infections. An example is *Listeria monocytogenes* infection, where γδ T cells play a non-redundant role. In the absence of γδ T cells, unusually large atypical abscesses in the liver of the *Listeria* infected mice are observed (148). γδ T cells are the major early source of IL-17 in the lungs of *Mycobacterium tuberculosis* infected mice. IL-17A from γδ T cells induce ICAM-1 and LFA expression in infected macrophages, leading to effective granuloma formation necessary for the containment of the bacteria (122). Vaccinia virus infection of *Tcrd*$^{-/-}$ results in increased viral titers and mortality. Innate γδ T cells can clear and control virus infection up to 8 days post infection without αβ T cells. However,

the response does not persist and the viruses eventually expand rapidly in the absence of adaptive T cell responses (149)

2.3. Tγδ17 cells: innate sources of IL-17

Tγδ17 cells found in adult mice include two subsets: the canonical TCR expressing, fetal-derived V4 cells and V2 cells continuously produced in the thymus after E17. While V4 cells are primarily generated in the fetal thymus and are thought to self-renew in tissues like V3 cells in the skin. Though the V4 and V2 subsets possess different developmental requirements and express distinct TCRs, they are functionally alike.

2.3.1. Cellular events during intrathymic differentiation of Tγδ17 cells

Tγδ17 cells are IL-7R^{+}CCR6^{+}IL-1R^{+}IL-23R^{+}TLR2^{+}CD27^{-} (150), (3), (117), (151). These cell surface markers are uniquely shared with Th17 cells and GALT IL-17/IL-22 producing ILCs (152), (153), (5). During thymic development the characteristic markers of Tγδ17 cells are first detected on the cell surface at the CD24^{lo} mature stage (Narayan et al, in press). Tγδ17 cells also acquire some unique receptors that are not shared with Th17 cells at the immature thymic stage (CD24^{hi}). These include the scavenger receptors SCART-1 (CD163L1) and 2 that are downregulated when γδTCR is crosslinked. The role of SCART-1 and 2 in Tγδ17 cells is not well understood (154).

It has been suggested that γδTCR^{+} thymocytes that recognize ligand and signal are selected to become IFNγ producers and those that do not interact with a ligand default to IL-17 producers (123). This conclusion was based on the analysis of the developmental

potential of T10/T22 non-classical MHC Class I specific γδ T cells. These γδ T cells form <0.5% of total γδ thymocytes as determined by the MHC tetramer assay and they can develop in the absence of their ligands in *b2m*$^{-/-}$, *T10*$^{-/-}$ or *T10*$^{-/-}$*T22*$^{-/-}$ mice. The absolute numbers of these cells were unaffected in the absence of their ligands and they did not express markers associated with TCR signaling (CD122 and lower expression of the TCR). However, stimulation of the T10/T22 cells that developed in the absence of their ligands resulted in IL-17 production, while those from WT mice produced IFNγ. Hence, these results suggested that Tγδ17 cells do not require ligand dependent stimulations to produce cytokines. This is likely an oversimplification. Immature V2 cells that are the immediate precursors of Tγδ17 cells require TCR-ITK signaling as the gene expression profile of *Itk*$^{-/-}$ immature V2 cells is divergent from that of WT V2, so much so that *Itk*$^{-/-}$ immature V2 cells resemble WT immature V1 cells that are destined to become IFNγ^{+} producers. While the absence of *Itk* does not alter the core transcriptional network associated with V2 cells (Narayan et al, in press) some form of TCR signaling is important for proper maturation of V2 Tγδ17 cells. Further, Tγδ17 cells express Src family kinase B lymphocyte kinase (BLK), best known for its function in BCR/FcR signaling in B cells (155). Tγδ17 cells are not generated efficiently in *Blk*$^{-/-}$ mice (156). While it is not known what activates BLK in γδ T cells, the specific requirement for BLK indicates unique biochemical properties of Tγδ17 cells and that the generation of most Tγδ17 cells involves signaling.

Another distinguishing marker that separates Tγδ17 cells and IFNγ producing γδ T cells is CD27, a TNF family member, expressed on all developing γδ cells, but not on

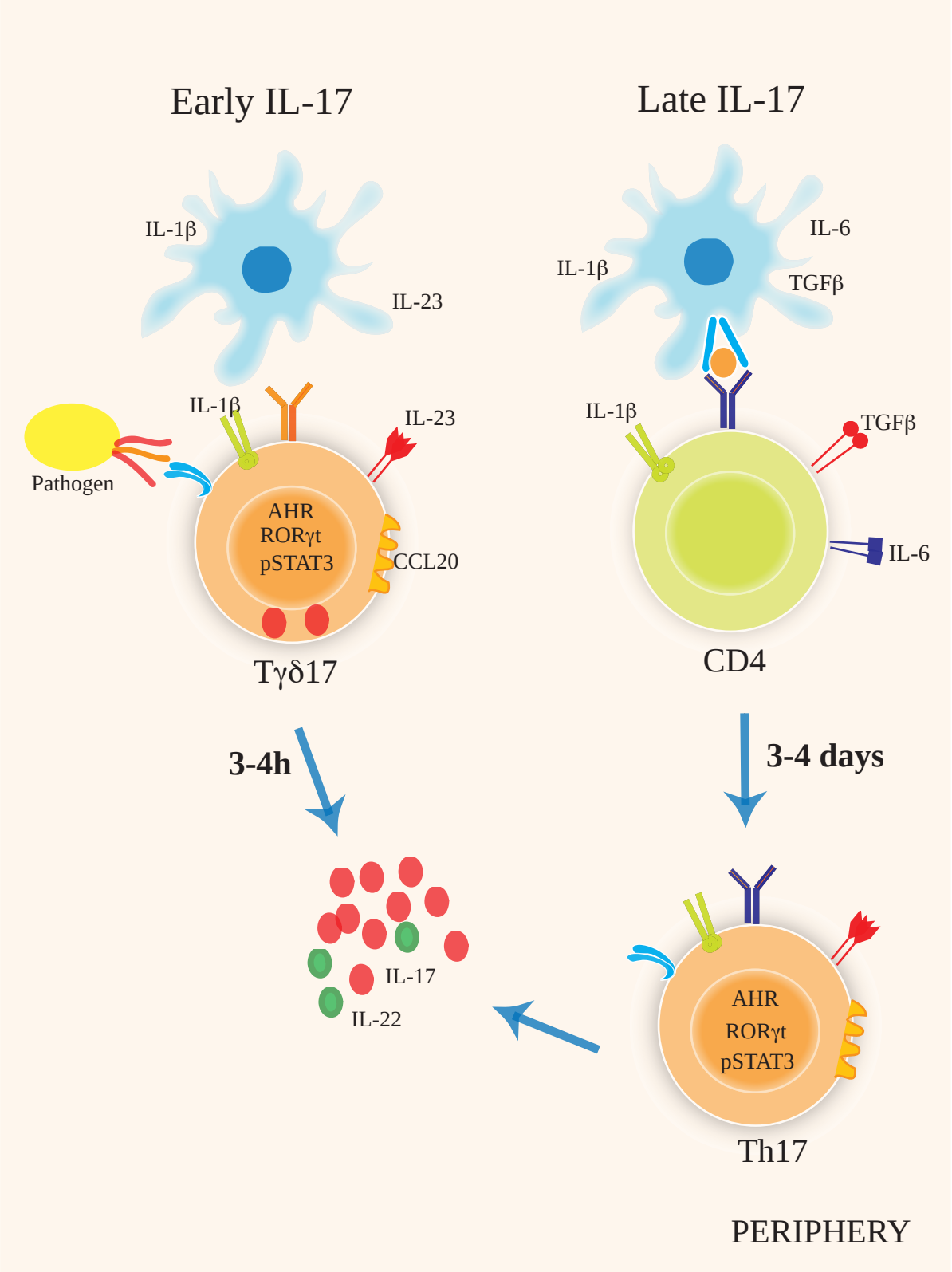
Tγδ17 cells (151). The ligation of CD27 with its ligand CD70 helps in the production of IFNγ, and survival and expansion of γδ T cells. The absence of CD27 reduces IFNγ production from γδ T cells but it does not affect the development of Tγδ17 cells (157). Furthermore, it has been shown that TCR stimulation of $CD27^{-}$ γδ T cells leads to apoptosis. The differential expression of CD27 on IL-17 versus IFNγ expressing cells reinforces the discrete developmental requirements of Tγδ17 cells. The unique gene network underpinning Tγδ17 differentiation is the subject of Chapter III.

2.3.2. Tγδ17 cell function

Similar to Th17 cells, Tγδ17 cells have protective and pathogenic roles in infections and autoimmune disorders, respectively. Tγδ17 cells participate in *Mycobacterium tuberculosis* (lung infections), *Candida albicans* (muco-cutaneous infections), *Escherichia coli* (gut infections) and a variety of other microbial infections (122), (158), (159). The early IL-17 production from these cells keeps the pathogen in check and creates a conducive environment for subsequent antigen specific responses by Th17 cells. Thus, it is the co-operation of early-innate Tγδ17 cells and adaptive Th17 cells that results in the control and elimination of pathogens (Fig.1.6). Although both innate and adaptive IL-17 producing arms play significant roles, the unique early contributions of innate Tγδ17 cells are often not appreciated in infection models due to very limited studies using proper animal models and in few reported cases, minimal changes were observed in the health and survival of an organism in their absence. A clear verdict on the essential function of

Figure 1.6. The early and late IL-17 producers. Innate-like Tγδ17 cells are preprogrammed effector cells residing in the peripheral tissues. They express TF RORγt, cytokine receptors for IL-1β and IL-23, and toll like receptors like TLR2. These cells can be activated in the presence of cytokines IL-1β and IL-23 alone or in combination with stimulation of their TLR by PAMPs. Due to their pre-activated feature, they can produce cytokines in a few hours after their activation. On the other hand CD4 T cell differentiation into Th17 cells requires antigen specific stimulation of their TCRs and the presence of cytokines like TGFβ, IL-1β and IL-6. Development of these adaptive effector cells takes 3-5 days. Once polarized, they migrate to their target tissues and take over the early IL-17 producers by their rapid proliferation abilities.

Figure 1.6


Early IL-17
Late IL-17
IL-1β
IL-23
IL-6
IL-1β
TGFβ
IL-1β
IL-23
IL-1β
TGFβ
Pathogen
AHR
RORγt
pSTAT3
CCL20
IL-6
Tγδ17
CD4
3-4h
3-4 days
IL-17
IL-22
AHR
RORγt
pSTAT3
Th17
PERIPHERY

Tγδ17 cells awaits infection studies using pathogens that elicit a strong innate IL-17 response necessary for their control.

The clonal expansion and strong IL-17 cytokine production makes γδ T cells key players in many autoimmune diseases such as EAE, psoriasis and IBD. In EAE, γδ T cells are the first T cell types to respond to IL-23, migrate to the CNS and secrete IL-17 and other inflammatory cytokines (160). It is suggested that the presence of γδ T cells in the CNS inhibits the suppressive functions of $FOXP3^{+}$ nTregs as well as the conversion of conventional αβ T cells to iTregs. The ineffective Treg function has been proposed to cause the expansion and infiltration of Th17 cells contributing to the pathology and inflammation in EAE. The increased resistance of *Tcrd*$^{-/-}$ mice to EAE development after MOG immunization further supports the role of γδ T cells in contributing to this autoimmune inflammatory disorder.

Similar to the EAE where Tregs are suppressed by Tγδ17 cells, interplay of Tregs and γδ T cells is also observed in IBD (161). It has been shown that in the absence of Phosphoinositide-dependent kinase 1 (PDK1) in T cells, mice succumb to colitis. The development of T cell dependent colitis seemed paradoxical, as PDK1 is important for the activation of conventional T cells. Subsequently, it was revealed that Tregs present in the *Pdk1*$^{-/-}$ deficient mice are dysfunctional in making regulatory cytokines such as IL-10 and TGFβ. The absence of IL-10 was suggested to cause the abnormal expansion of V2 and V5 γδ T cells in the IEL compartment of the colon of these mice. Further, V2 γδ T cells in the colon induced IL-17 dependent inflammation.

Through their presence in the epithelia of several distinct tissues, Tγδ17 cells serve as sentinels of the immune system, poised to serve the first line of defense. But, the presence of the armed effector cells in these tissue interfaces can be dangerous under an inflammatory condition. Psoriasis is one example in which the V2 cells residing in the dermal layer cause skin inflammation by producing IL-17 (162). IL-17R signaling in epithelial cells mediates recruitment of neutrophils, epidermal thickening and hyperplasia. The other inflammatory cytokines released by Tγδ17 cells, IL-22 and IL-6, further enhance this inflammation.

In sum, Tγδ17 cells are strong inducers of inflammation in many organ specific autoimmune disorders and understanding the development and maintenance of these innate cells is crucial for providing effective treatment options in clinics. Although Tγδ17 cells are the major innate IL-17, other ILCs also contribute significantly to early IL-17 production, particularly in the gut (109). Thus, understanding how innate IL-17 is made requires more integrated analysis of all sources of innate IL-17 in autoimmune models and in infectious settings.

2.4. Other innate lymphoid sources of IL-17

2.4.1. iNKT cells

A small proportion of iNKT cells that are $CD44^{hi}$ $NK1.1^{-}$ $ROR\gamma t^{+}$ produce IL-17 upon their TCR stimulation (163). Similar to Tγδ17 cells, IL-17 producing iNKT cells are generated in the thymus in an IL-6 independent manner. It is not known if the developmental pathways of Tγδ17 cells and IL-17 expressing iNKT cells are linked.

Though these cells represent a minute IL-17 producing population, they are shown to play important roles in airway hyperreactivity and asthma like lung inflammatory responses (164).

2.4.2. GALT ILCs

ILCs comprise diverse groups of lymphocytes that do not express somatically rearranged antigen specific receptors and have innate abilities to sense altered tissue environments and pathogens. The prototypes of the family are NK cells and Lymphoid Tissue initiator (LTi) cells (4). Fetal LTi cells are generated starting 12.5 days post coitus from common lymphoid progenitors (CLP) in the fetal liver. Their development and maintenance requires signaling from IL-7 and TFs ID2 and RORγt. Subsets of CLPs that express integrin α4β7 and have lost the potential to develop into B cells generate immature RORγt^{+} cells that then mature into LTi cells (165). The release of Lymphotoxins (LT) from LTi cells mediates their interactions with the stromal mesencymal tissue organizer cells that express LTβ receptor. These interactions form the basis for the development of LNs and Peyer's patches. Other than being a major source of LT, LTi cells also secrete IL-17 and IL-22 cytokines in the sterile fetal environment. In adults, LTi related ILCs secrete IL-17 and/or IL-22. A major adult ILC cells that are capable of IL-22 secretion is referred to as NK22 cells since they express the NK cell marker NKp46 (166). These cells develop after birth and constitute the major ILC subset after weaning. Similar to IL-22 deficient mice, *Citrobacter rodentium* infection induces severe debilitating disease in

the deficiency of RORγt^{+} ILCs, suggesting their non-redundant requirements in the GALT (167).

3. Gene networks controlling IL-17 production

Literature summarized so far described the unique and contrasting features of adaptive Th17 and innate Tγδ17 cells. A set of TFs positively regulates the expression of IL-17 produced by both of these lineages. Dynamic fluctuations in the cytokine milieu can impact the peripheral activation and differentiation of αβ T cells towards iTregs or Th subsets. Therefore, the TF network for adaptive Th17 cells is embedded with TFs activated by TCR signaling and cytokines. In contrast, Tγδ17 cells arise from the thymus as pre-programmed effectors. The TFs that regulate thymic programming of γδ T cells are those involved in generating T cells from T cell progenitors. The most prominent are those belonging to the High Mobility Group box TF family that includes, TCF1, LEF1, SOX13 and Sox4. These TFs are the focus of Chapter III.

3.1. RORγt: *the master regulator of all IL-17 producing cells*

RORγt commonly marks all IL-17 producing innate and adaptive lymphocytes (71). It belongs to the retinoic acid orphan receptors (RORs) superfamily of steroid hormone receptors (168). RORs exhibit a typical nuclear receptor domain structure consisting of four major functional domains: an N-terminal domain, followed by a highly conserved DNA binding domain (DBD), a hinge domain, and a C-terminal ligand-binding domain (LBD). They regulate gene transcription by binding to specific DNA response elements

(ROREs) in the regulatory region of target genes. There are three members in ROR family, RORα, RORβ and RORγ. From *Rorg* locus two distinct transcripts are generated by alternative transcription start site utilization, the longer form, *Rorg* is expressed mainly in the liver, adipose tissue and kidney, while a marginally shorter transcript *Rorc* encoding for RORγt is expressed predominantly in the immune system.

RORγt is highly expressed during thymic T cell development. *Rorc*$^{-/-}$ mice have smaller thymi with drastically reduced DP and SP T cell numbers, due to an arrest in thymocyte transition to DP stage, and massive apoptosis of the residual DP cells. It was subsequently shown that RORγt is required for the expression of the anti-apoptotic TF BCL-XL in thymocytes (169). As described earlier, RORγt is important for the development of LTi cells. Thus, *Rorc*$^{-/-}$ mice lack functional LTi cells and most secondary lymphoid organs (169). All ILCs related to LTi cells, including NK22 cells, are also lost when RORγt is absent (166).

After the discovery of Th17 cell lineage, gene expression profiling analysis of Th17 cells identified RORγt as one of the most lineage-specific TFs. Subsequently, with a RORγt reporter mouse line, it was shown that the RORγt^{+}, but not RORγt^{-}, αβ T cells isolated from the LP expressed IL-17 (71). The in vivo analysis of LP cells from RORγt deficient mice showed significant loss of Th17 cells. Conversely, retroviral overexpression of RORγt in T cells was shown to be sufficient to turn on IL-17 production. These results suggested that RORγt is necessary and sufficient to induce Th17 cell differentiation. Subsequent studies showed that other TFs such as STAT3 and IRF4 are also critically required for Th17 cell differentiation.

It is proposed that RORγt enables IL-17 transcription by binding to the conserved non-coding sequence 2 (CNS2) of the *Il17* gene locus (170). The CNS2 of *Il17* gene locus has been shown to undergo active chromatin remodeling in Th17 polarization conditions (171) and in its absence T cells do not convert efficiently to Th17 lineage.

In Th17 cells, the function and expression of RORγt is antagonized by a variety of TFs that reciprocally promote iTreg differentiation. In the LP of the small intestine, a small subset of CD4 T cells that co-express FOXP3 and RORγt have been observed using confocal microscopy (78). This result suggested that the T cells are poised for two distinct cell fates and tipping the fine balance between these two factors could lead to generation of Treg cells vs. Th17 cells. Further, it was shown that FOXP3 could interact with RORγt and compete with its co-activators to inhibit the RORγt mediated transcription of the *Il17* gene (113). The presence of cytokines like IL-6, IL-21 or IL-23 relieves the FOXP3 mediated inhibition of RORγt, thereby promoting Th17 differentiation.

Another recently described TF that inhibits the Th17 lineage while promoting iTreg differentiation is the TF Inhibitor of DNA binding 3, ID3 (172). ID3 belongs to the Helix Loop Helix TF family and inhibits the function of E protein family members such as E2A and E47 critical for T and B cell development at multiple stages of early differentiation processes. In the absence of ID3, there is a defect in the generation of TGFβ dependent iTregs. The loss of iTregs enhanced the generation of Th17 cells in the LP and Peyers patches of *Id3*$^{-/-}$ mice. Molecular analysis of the *Rorc* promoter led to the identification of four E boxes that can be recognized by E proteins. These sequence

elements were enriched with E2A in the TGFβ stimulated ID3 deficient T cells and the knock down of E2A abrogated *Rorc* transcription and IL-17 production. Thus, ID3 functions as an inhibitor for TGFβ dependent RORγt expression by antagonizing E proteins.

3.2. RORα

Another member of the orphan receptor family of TFs, Retinoic Acid related Orphan Receptor alpha (RORα), regulates Th17 cell lineage by synergistic co-operation with RORγt (173). Of the known isoforms of *Rora* the isoform *Rora4* is expressed in the Th17 cells. Forced expression of RORα in T cells can differentiate T cells to Th17 lineage cells. However, co-transduction of RORα and RORγt greatly increases *Il17a, Il17f* and *Il23* expression and induces histone acetylation at the CNS2 element of the *Il17* gene locus. In the context of Th17 cell differentiation, the deficiency of RORα only partially mimics the deficiency of RORγt. However, the absence of both of these TFs completely abrogates Th17 cell differentiation. These results suggested a non-redundant requirement of RORγt and RORα in regulating Th17 cell differentiation. To date, the exact mechanism of how RORγt and RORα work synergistically is not well elucidated. Although RORα is also expressed in Tγδ17 cells, whether its absence affects their IL-17 production is not known (159).

3.3. Aryl hydrocarbon receptor (AhR), *a sensor of toxins and dietary byproducts*

Another TF important for Th17 cell lineage and innate IL-17 producing cells is AhR. AhR is a cytosolic sensor of small synthetic compounds (xenobiotics) and natural chemicals. In the absence of AhR, Th17 cells can produce IL-17A and IL-17F, but not IL-22 (26). Further, IL-22 production from adult GALT ILCs is reduced in *Ahr*$^{-/-}$ mice (27). Conversely, AhR overexpression promotes Th17 cell differentiation and IL-22 production. Although in vitro and in vivo studies performed using high concentration of AhR agonists have indicated a function of AhR in both Th17 and Tregs regulation, its role in vivo in controlling the balance of Th17 and Tregs remains to be determined (174). The uniqueness of AhR lies more in how it directly regulates all IL-22 production. Similar to Th17 cells, AhR activation also promotes IL-22 secretion from a subset of Tγδ17 cells (3).

3.4. RUNX1

RUNX1 plays a dual role in Treg and Th17 cell differentiation (175). RUNX1 belongs to the family of RUNX proteins, all of which contain a runt domain (identified in Drosophila) for DNA binding. RUNX proteins function by pairing with their non-DNA binding partner, core binding factor β (CBFβ), which stabilizes their binding to the DNA (176). It has been shown that TGFβ can upregulate *Runx1* in CD4 T cells, which promotes *Foxp3* transcription. For Th17 cell differentiation, RUNX1 increases the expression of *Rorc* as well as it interacts with RORγt at the *Il17* gene locus. Importantly, RUNX1 can form an inhibitory complex with T-BET, which results in the blockade of RUNX1 mediated transactivation of *Rorc* promoter, leading to inhibition of *Il17a* and

Il17f expression (177). Details of this interaction, however, remain to be uncovered as IL-17 and IFNγ double producing T cells are commonly found in inflammatory environments, clearly indicating that not all RUNX1 is bound by T-BET and vice versa (80).

3.5. TCR signaling activated IRF4

The interferon regulatory factors (IRF), named for their ability to induce type I IFNs, have multiple roles in T cell differentiation. The absence of IRF4 abolishes in vitro Th17 cell differentiation (48). Activation of TCR induces the expression of IRF4 (178). Further, it is also strongly uprgeulated downstream of IL-1β signaling in T cells (79). In vivo models to study Th17 cell development have shown that IRF4 deficient mice are completely resistant to development of EAE despite mounting a normal Th1 response. A part of the resistance to EAE in these mice may be accounted for by an increase in the number of Tregs. The absence of IRF4 affects *Rorc* transcription, but overexpression of *Rorc* in IRF4 deficient T cells only marginally restores IL-17 production, suggesting that IRF4 and RORγt act in parallel, although the mechanism of IRF4 mediated regulation of Th17 cell differentiation is not known. Importantly, unlike Th17 cells, Tγδ17 cells do not require the activation of IRF4 for their development or IL-17 production (159).

3.6. STAT3

Signal Transducer and activator of transcription 3 (STAT3) regulates signaling downstream of cytokines IL-6, IL-21 and IL-23 in Th17 cell differentiation pathway (6).

STAT3 is activated by its phosporylation by JAKs recruited downstream of the IL-6R complex. Once activated, STAT3 proteins form homodimers or heterodimers and are translocated to the nucleus where they affect transcription of hundreds of target genes. As one would expect, deficiency of STAT3 in T cells abrogates T cell programming to the Th17 cell lineage (179). T cell specific deficiency of STAT3 protects mice from IBD due to stunted Th17 cell responses and decreased concentrations of other pro-inflammatory cytokines (98). For programming of Th17 cells, chromatin immunoprecipitation coupled with massive parallel sequencing (ChiP-Seq) experiments have revealed that STAT3 binds to *Il17a, Il17f and Il21* loci. Moreover, STAT3 directly regulates the expression of major TFs that drive Th17 cell differentiation such as *Rorc*, *Rora*, *Batf, Irf4*, *AhR* and *cMaf*. STAT3 deficiency does not affect the generation of Tγδ17 cells (180).

Intriguingly, ablation of STAT3 specifically in Tregs (*Foxp3-creStat3*$^{fl/fl}$ mice) induces fatal, intestinal inflammation associated with a massive increase in the number of Th17 cells in the gut of these mice. It was further shown that in the absence of STAT3, Tregs expressed transcripts of inflammatory cytokine genes like *Il6* and *Vip*, as well as other genes like *Tgfb* whose protein products promote Th17 cell development (181).

STAT3 signaling is inhibited by IL-2, which has a reciprocal role in promoting the Treg differentiation while inhibiting Th17 cell induction (182). It is proposed that IL-2 activated STAT5 interferes with STAT3 binding at *Il17a* and *Il17f* gene promoters (183). Thus, Th17 commitment not only requires equilibrium between FOXP3 and RORγt, but also a dynamic balance between TFs upstream in this pathway, STAT3 and STAT5.

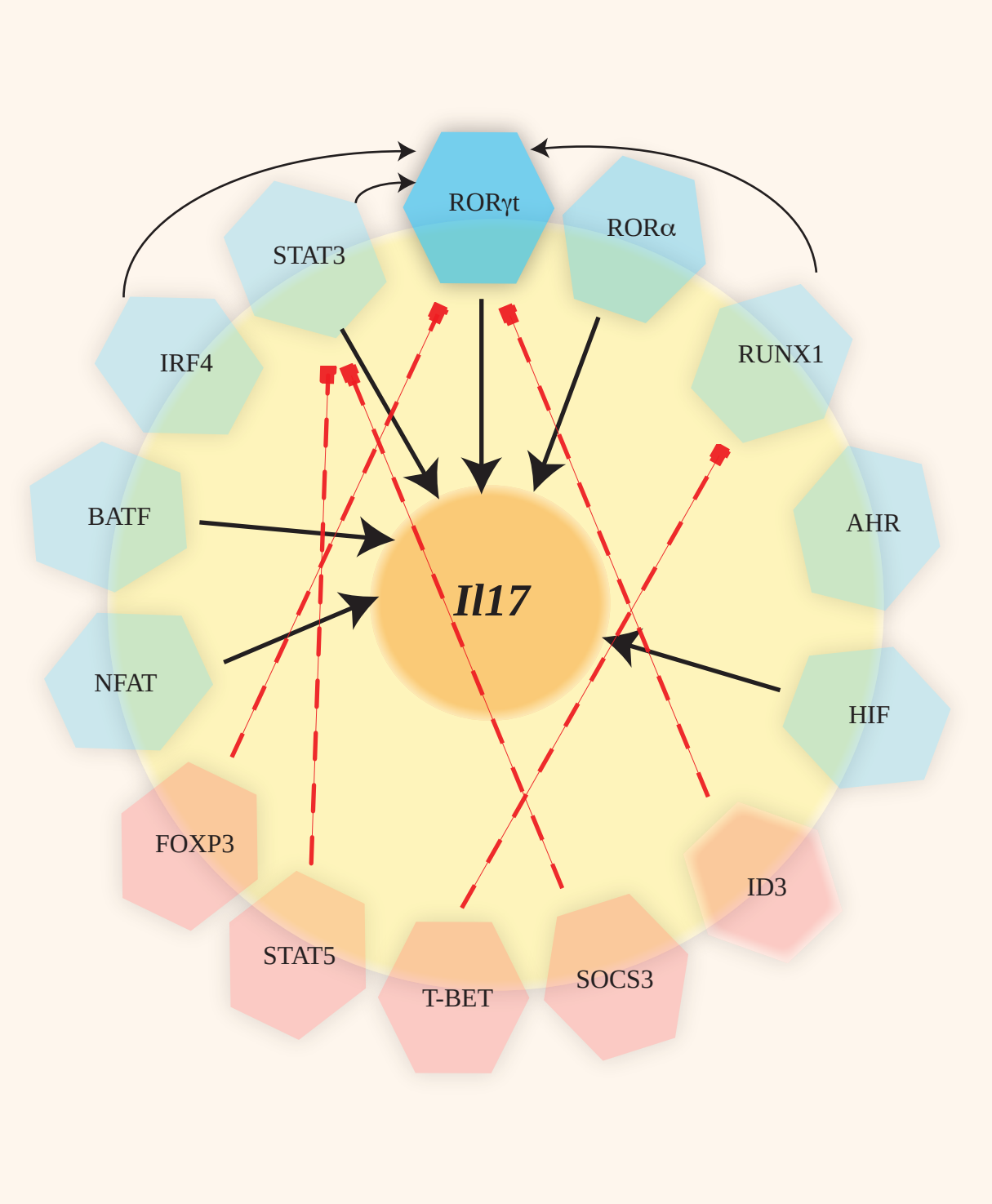
The other important regulator that prevents overt STAT3 signaling is Suppressors of Cytokine Signaling 3 (SOCS3). SOCS members are thought to be classic negative feedback regulators that restrain cytokine signaling by degrading cytokine receptors, affecting activation of JAKs and STATs, and by targeting JAKs or STATs for degradation (184). Of multiple SOCS proteins known to affect T cell development and effector cell differentiation, SOCS3 specifically limits Th17 cell generation. SOCS3 deficiency is associated with hyperactivation of STAT3 that leads to an increased generation of inflammatory cytokines IL-21 and IL-17 (185).

In addition to these prototypic TFs of Th17 cells there are others, less characterized, which are essential for Th17 differentiation. BATF, an AP-1 family TF, promotes Th17 cell differentiation by increasing the transcription of *Il17a, Il17f,* and *Il21* genes (186). Another TF that BATF can regulate is c-Maf, which has been shown to be important for inducing IL-21 expression (187). Furthermore, the NF-κB family member Iκbζ and hypoxia-induced factor-1 (HIF-1) are necessary for optimal transcription of *Il17a* or *Rorc* (188), (189). These findings reveal that IL-17 production is a highly regulated process involving diverse signal transduction pathways downstream of TCR and cytokines, with multiple feedback loops, some like AhR, involved in sensing environmental alterations to fine tune cytokine production (Fig.1.7).

In this chapter I have presented the key characteristics of adaptive Th17 cells and innate-like Tγδ17 cells. Innate IL-17 producers develop early in the embryos, whereas the gradual post-natal microbial colonization of the gut drives LP Th17 cells differentiation.

Figure 1.7. Positive and negative regulators of IL-17 production. A schematic of the TFs that regulate Th17 cell differentiation is shown. The expression of the TF RORγt is central to differentiation to IL-17 producing innate and adaptive cells. Signals from the TFs STAT3 and IRF4 contribute to the generation of Th17 cells but they are not required for Tγδ17 cells. Some of these TFs increase the expression of RORγt to enhance the Th17 cell differentiation (shown in arrows outside the circle). Other TFs that play important role in Th17 cell differentiation are RORα, RUNX1, NFAT, BATF, Iκbζ and HIF1. The TFs FOXP3, ID3, STAT5, SOCS3 and others (shown in red bars) inhibit the expression or transcriptional ability of RORγt and STAT3. Thus, a balance of cytokines and TFs helps in maintaining the equilibrium of Th17 and iTregs.

Figure 1.7


RORγt
RORα
STAT3
IRF4
RUNX1
BATF
AHR
Il17
NFAT
HIF
FOXP3
ID3
STAT5
T-BET
SOCS3

IL-17 producing innate and adaptive cells are also different in their lineage plasticity. While Th17 cells can shut down IL-17 expression and switch to become Th1-like IFNγ producers under specific inflammatory settings, innate Tγδ17 cells do not deviate from their programmed function (158). Moreover, IL-23 has been proposed to induce this conversion of Th17 cells to Th1 cells (190), while IL-23 is a potent activator of IL-17 production by Tγδ17 cells. The flexibility to acquire different effector function is probably advantageous in infectious settings where extracellular pathogens attempt to evade the host immune system by invading target cells. For eliminating these pathogens Th1 cell responses would be beneficial over Th17 cells.

The most important distinguishing feature of Th17 versus Tγδ17 differentiation is that Th17 cells differentiate under the control of TCR and cytokine signaling at the time of the T cell recognition of pathogen derived antigens presented by APCs. This entails TFs activated or induced by TCR and cytokine receptor signaling. In contrast, Tγδ17 cells are produced in the thymus, in the absence of inflammation. How innate Tγδ17 function is programmed was largely unknown, but given its reliance of RORγt, a common as well as unique gene networks in comparison to those responsible for Tγδ17 differentiation was expected.

The experiments that follow in this thesis further illustrate the distinct biochemical pathways and gene networks that regulate the differentiation of adaptive Th17 cells versus innate-like Tγδ17 cells. Although the importance of TGFβ in Th17 cell differentiation was realized soon after the discovery of this effector lineage, the mechanism by which TGFβ promotes Th17 was unclear. In Chapter II, I determined

whether TGFβ regulates Th17 cell differentiation in a SMAD2 dependent manner. We identified that effector functions of γδ subsets are molecularly programmed in the thymus. Experiments in Chapter III determined the functions of TFs selectively expressed in developing Tγδ17 cells. I show that HMG TFs TCF1/LEF1 and SOX13 are central regulators of Tγδ17 cells.

CHAPTER II

SMAD2 is essential for Th17 cell differentiation

Attributions and Copyright information

All the experiments described in this chapter were performed by me. This data was published in the *Journal of Biological Chemistry* in 2010.

Introduction

The importance of the cytokine TGFβ in regulating Th17 cell differentiation came as an unexpected discovery as it linked the generation of regulatory T cells to the inflammatory IL-17 producing cells (58), (67) After multiple convincing publications illustrating the substantial role of TGFβ, it became imperative to understand how TGFβ regulates the Th17 cell differentiation pathway.

The TGFβ superfamily includes TGFβ, Activin and Bone Morphogenic Proteins (BMP) proteins, all of which have pleiotropic roles in the development and differentiation of immune cells of the lymphoid and myeloid lineages (82). TGFβ is synthesized as a precursor, which is processed in the golgi complex by a furin like peptidase for its maturation. Following processing, it exists as a homodimer bound non-covalently to another homodimer protein, called the latency-associated protein (LAP) (191). Once released into the extracellular matrix, LAP is proteolysed by TGFβ activator (TA) enzyme releasing active TGFβ. TGFβ signals through the TGFβ type I (TGFβRI) and TGFβ type II (TGFβRII) transmembrane serine/threonine protein kinase receptors. It first binds to the constitutively active kinase receptor TGFβRII that recruits TGFβRI by phosphorylating its glycine-serine rich domain and thus forming a heterotetrameric complex. TGFβRI further uses its kinase domain to phosphorylate Small Mothers against Decapentaplegic (SMAD) proteins, ultimately transducing the signals to the nucleus (191).

SMADs are divided into three functional classes: receptor regulated SMADs (R-SMADs which include SMAD2 and SMAD3), common mediator SMADs (Co-SMADs, SMAD4), and inhibitory SMADs (I-SMADs which include SMAD6 and SMAD7). SMAD2 and SMAD3 are subject to TGFβ Receptor I mediated phosphorylation and subsequent activation (192). Phosphorylated R-SMADs form homotrimers and interact with SMAD4, which mediates translocation of R-SMADs into the nucleus. Another member of the TGFβ superfamily, Activin, also shares the R-SMADs (SMAD2 and SMAD3) and a co-SMAD (SMAD4) with TGFβ, to transduce signals downstream of ActivinR complexes, which exist as a complex of Activin type II and Activin type I receptors (192). The kinetic differences of SMAD2/3 activation and a selective expression of Activin Receptors as compared to the ubiquitously expressed TGFβ Receptors are some of the features that distinguish these two morphogen pathways. However, when expressed on the same cell types, Activin and TGFβ can synergize with each other. For instance, ActivinA, a member of the Activin family can induce *Foxp3* expression in CD4 T cells, but when added to TGFβ, its synergistic activity with TGFβ potentiates a significant increase in the expression of *Foxp3* (193).

The receptor SMADs, SMAD2 and SMAD3 are comprised of two amino (N) terminus Mad homology domains known as MH1 and MH2 domains, which are linked by a poorly conserved linker region. The MH1 domain of these proteins is important for DNA binding, nuclear import and for activating transcription, while the MH2 domain helps in protein oligomerization that is required for efficient transcriptional activation processes. SMAD2 and SMAD3 proteins share around 60% homology in their MH1

domains while their MH2 domains are highly identical in their amino acid sequence (194).

SMADs constantly shuttle between the cytoplasm and nucleus but they are retained longer in the nucleus upon their activation, which requires phosphorylation at their Carboxyl terminus by the serine-threonine kinase activity of TGFβRI. A number of proteins have been identified that mediate the interactions of R-SMADs with their membrane receptors. An example of such a helper protein is SMAD anchor for receptor activation (SARA), which tethers unphosphorylated SMADs to the TGFβRI kinase in the cytoplasm and dissociates from them upon SMAD activation (194). In the nucleus, SMAD2 and SMAD3 function as weak TFs by forming complexes with each other, often associating with SMAD4 or other TFs and thus regulate transcription of a plethora of genes involved in cell cycle, cell development and cell death. Some genes in the TGFβ pathway are redundantly regulated, but unique targets of SMAD2 and SMAD3 also exist, suggesting that each SMAD possesses distinct functions. For example, SMAD3 but not SMAD2 binds to the FOX family members FOXO1, FOXO3 and FOXO4 proteins in a TGFβ dependent manner to activate transcription of p21 (195). Similarly, SMAD3 interacts with Vitamin D Receptor (VDR) and its overexpression induces transactivation of VDR associated target genes (196).

A major difference between the functional mechanism of SMAD2 and SMAD3 is introduced by an extra exon, Exon3 in the MH1 domain of SMAD2, which restricts its binding to the DNA. Therefore, SMAD2 interacts with proteins such as SMAD3 and/or SMAD4, other TFs and co-activators to mediate its transcriptional effects (194). The N-

terminal MH1 domain of SMAD3 and SMAD4 recognizes the sequence 5'-GTCT-3' and its reverse complement, 5'-AGAC-3', as SMAD binding elements on DNA. Due to their weak binding, SMADs work as oligomeric complexes by binding to these inverted or direct repeats and often requiring synergistic actions from other TFs. For example, for TGFβ dependent peripheral Treg induction, SMAD3 binds along NFAT at *Foxp3* enhancer region to enable gene expression (89). Hence, due to their indirect or direct interactions with several other TFs, SMADs modulate transcription of a large number of genes. Importantly, in contrast to most TFs that can bind to naked DNA and directly recruit transcription activation complexes to proximal promoters, SMAD TFs require chromatin to be able to activate transcription (197). Therefore, they predominantly act through chromatin remodeling by recruiting the histone acetylase p300 (which acetylates histone H3) and the SWI/SNF component Brg1 among other histone modifying enzymes.

Though most of the SMAD-DNA binding interactions have been studied in the context of R-SMAD-SMAD4 complexes, some of the genes do not require SMAD4 for their regulation (198). Ubiquitin ligase- TRIM33/TIF1γ is one such protein that competes with SMAD4 to associate with SMAD2/3 complexes (199). Interestingly, SMAD2/3-TIF1γ complexes possess complementary roles to SMAD2/3-SMAD4 complexes in mediating erythroid cell differentiation. Furthermore, in the immune system, SMAD2/3-TIF1γ and SMAD2/3-SMAD4 complexes function in a stage specific manner in regulating iNKT cell differentiation, where the TIF1γ branch controls lineage expansion while the SMAD4 branch maintains the maturation of iNKT cells (200).

Although SMADs are the significant transducers of TGFβR signaling, TGFβ also activates other signaling cascades including Erk, JNK and p38 MAPK pathways (201). Unlike slower SMAD dependent responses, SMAD independent activation is marked by rapid kinetics of activation (201). The mechanisms of activation of these SMAD independent pathways and their biological consequences are poorly characterized. For instance, MAPK pathways are also triggered upon TCR stimulation, thus whether their concurrent or subsequent activation downstream of TGFβR has unique independent effects is difficult to elucidate (202).

As described in the previous chapter, TGFβ plays important roles in the development/maintenance of CD8 T cells and NKT cells in the thymus. It is indispensable for the maintenance of peripheral tolerance by regulating survival of nTregs, controlling activation of CD4 and CD8 T cells and inducing T cell effector differentiation into iTregs (191). Due to its central role in maintaining T cell tolerance, TGFβ was known as regulatory cytokine but its key reqirement for Th17 cell differentiation changed this paradigm. The importance of TGFβ in T cells was elucidated by the usage of mice that lacked TGFβ or its receptors on T cells. To dissect further the mechanism by which TGFβ functions in T cells, *Smad2*, *Smad3* or *Smad4* deficient mouse genetic models have been utilized.

The disruption of *Smad3* in embryonic stem cells generates viable mutant mice, which develop a progressive illness with onset around time of weaning. Symptomatic *Smad3* mutant mice die between 1 and 3 months of age due to a wasting syndrome that is associated with formation of pyogenic abscesses around the eyes and within the walls of

the stomach and intestine (203). The initially characterized *Smad3* deficient mice were associated with enlarged peripheral lymph nodes due to hyperplasic T cells that were unresponsive to TGFβ. However, subsequently characterized, *Smad3* deficient mice, generated by another group, were healthy and had a relatively normal immune system (204). Some of these differences in the phenotype of *Smad3*$^{-/-}$ mice could be due to different genetic backgrounds or different targeting mutations in the *Smad3* gene. Chen Dong's lab studied the role of SMAD3 in T cell differentiation and showed that *Smad3* deficient nTregs were functional in suppressing proliferation of effector T cells in the in vitro Treg suppression assays. But, CD4 T cells from *Smad3* deficient mice exhibited a profound defect in differentiation into FOXP3^{+} iTregs (205). Other Treg associated genes such as *Gpr83* and *Ecm1* were also affected in the *Smad3* deficient iTreg cells. These results supported the previous studies, which showed that binding of TFs SMAD3 and NFAT at *Foxp3* enhancer is essential to induce efficient transcription of *Foxp3* gene (89).

Reciprocal to the defects in iTreg induction, SMAD3 deficient T cells increasingly differentiated into Th17 cells (205). However, the expression of RORγt remained unchanged in these Th17 cells. Further, it was suggested that the interaction of SMAD3 with RORγt potentially inhibits RORγt dependent transcriptional activation of *Il17* gene. Thus the authors concluded that SMAD3 reciprocally regulates Th17 and iTreg differentiation of T cells. These results further indicated that TGFβ signaling utilizes SMAD3 independent mechanisms to induce Th17 cell differentiation.

Unlike *Smad3*$^{-/-}$ mice, *Smad4*$^{-/-}$ mice are embryonic lethal due to arrest in gastrulation during embryonic development (206). Therefore, the role of SMAD4 in T

cell differentiation has been studied by the usage of *Cd4-Cre-Smad4*$^{fl/fl}$ (CD4:SMAD4) mice (113) and *Lck-Cre-Smad4*$^{fl/fl}$ (Lck:SMAD2) mice (207). The activity of Cre recombinase excises *Smad4* gene at the DP and DN T cell developmental stages, in these transgenic mice models respectively. Though CD4:SMAD4 mice remained healthy, the Lck:SMAD4 mice developed gut lesions (adenomas) in the sub-pyloric duodenum. In the LP of the Lck:SMAD4 mice, there was an increase in IL-17A expressing CD4 T cells that correlated with an increase in Th17 polarizing cytokines, TGFβ, Activin, IL-23, IL-6, and IL-1β. Despite an increase in Th17 cells in the gut of these mice, *in vitro* differentiation of Lck:SMAD4 deficient T cells towards Th17 lineage was similar to their wild type counterparts suggesting a T cell extrinsic role of SMAD4 in Th17 cell differentiation in this mouse model. Furthermore, no defects in in vitro Th17 cell differentiation were observed in CD4:SMAD4 mice. The differences in the phenotype of these mutant mice could arise due to differences in the time of deletion of SMAD4 in the developing thymocytes or due to different animal housing conditions. Interestingly, in both of these mutant mouse models, differentiation of CD4 T cells to iTregs was significantly reduced.

Combined, these studies illustrated that T cells derived from *Smad3*$^{-/-}$ or *Smad4* CKO mice possess defects in iTreg induction. But, unlike mice with defective TGFβ signaling in T cells (90), none of these models showed an impaired Th17 cell differentiation response. There could be multiple interpretations of these observations. First, it is possible that SMADs show a higher redundancy in regulating Th17 cell differentiation (which requires co-operative actions of both TGFβ and IL-6) but have

stronger non-redundant requirements in regulating iTreg differentiation (which is dependent on TGFβ alone). Importantly, unlike TGFβ deficient mice that suffer from spontaneous multi-organ autoimmunity (85), *Smad3*$^{-/-}$ and *Smad4* CKO mice are relatively healthier with delayed partial penetrance of organ-specific inflammatory disorders. Furthermore, the importance of SMADs in reinforcing tolerance is reemphasized by the loss of both SMAD2 and SMAD3 in T cells, which develop the autoimmunity observed with TGFβ deficiency (208). Hence, these observations illustrate the existence of strong compensatory mechanisms in the single *Smad* gene deficient mouse models. Second, non-SMAD pathways could control some aspects of these differentiation pathways. Activation of p38 MAPK, JNK and Erk kinase pathways downstream of TGFβ has been suggested to affect in vitro Th17 cell differentiation (209). A recent study showed that TF Eomesodermin (Eomes) is suppressed by TGF in a SMAD independent way. Eomes was further shown to inhibit transcription of *Rorc* and *Il17* (210). Though it is conceivable that the non-SMAD pathways contribute in Th17 cell differentiation, but due to the multiple routes for MAPK-JNK activation including the TCR activation itself, it is difficult to know if these are the dominant pathways downstream of TGFβ. Last, though it has been shown that SMAD4 is not uniquely required for Th17 cell differentiation, the other R-SMAD, SMAD2, could still be an important player in driving these processes.

The experiments described in this chapter determined if TGFβ utilizes SMAD2 to regulate Th17 cell differentiation. *Smad2*$^{-/-}$ mice are embryonic lethal due to defects in mesoderm formation (211). Here, by analysis of T cell-specific *Smad2* deficient mice we

show, that in contrast to SMAD3 and SMAD4, SMAD2 plays a non-redundant role in the generation of Th17 cells in vitro and in vivo. The diminution in IL-17 production by $CD4^+$ T cells correlates with an accelerated loss of *Il6ra* expression and a corresponding decrease in STAT3 activation in *Smad2*-deficient T cells, suggesting that SMAD2 specifically modulates the crosstalk between TGFβ and IL-6 in Th17 cell differentiation.

Results and Discussion

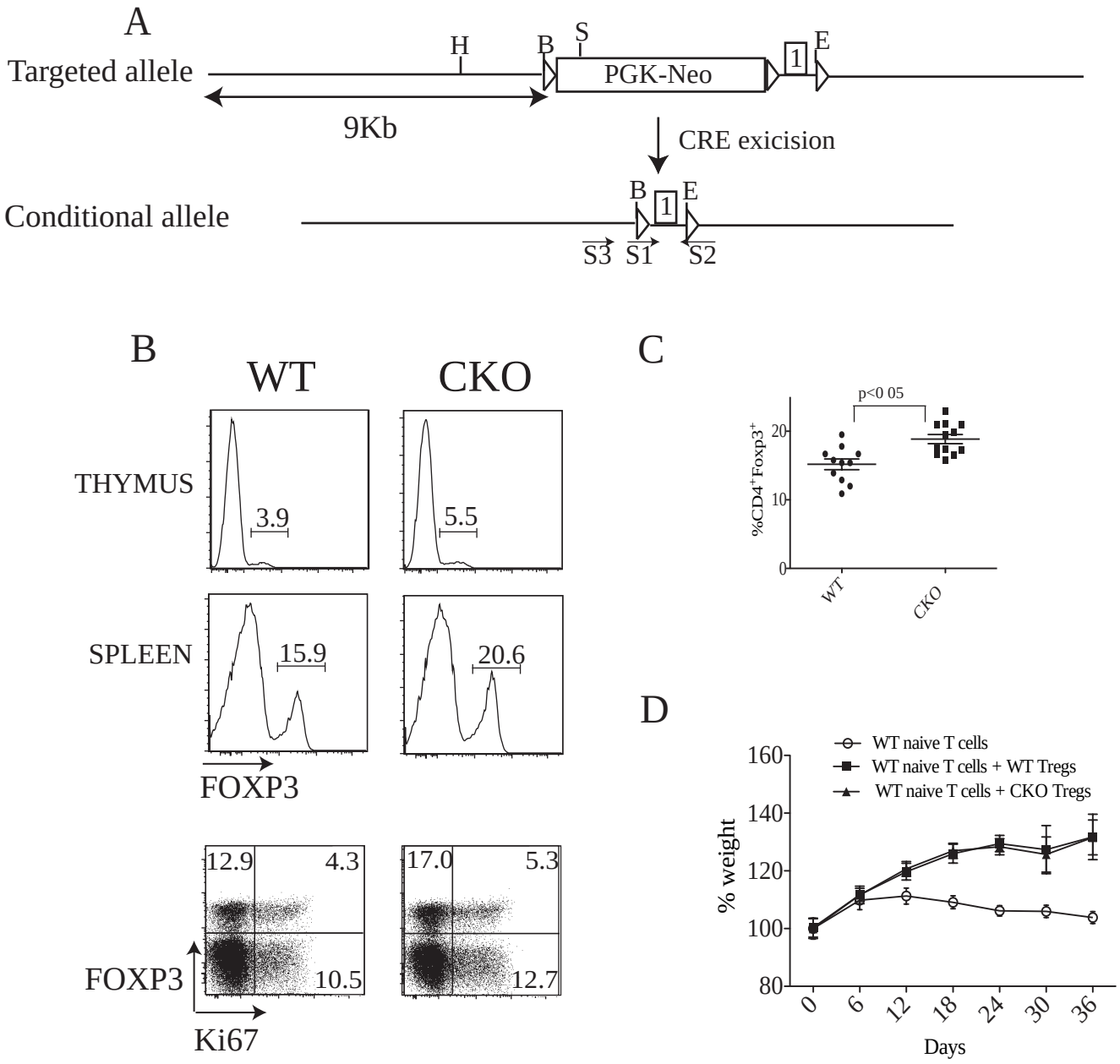
Smad2 deficiency in T cells does not impair immune homeostasis

To determine the role of SMAD2 in T cells, *Smad2* conditional knock out mice were generated by crossing CD2 promoter Cre transgenic mice to *Smad* $^{fl/fl}$ mice (referred to as *Smad2* CKO mice) (Fig.2.1A). In these mice, *Smad2* is deleted from the genome of developing T cells at DN2-DN3 stages of thymic maturation.

Smad2 CKO mice appeared healthy and showed normal development of T cells in the thymus, normal frequency and cellularity of conventional αβ T cells in the peripheral lymphoid organs, LN and spleen (Fig.2.1B). A subtle but consistent increase in the frequency and numbers of nTregs was observed in the thymus and spleen of *Smad2* CKO mice, although their rate of proliferation as observed by Ki67 staining among Treg cells was not altered (Fig.2.1B and 2.1C). In vivo, Treg cells from *Smad2* CKO functioned normally as they could control the colitogenic T cells in lymphopenic *Rag*$^{-/-}$ mice and were able to inhibit the proliferation of conventional T cells *in vitro* (Fig.2.1D).

Figure 2.1. T cell homeostasis is maintained in *Smad2* CKO mice. (A) *Smad2* Targeted Allele and Conditional Allele is shown. Exon1 of the *Smad2* gene was flanked by the loxp sites, introduced by the homologous recombination (EJ Robertson et al). B (BamH1), E (EcorI),S (Spe1), H (HindIII) are some of the restriction enzyme sites used for the generation of the targeting allele. Primer locations for genotyping the mice are shown, and primers are abbreviated as S1, S2 and S3. (B) Representative flow cytometric profiles of frequencies of nTreg cells in the thymus and spleen of WT and *Smad2* CKO mice, and proportion of splenic nTreg cells in cell cycle as determined by Ki67 staining. (B) Average frequency of nTreg cells in the spleen from 4 independent experiments. Statistical significance was determined by Student's *t* test. (C) WT and *Smad2* CKO nTreg cells can equally suppress colitis precipitated by the transferred naïve CD4 T cells in *Rag1*$^{-/-}$ recipients, as indicated by normal weight gains over time in mice that were co-injected with nTreg cells of either genotype. X-axis, % weight change in recipients after T cell transfer, with the starting weight set at 100%. Error bars represent standard error (S.E.).

Figure 2.1


A
Targeted allele
H
B
S
PGK-Neo
1
E
9Kb
CRE exicision
Conditional allele
B
1
E
S3
S1
S2
B
WT
CKO
THYMUS
3.9
5.5
SPLEEN
15.9
20.6
FOXP3
12.9
4.3
10.5
17.0
5.3
12.7
FOXP3
Ki67
C
p<0 05
%CD4+Foxp3+
20
10
0
WT
CKO
D
WT naive T cells
WT naive T cells + WT Tregs
WT naive T cells + CKO Tregs
% weight
160
140
120
100
80
0
6
12
18
24
30
36
Days

Smad2 regulates T cell differentiation into iTreg and Th17 cells

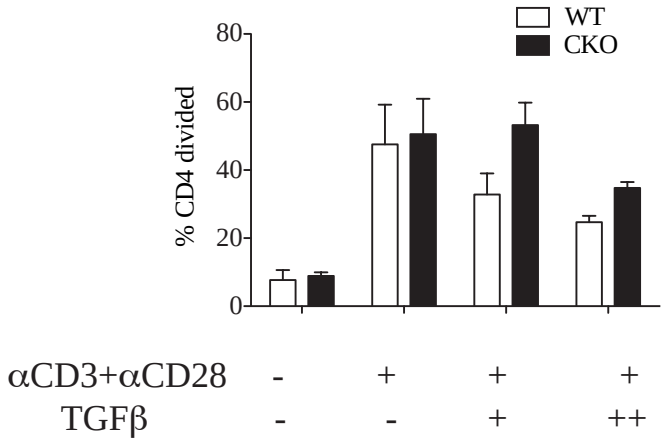
TGFβ suppresses the cell division of conventional T cells by inducing cell cycle arrest Before testing *Smad2* CKO T cells for TGFβ mediated effector T cell differentiation, their responsiveness to TGFβ mediated suppression in proliferation was tested. To assay this, cells were labeled with the cell cycle dye CFSE and activated in the presence or absence of TGFβ. As expected, WT $CD4^+$ T cells showed diminished proliferation in the presence of TGFβ, with the proportion of divided cells ($CFSE^{lo}$) decreased by ~50% as compared with cultures without TGFβ (Fig.2.2A). In contrast, *Smad2* CKO T cells were relatively insensitive to TGFβ as indicated by the limited difference in the proportion of divided cells with TGFβ (Fig.2.2A). However, when the concentration of TGFβ was increased, *Smad2* CKO T cells responded to TGFβ, and their proliferation was reduced (Fig.2.2A). These results suggested that there is a dose-dependent impairment in the TGFβ signaling in *Smad2* CKO T cells. $CD4^+$ T cells stimulated with TGFβ in vitro convert to FOXP3 expressing $CD4^+$ T cells that resemble nTreg cells. We observed that in *Smad2* CKO T cells, there is a partial, but significant, decrease in the TGFβ induced differentiation to $FOXP3^+CD4^+$ T cells (Fig.2.2B). These results demonstrated a non-redundant role of R-SMADs, SMAD2 and SMAD3, for differentiation of CD4 T cells to iTregs.

Th17 cells can be generated in the presence of TGFβ and IL-6 as well as in the presence of IL-1β and IL-6. To study if SMAD2 functions downstream of TGFβ in regulating Th17 cell induction, *Smad2* CKO naïve $CD4^+$ T cells were cultured in different culture conditions under varying concentrations of both TGFβ and IL-6. Unlike

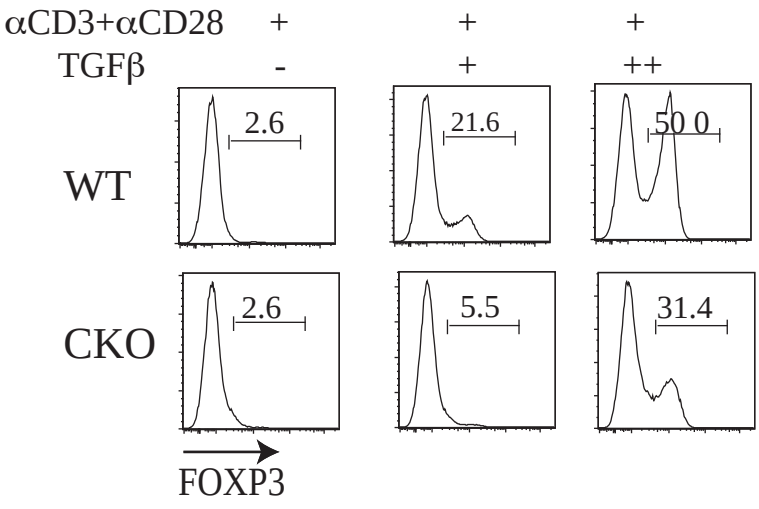
Figure 2.2. SMAD2 is necessary to efficiently induce TGFβ dependent FOXP3^{+} CD4^{+} T cell subsets. (A) CFSE-labeled WT and *Smad2* CKO naive CD4 T cells were activated (anti-CD3/CD28 crosslinking in all panels) with varying concentrations of TGFβ for 2 days. Extent of proliferation was measured by the loss of CFSE using flow cytometry. Data shown is representative of three independent experiments (a minimum of 3 mice/ genotype/experiment) with similar results. Error bars are S.E. (B) WT and *Smad2* CKO naive CD4 T cells were cultured under iTreg conditions with varying concentrations of TGFβ for 3 days. FOXP3 expression was analyzed by intra-nuclear staining. Data are representative of three independent experiments with similar results.

Figure 2.2

A


WT
CKO
% CD4 divided
80
60
40
20
0
αCD3+αCD28 - + + +
TGFβ - - + ++

B

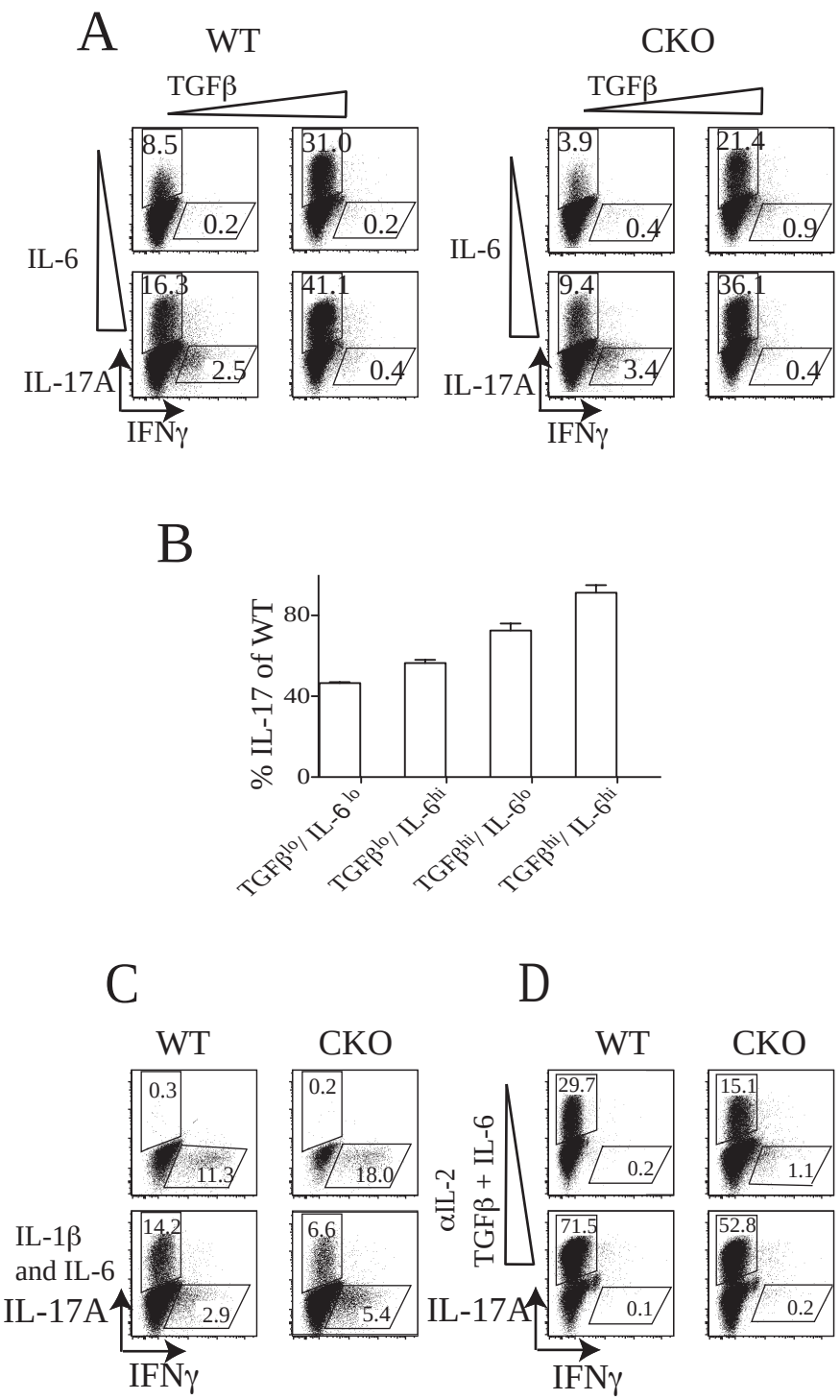

αCD3+αCD28 + + +
TGFβ - + ++
WT
CKO
2.6
21.6
50 0
2.6
5.5
31.4
FOXP3

the previously described *Smad3*$^{-/-}$ and *Smad4* CKO T cells, *Smad2* CKO T cells were significantly impaired in Th17 cell differentiation (Fig.2.3A). Critically, the differentiation of *Smad2* deficient CD4 T cells in converting to the Th17 lineage was dictated by the concentration of both TGFβ and IL-6. *Smad2* CKO T cells showed an increased impairment in converting to the Th17 cell lineage at lower concentrations of both TGFβ and or/ IL-6 than at higher concentrations of both of these cytokines (Fig.2.3A and 2.3B). This suggested that alterations in both TGFβ and IL-6 signaling pathways in *Smad2* CKO T cells were responsible for the reduced efficiency in the Th17 cell generation.

Further, *Smad2* CKO T cells were cultured in the presence of IL-1β and IL-6 to acquire an alternative Th17 cell fate. Surprisingly, the IL-17 induction in these culture conditions was also less efficient in the *Smad2* deficient T cells than with the control T cells (Fig.2.3C). Adding IL-2 to Th17 cultures strongly inhibited IL-17 expression and its blockade resulted in an extremely efficient conversion to Th17 lineage with more than 60-70% cells expressing IL-17. *Smad2* CKO T cells were cultured using classical (with TGFβ and IL-6) Th17 culture conditions and IL-2 signaling was blocked to determine if it would restore IL-17 induction in *Smad2* deficient T cells to the control levels. Although blocking IL-2 signaling did considerably increase IL-17 induction in *Smad2* CKO T cells, it did not restore the expression to control cell levels suggesting that impairment observed in *Smad2* CKO T cells was not due to an alteration in the IL-2 pathway due to *Smad2* deficiency (Fig.2.3D).

Figure 2.3. SMAD2 is necessary to efficiently induce IL-17A$^+$ CD4$^+$ T cell subsets. (A and B) WT and *Smad2* CKO naive CD4 T cells were cultured in varying doses of TGFβ and IL-6 with mitomycin treated splenocytes in Th17 conditions for 4 days. Intracellular staining (ics) for IL-17A and IFNγ was performed after restimulation for 5h with PMA and ionomycin. Flow cyotmetric profiles shown in (A) are representative of three independent experiments, and (B) shows average reductions in IL-17A$^+$ *Smad2* CKO T cells relative to controls in varying culture conditions. (C) WT and *Smad2* CKO CD4 naive T cells were cultured with IL-1β and IL-6 in activating conditions for 4 days followed by ics. Top row, anti-CD3/CD28 mAb without cytokines. (D) WT and *Smad2* CKO naive CD4 T cells were cultured with increasing doses of TGFβ and IL-6 in the presence of fixed amount of IL-2 blocking mAb for 4 days, followed by ics for the cytokines. Data shown is representative of three experiments.

Figure 2.3


A
WT
CKO
TGFβ
TGFβ
8.5
31.0
0.2
0.2
3.9
21.4
0.4
0.9
IL-6
IL-6
16.3
41.1
9.4
36.1
2.5
0.4
3.4
0.4
IL-17A
IL-17A
IFNγ
IFNγ
B
% IL-17 of WT
80
40
0
TGFβlo/ IL-6 lo
TGFβlo/ IL-6hi
TGFβhi/ IL-6lo
TGFβhi/ IL-6hi
C
D
WT
CKO
WT
CKO
0.3
0.2
29.7
15.1
11.3
18.0
0.2
1.1
αIL-2
TGFβ + IL-6
IL-1β
and IL-6
14.2
6.6
71.5
52.8
IL-17A
2.9
5.4
IL-17A
0.1
0.2
IFNγ
IFNγ

It was next determined if other cytokines that activate SMAD2 were also impaired in their function in *Smad2* deficient T cells. Activin A, a member of the TGFβ family of cytokines, has been shown to exhibit a marked preference for SMAD2 activation over SMAD3 in $CD4^+$ T cells (193). Analysis of Activin Receptor II on CD4 T cells showed that it was not expressed on naïve $CD4^+$ T cells but was upregulated upon T cell activation. Activin A with IL-6 could promote the generation of IL-17 secreting $CD4^+$ T cells without TGFβ addition, even though only ~15% of activated conventional T cells detectably expressed ActRII (Fig.2.4A). Activin-mediated Th17 generation from naïve *Smad2* CKO $CD4^+$ T cells was also significantly impaired compared to control $CD4^+$ T cells (Fig.2.4A).

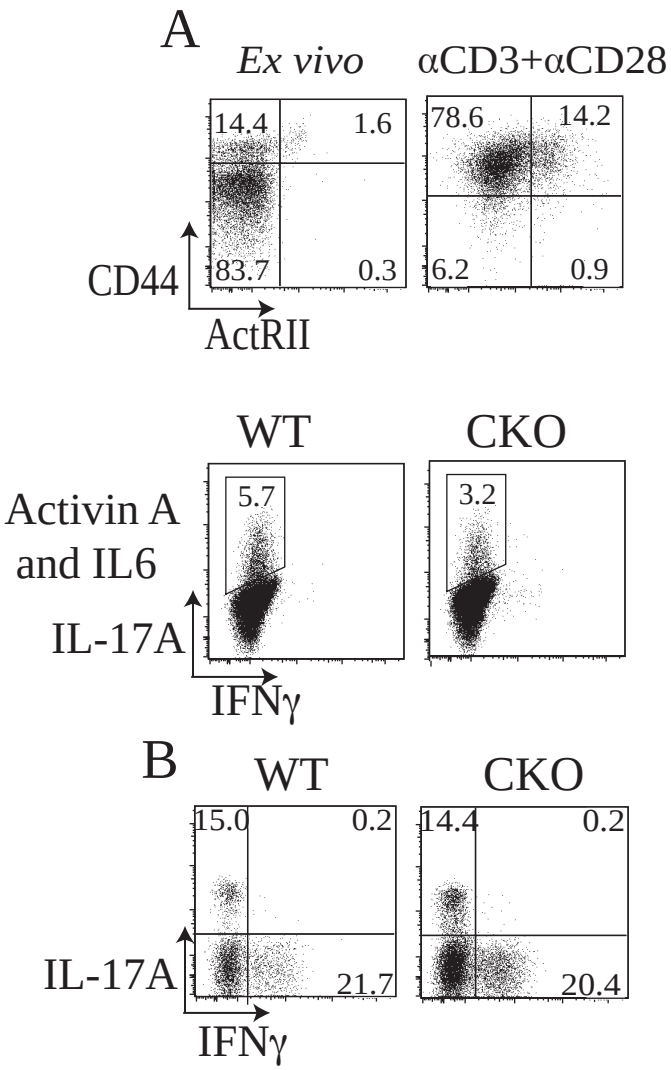
Finally, it was determined if IL-17 production dependent on SMAD2 in CD4 T cells affected IL-17 production in innate γδ T cells, which constitute the major early source of IL-17 in vivo. It had been shown that in contrast to αβT cells, IL-17 production in γδT cells did not require TCR signaling or IL-6, but was dependent on TGFβ and SMAD3 (110). Ex vivo γδ T cells from the LNs of *Smad2* CKO mice were not different from control γδ T cells in IL-17A secretion, indicating that SMAD2 was dispensable for innate IL-17A production (Fig.2.4B). In sum, SMAD2 was found to be uniquely required to efficiently induce IL-17 in adaptive αβT cells but not in innate γδ T cells.

Smad2 regulates IL-6Ra expression and STAT3 phosphorylation in T cells

Since both IL-6 and TGFβ dictate Th17 cell differentiation in a SMAD2 dependent manner, the alterations in the IL-6 signaling cascade in SMAD2 deficient T cells were

Figure 2.4. SMAD2 is necessary to efficiently induce Th17 cell differentiation but it is not required for Tγδ17 cell generation. (A) Activin receptor II expression on B6 CD4 T cells ex vivo and after activation for 2d. B. IL-17A^{+} cells generated with Activin A and IL-6 in 3d activated CD4 T cells. (B) WT and *Smad2* CKO peripheral lymph node cells were stimulated with PMA and Ionomycin for 5h and analyzed for cytokine expression in γδ T cells. Data are representative of 2 independent experiments

Figure 2.4


A
Ex vivo
αCD3+αCD28
14.4
1.6
83.7
0.3
78.6
14.2
6.2
0.9
CD44
ActRII
WT
CKO
Activin A
and IL6
5.7
3.2
IL-17A
IFNγ
B
WT
CKO
15.0
0.2
21.7
14.4
0.2
20.4
IL-17A
IFNγ

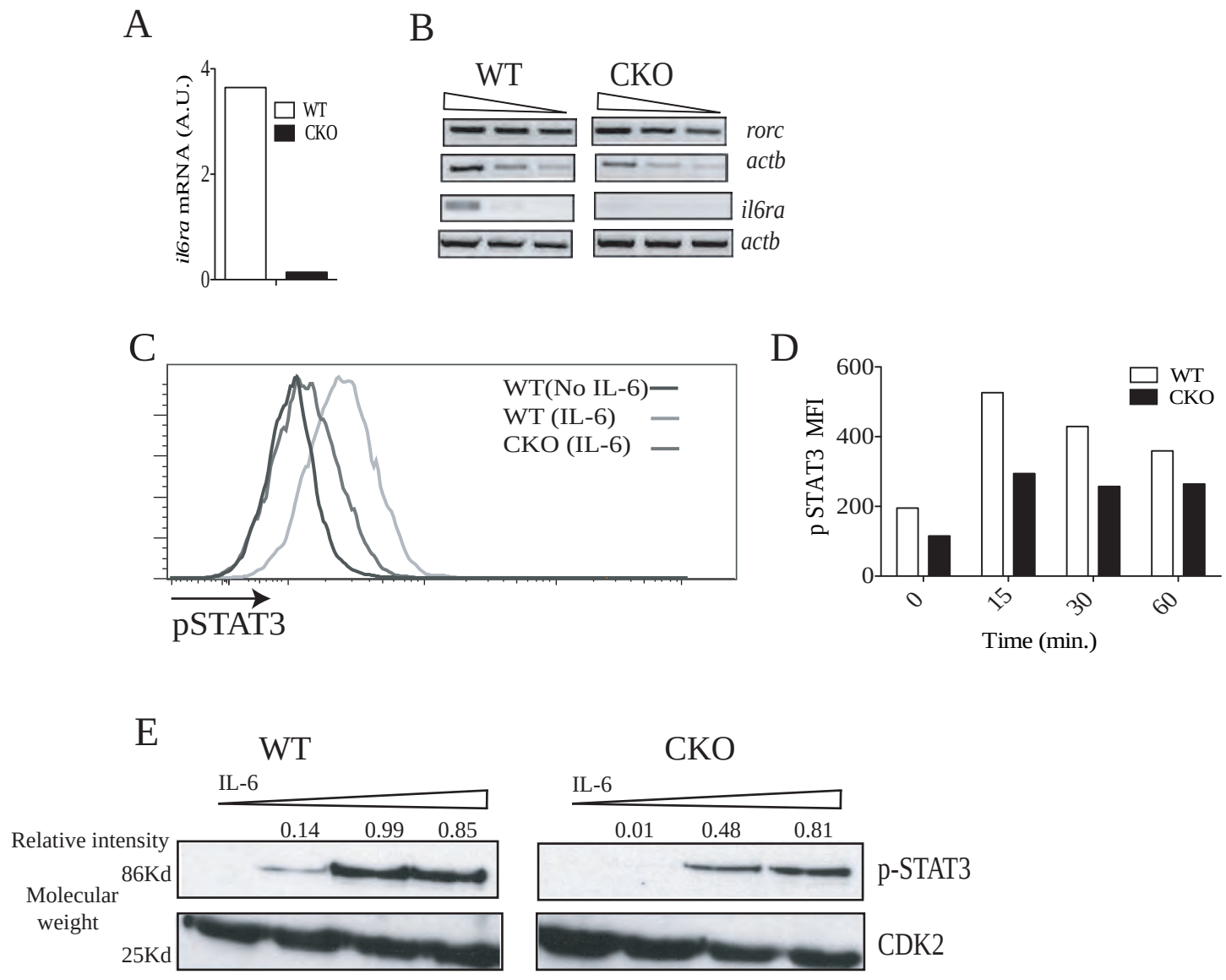
examined. A previous publication had showed that TGFβ upregulates the expression of IL-6Rα in activated $CD4^+$ T cells (92). Further, increased and sustained STAT3 phosphorylation in activated $CD4^+$ T cells was observed in the presence of both TGFβ and IL-6, compared to IL-6 alone (93). These results suggested that one function of TGFβ in promoting Th17 cell generation is to enhance and/or prolong IL-6 signaling in T cells. To investigate whether the IL-6 signaling pathway was altered in *Smad2* CKO T cells, the amounts of *Il6ra* transcripts in stimulated SMAD2 deficient T cells were measured. QPCR analysis showed a dramatic down-modulation of *Il6ra* mRNA expression in *Smad2* CKO T cells that were activated or cultured in Th17 conditions (Fig.2.5A and 2.5B). Furthermore, when SMAD2 deficient CD4 T cells were cultured ex vivo with IL-6 alone, a significant decrease in phosphorylated STAT3 was observed at early time points (15 and 30 min) compared to phosphorylated STAT3 levels in control $CD4^+$ T cells, in an IL-6 concentration dependent manner (Fig.2.5C, 2.5D and 2.5E). In contrast to the alteration in IL-6 signaling, no change in the expression of Th17 cell differentiation central transcription factor, *Rorc* was observed in *Smad2* CKO Th17 cells (Fig.2.5B). These results suggest that *Smad2* CKO $CD4^+$ T cells have a decreased capacity to respond to IL-6 and that the synergy between TGFβ and IL-6 in promoting Th17 differentiation likely involves SMAD2 regulation of IL-6R expression.

Smad2 deficient conventional T cells cause more severe colitis

It has been shown that IL-17A is protective during colitis induction and $CD4^+$ T cells that cannot produce IL-17A cause more aggressive colitis in $Rag1^{-/-}$ recipients (212). The *in*

Figure 2.5. SMAD2 modulates IL-6Rα expression on CD4 T cells. (A) Real time RT-PCR analysis for *Il6ra* mRNA expression in activated CD4 T cells from WT and *Smad2* CKO mice (pooled cells from 3 mice/genotype) relative to *Actb* expression. (B) Semi-quantitative RT-PCR analysis for *Il6ra* and *Rorc* with *Actb* as control. A 4-fold dilution series is represented. One of two experiments with similar results is shown. (C) Naïve CD4 T cells from WT and *Smad2* CKO mice were activated for 30 min in the presence of IL-6. Expression of phosphorylated STAT3 (pSTAT3) was analyzed by ICS and flow cytometry. (D) Mean fluorescent intensity of pSTAT3 expression in WT and *Smad2* CKO T cells at various time points after IL-6 stimulation. Data are representative of three independent experiments with similar results. (E) Western blot for pSTAT3 expression in WT and *Smad2* CKO $CD4^{+}CD25^{-}$T cells stimulated with IL-6 at 1,5 and 10ng/ml for 30 minutes. Relative band intensities of phsospho-STAT3 normalized to loading control CDK2 is shown.

Figure 2.5


A
il6ra mRNA (A.U.)
4
2
0
WT
CKO
B
WT
CKO
rorc
actb
il6ra
actb
C
WT(No IL-6)
WT (IL-6)
CKO (IL-6)
pSTAT3
D
p STAT3 MFI
600
400
200
0
WT
CKO
0
15
30
60
Time (min.)
E
WT
CKO
IL-6
IL-6
0.14
0.99
0.85
0.01
0.48
0.81
Relative intensity
86Kd
Molecular weight
25Kd
p-STAT3
CDK2

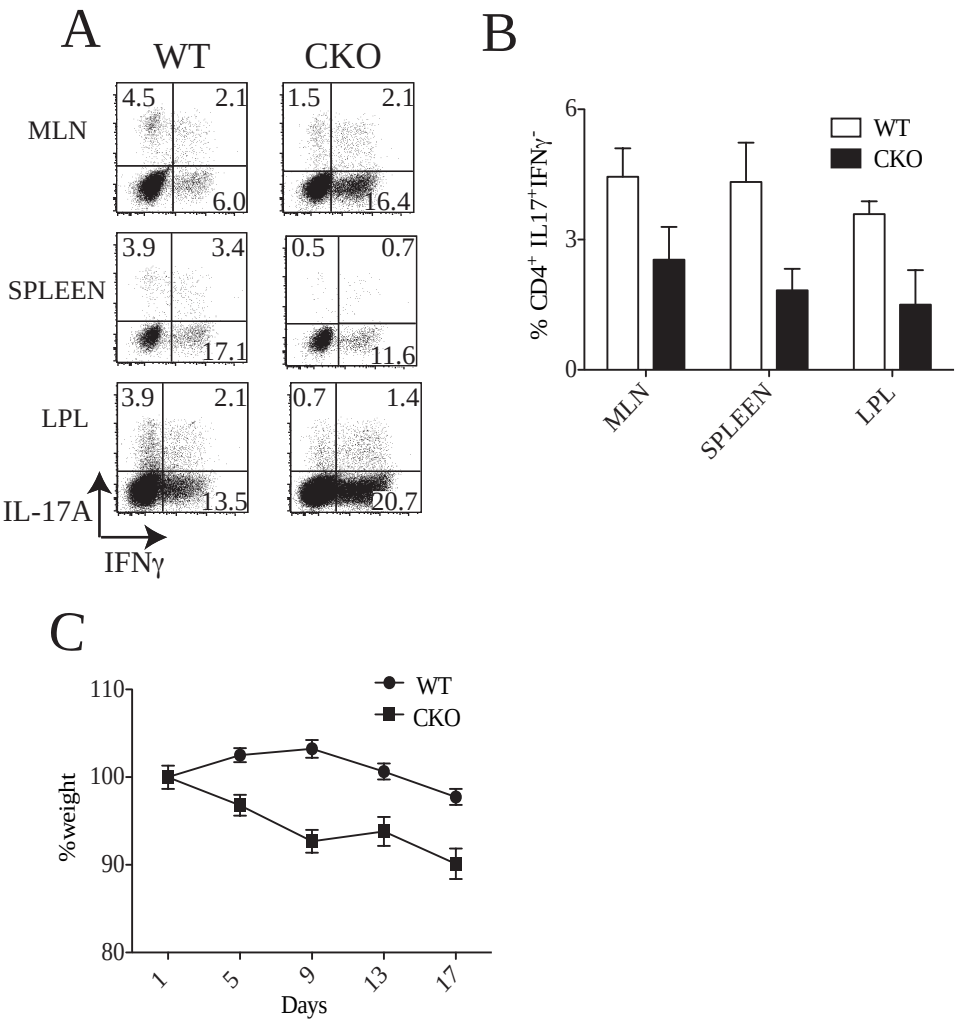
vivo relevance of the *in vitro* defects in IL-17 production by *Smad2* CKO $CD4^+$ T cells was tested by determining if naïve *Smad2* CKO $CD4^+$ T cells produced IL-17 when transferred to lymphopenic *Rag1*$^{-/-}$ recipients. Three weeks after T cell transfer there was a significant decrease in IL-$17A^+CD4^+$ T cells isolated from the peripheral lymphoid organs and colonic LP of *Rag1*$^{-/-}$ recipients that had been reconstituted with naïve *Smad2* CKO $CD4^+$ T cells as compared to control $CD4^+$ T cells (Fig.2.6A and 2.6B). Interestingly, the decrease in IL-17 production was not consistently observed in *Smad2* CKO $CD4^+$ T cells that were secreting both IL-17A and IFNγ (Fig.2.6A). The decrease in overall IL-17 production by activated *Smad2* CKO $CD4^+$ T cells in lymphopenic *Rag1*$^{-/-}$ mice was correlated with the more severe colitis induced by the transferred *Smad2* CKO T cells as revealed by more rapid and severe weight loss in the recipients (Fig.2.6C). These results demonstrated that SMAD2 was necessary for normal production of IL-17 by $CD4^+$ T cells in a lymphopenic environment.

C. rodentium infection in Smad2 CKO mice elicits diminished Th17 cell induction

The IL-17 family of cytokines is required for efficient clearance of the gut pathogen *Citrobacter rodentium* (13). To determine whether pathogen-driven IL-17 production by $CD4^+$ T cells also required SMAD2, *Smad2* CKO mice were infected with *C. rodentium*. Earlier studies have shown that *Citrobacter* infection in C57BL/6 mice reaches maximal pathogen load by a week, and it is resolved in two weeks. Ten days after infection *Smad2* CKO mice had comparable numbers of activated T cells in the mesenteric lymph nodes and spleen as WT infected mice (Fig.2.7A). However, there was a significant reduction in

Figure 2.6. SMAD2 is required *in vivo* for the generation of IL-17A^{+} CD4 T cells. (A and B). *Smad2* CKO CD4 T cells cause more severe colitis in *Rag1*$^{-/-}$ hosts. Naive WT and *Smad2* CKO CD4 T cells were transferred to *Rag1*$^{-/-}$ recipients (n=5/genotype). 3 weeks post transfer, T cells from the lymphoid organs were analyzed for IL-17A and IFNγ expression by ics. Flow cytometric profiles of individual mice in A, and averages in B are representative of two independent experiments with similar results. (C) The onset and severity of colitis were monitored by weight measurements, visual signs of distress and gut histology. Data shown is an average of two independent experiments.

Figure 2.6


A
WT
CKO
MLN
SPLEEN
LPL
4.5
2.1
6.0
1.5
2.1
16.4
3.9
3.4
17.1
0.5
0.7
11.6
3.9
2.1
13.5
0.7
1.4
20.7
IL-17A
IFNγ
B
% CD4+ IL17+IFNγ-
6
3
0
WT
CKO
MLN
SPLEEN
LPL
C
110
100
90
80
%weight
WT
CKO
1
5
9
13
17
Days

the frequency of Th17 cells in the lymphoid organs of infected *Smad2* CKO mice (Fig.2.7B). In *Smad2* CKO mice IL-17^{+}CD4^{+} T cells accumulated on average to ~50% of the numbers seen in control infected mice (Fig.2.7C). These results demonstrated that during *Citrobacer rodentium* infection optimal Th17 cell generation required SMAD2.

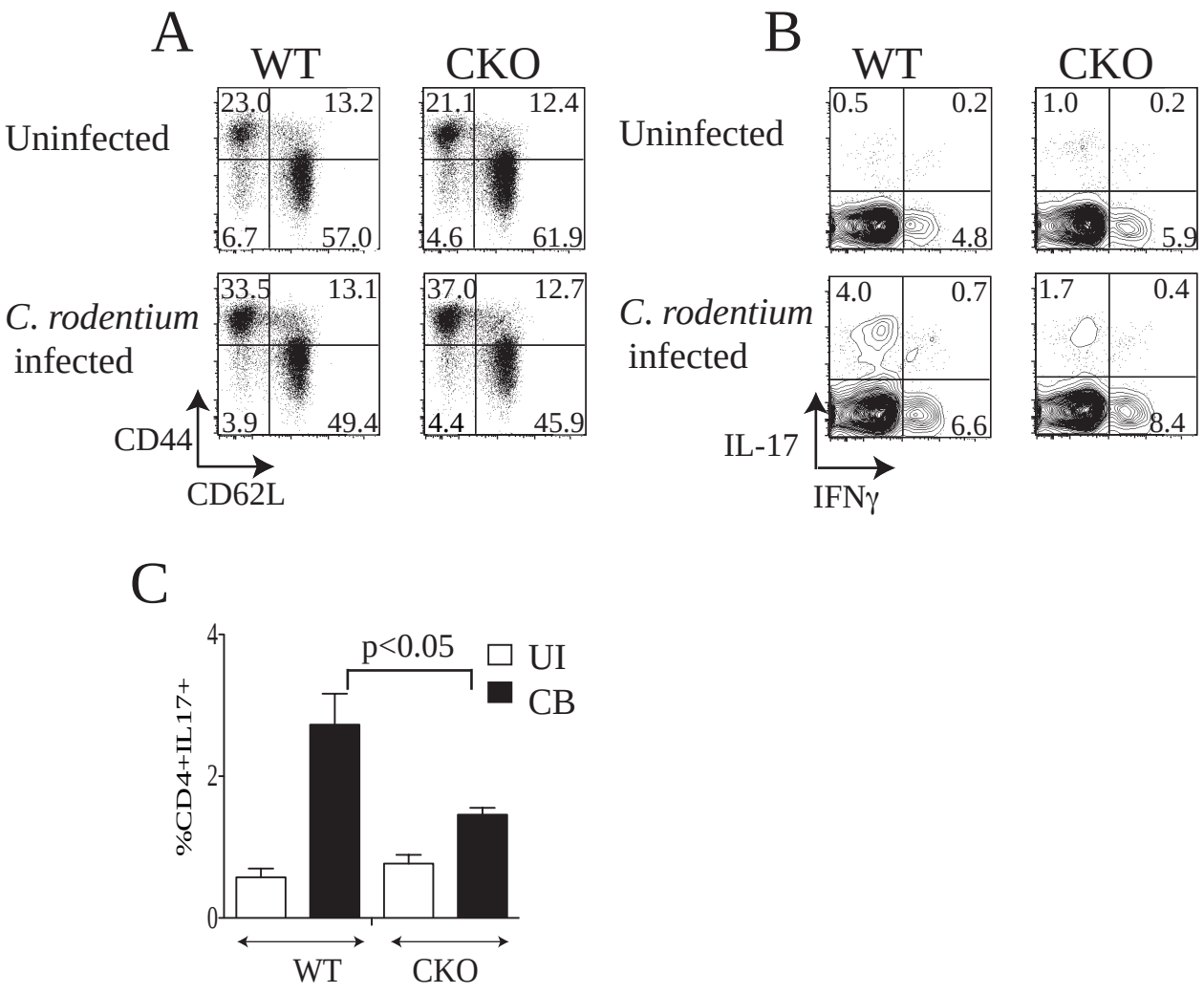
Summary and future directions

The work in this chapter illustrated a previously unidentified role of R-SMAD- SMAD2 in regulating both iTreg and Th17 cell differentiation pathways. Combining the results obtained from the analysis of *Smad3*$^{-/-}$ and *Smad4* CKO T cells, it can be concluded that all SMADs are necessary for the induction of iTregs. Though SMAD3 has been shown to bind at *Foxp3* enhancer to increase its transcription, the interaction of SMAD2 at this gene locus was not observed (89). Since, SMAD2 does not directly bind to DNA, its affect in gene transcription is generally mediated through its interaction with other TFs. Thus, future work will elucidate if SMAD2 regulates transcription of *Foxp3* by interacting with SMAD3/4 or in an independent manner.

The defects in Th17 cell differentiation observed in the absence of SMAD2 are dependent on the concentration of both TGFβ and IL-6. Further, in the absence of *Smad2*, the expression of alpha chain of *Il6r* gene was diminished with an associated reduction in the phosphorylation of STAT3, the central TF in Th17 cell differentiation. Forthcoming studies will help us understand how SMAD2 modulates the expression of *Il6ra* upon T cell activation. The importance of SMAD2 in regulating the in vivo Th17 cell differentiation was realized by the relatively reduced frequency of CD4^{+} IL-17^{+} cells

Figure 2.7. SMAD2 is required for the generation of IL-17A$^+$ CD4 T cells during *Citrobacter rodentium* infection. (A) Diminished Th17 response to *C. rodentium* infection in *Smad2* CKO mice. WT and *Smad2* CKO mice infected with *C. rodentium* were sacrificed at 2 weeks post-infection. Representative flow cytometric profiles of CD4$^+$ T cells expressing the activation markers CD44 and CD62L in the spleens of WT and *Smad2* CKO mice are shown. Data are representative of two independent experiments (minimum 3 mice/genotype). (B) CD4$^+$ T cells from lymphoid tissues (splenocytes shown here, but a similar pattern observed in all tissues) of mock-infected and infected WT and *Smad2* CKO animals were analyzed for the expression of IL-17A and/or IFNγ by ics. (C) Averages (n=5/genotype) of the frequencies of CD4+IL-17A+ splenocytes represented in B are shown. UI, uninfected; CB, *Citrobacter* infected. Data are representative of two independent experiments.

Figure 2.7


A
WT
CKO
Uninfected
23.0
13.2
6.7
57.0
21.1
12.4
4.6
61.9
C. rodentium infected
33.5
13.1
3.9
49.4
37.0
12.7
4.4
45.9
CD44
CD62L
B
WT
CKO
Uninfected
0.5
0.2
4.8
1.0
0.2
5.9
C. rodentium infected
4.0
0.7
6.6
1.7
0.4
8.4
IL-17
IFNγ

C
p<0.05
UI
CB
%CD4+IL17+
4
2
0
WT
CKO

observed upon *C. rodentium* infection in *Smad2* CKO mice and also upon transfer of naïve *Smad2* deficient CD4 T cells in the lymphopenic mice. Interestingly, SMAD2 mediated Th17 cell differentiation was altered not only downstream of TGFβ but also downstream of ActivinA or IL-1β cytokines. Since SMAD2 is known to mediate signals specifically downstream of TGFβ and Activin, the defect of SMAD2 deficient T cells in IL-1β dependent Th17 cell differentiation was surprising. This could be interpreted in the following ways. First, in these in vitro cultures TGFβ was not neutralized. The endogenous T cell produced TGFβ or low levels of TGFβ present in the culture media could synergize with IL-1β for Th17 cell differentiation. It is possible that this co-operation between TGFβ and IL-1β is altered in the absence of SMAD2 leading to decreased IL-17 production. Second, since SMAD2 deficient T cells respond weakly to IL-6 stimuli and show defects in STAT3 phosphorylation, this could also lead to their lower Th17 cell differentiation in the presence of IL-1β and IL-6 cytokines.

It has been shown that Th17 cells can be generated in vivo in an IL-6 independent manner (101). Future work will determine if these IL-6 independent Th17 cells are present in normal proportions in the absence of SMAD2. Interestingly apart from regulating IL-17 induction, TGFβ has been shown to inhibit IL-22 expression from Th17 cells (213). We will further investigate if the TGFβ signaling via SMAD2 negatively regulates IL-22 production from Th17 cells. It is possible that SMAD2 deficient Th17 cells could be qualitatively different if they produce less IL-17A and more IL-22.

One of the major unresolved issues in understanding the role of SMAD2 in Th17 cell differentiation is the lack of knowledge on its molecular mechanism. The nuclear

translocation of SMAD2 had been thought to require its interaction with SMAD4. But, SMAD4 deficiency does not affect Th17 cell differentiation. How SMAD2 functions independent of SMAD4 in regulating Th17 cell differentiation requires additional investigations. Interestingly, TF TIF1γ has been shown to associate with SMAD2/3 in a SMAD4 independent manner to regulate differentiation of NKT cells (200). Hence, it will be determined if SMAD2 interacts with TIF1γ to regulate Th17 cell differentiation.

Together, we propose that TGFβ signaling regulates T cell differentiation via its qualitative and quantitative effects. For Th17 cell differentiation, we observe a biased role of SMAD2 over SMAD3 but for the generation of Tγδ17 cells SMAD2 did not play an essential role indicating that different pathways contribute toward differentiation of innate and adaptive IL-17 producing cells.

Materials and methods

Mice and infection: *Smad2* $^{fl/fl}$ mice generated in the laboratory of Dr. Liz Robertson were provided by Dr. Richard Flavell. These mice were backcrossed six times to C57BL/6 background before analysis. *Smad2* CKO mice were generated by crossing *hCD2 Cre* Tg^{+} mice with *Smad2*$^{fl/fl}$ mice. *Rag1*$^{-/-}$ and C57BL/6 mice were purchased from the Jackson laboratory. For *Citrobacter rodentium* infection strain DSS 100 was obtained from Dr. John Leong (UMMS). 10^{10} CFU of *Citrobacter* in 10% sodium bicarbonate was administered by oral gavage. All experiments were approved by The University of Massachusetts Institutional Animal care and Use Committee.

Abs, flow cytometry and cell sorting: Cells were stained for surface markers, and intracellular cytokines and transcription factors using monoclonal antibodies (mAbs) and intracellular kits purchased from BD Biosciences and ebioscience. Samples were acquired on a BD LSRII cytometer and data post acquisition was analyzed using Flowjo software (Treestar). Naïve T cells and nTreg cells were sorted to >95% purity using a MoFlow cytometer (Dako Cytomation).

RT-PCR and Real time PCR: RNA was prepared using Trizol reagent and cDNA was made using Omniscript RT-PCR kit (Qiagen). For semi-quantitative RT PCR, four fold serial dilutions of cDNA were used. Following PCR primers were used: *Smad2* ATGTCGTCCATCTTGCCATT and GTCCCCAAATTTCAGAGCAA; *Il6ra* ACAGTGTGGGAAGCAAGTCC and ATGGTCAAAGGAGTTCACGG3; Rorc: CCGCTGATAGGGCTTCAC and TGTAATGTGGCCTACTCCTGCA. Real time PCR amplification was performed by using iQ SYBR Green supermix (BioRad). All data was normalized to *Actb* or *Gapdh* mRNA expression.

Western Blotting: Lysates from WT CD4 T cells and *Smad2* deficient CD4 T cells were separated on a 10% SDS-polyacrylamide gel. Western blot for pSTAT3 (Cell Signaling Technology) and CDK2 (Santa Cruz) loading control was developed using Enhanced Chemiluminiscence (Pierce). Relative band intensity was calculated using ImageJ software.

T cell culture: For *Foxp3* induction, sorted naïve $CD4^+CD25^-CD44^{hi}$ $CD62L^{lo}$ T cells were activated with plate bound anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml) mAbs in 96 well plates for 3d in the presence of 2ng/ml or 5ng/ml rTGFβ, Peprotech) and rIL-2 (ebioscience). *Th17 differentiation*: Naïve $CD4^+$ T cells were co-cultured with mitomycin C treated, T cell depleted splenocytes (1:5 ratio) for 4d in the presence of anti-CD3 (1mg/ml) and CD28 (3mg/ml) mAb with cytokines at various concentrations: TGFβ (2 and 5 ng/ml), rIL-6 (20 and 40ng/ml, Peprotech), rIL-1β (10ng/ml, Peprotech). For blocking IL-2 in Th17 cultures, cocktails containing anti-IL2, anti-CD122 and anti-CD25 mAbs were added at 10ng/ml each. Anti- IFNγ and anti IL-4 mAbs were also used at 10ng/ml each to block Th1 and Th2 differentiation, respectively (all blocking mAbs from BD bioscience).

CFSE labeling to measure cell proliferation: Sorted naïve $CD4^+$ T cells were incubated in PBS containing 1mM CFSE for 15 min at 37^0C. Cells were washed and activated with plate bound anti-CD3/CD28 mAb in the absence or presence of TGFβ for 2 days.

Colitis: To induce colitis, naïve $CD4^+$ T cells (3×10^5) sorted from WT or *Smad2* CKO mice were intraperitoneally injected in *Rag1*$^{-/-}$ recipients and analyzed at indicated days. For prevention of colitis, sorted $CD4^+CD25^+$ Treg cells (2×10^5) were co-injected with naïve T cells from WT mice in *Rag1*$^{-/-}$ recipients. General health and weight of animals were monitored and colonic pathology was determined 4wks after injections by H&E staining of formalin fixed tissues. To isolate colonic lymphocytes, colons excluding

cecum were isolated, cleaned and incubated at 37^0C with media containing 1.5mg/ml Dispase (Invitrogen) for 45 minutes. Intestines were then finely cut and incubated in media containing 0.8mg/ml Collagenase II (Invitrogen) and 1mg/ml Dispase for 30 minutes at 37^0 C. Digested intestines were washed and passed through glass wool column followed by lymphocyte separation using percoll gradient.

CHAPTER III

HMG box TFs SOX13 and TCF1/LEF1 program the development of Tγδ17 cells

Attributions and Copyright information

Some results in this chapter are submitted for publication and some results are derived from the upcoming publication:

Intrathymic programming of effector fates in three molecularly distinct γδ T cell subtypes **Nature Immunology (in press)**

Kavitha Narayan*, Katelyn E. Sylvia*, Nidhi Malhotra, Catherine C. Yin, Gregory Martens, Therese Vallerskog, Hardy Kornfeld, Na Xiong, Nadia R. Cohen, Michael B. Brenner, Leslie J.Berg and Joonsoo Kang, The Immunological Genome Project Consortium

* Contributed equally

Limited influence of αβTCR+ thymocytes on thymic γδ effector subset differentiation **Cutting Edge Journal of Immunology (under revision)**

Kavitha Narayan, Nidhi Malhotra, Katelyn E. Sylvia and Joonsoo Kang

Specific contributions to figures:

Figure 3.1, 3.2 KN

Figure 3.3 KS, NM

Figure 3.8 KN

Figure 3.9 NM, KN, KS

Figure 3.13 KN

Figure 3.14 KN, NM

Specific contribution to methods:

Microarray data analysis, KN

Introduction

The developmental programming and differentiation of T cells into diverse functional subsets begins with the migration of pluripotent hematopoietic precursors from the bone marrow or the fetal liver into the thymus. After seeding the thymus, the T cell progenitors rapidly proliferate, differentiate, undergo T cell receptor (*Tcr*) gene rearrangements and finally assemble TCR complexes that dictate their further survival and function. The cells go on to give rise to distinct T cell lineages including γδ T cells, αβ T cells and NKT cells that undergo thymic maturation and subsequently migrate into the peripheral lymphoid and non-lymphoid organs. The step-wise thymic differentiation process includes successive intermediates $CD4^{-}CD8^{-}$ (double negative, DN), $CD4^{+}CD8^{+}$ (double positive, DP) and $CD4^{+}$CD8- or $CD8^{+}CD4^{-}$ (single positive, SP) stages. The DN stages are further subdivided into DN1, DN2, DN3 and DN4 developmental stages (as described earlier in the introduction (125). The γδ T cells arise between DN1 and DN3 stages. The DN3 stage αβ lineage committed cells rapidly pass through DN4 to DP stage. The immature DP thymocytes give rise to conventional CD4SP and CD8SP αβ T cells and innate-like αβ NKT cells.

This T cell commitment process is an irreversible forward progression of distinct developmental stages, which are marked by stage specific gene expression patterns that are regulated by core TF networks that often act at multiple stages. These TFs act in concert with the regulatory influences of thymic epithelium, which provides a potent combination of growth factors and receptor ligands to trigger and support the T cell commitment, survival and maturation processes. The complex TF networks specifiying T

cell fate and differentiation includes WNT, Notch and other classical morphogen pathways (214).

One of the major goals of this thesis work has been to understand the role of WNT signaling components in thymic γδ T cell effector fate determination. *Wnt* is a combined abbreviation from *Drosophila melanogaster*'s segment polarity gene *Wingless* and *Integrase-1* (215), which is the mammalian homolog of *Wingless* gene. WNT proteins are a family of secreted lipid modified glycoproteins that are crucial for developmental processes such as cell fate specification, progenitor cell proliferation and control of asymmetric cell division (216). As morphogens, they function in a dose dependent gradient manner to provide positional cues to specify cell fate. In the hematopoietic system, WNT family proteins regulate renewal of hematopoietic stem cells and their differentiation into T and B cell lymphocyte lineages. The thymic epithelial cells primarily release WNT proteins, while their receptors (Frizzled) are expressed on both stromal cells and developing thymocytes. Different frizzled receptors and diverse WNT family members are expressed in thymocytes in a developmentally regulated pattern (217).

There are at least three different pathways characterized for WNT signal transduction, of which the canonical WNT signaling pathway involving β-catenin and members of the T cell factor (TCF)/lymphocyte-enhancer-binding factor (LEF) family is most well studied in the immune system (218), (219). In the canonical WNT pathway, β-catenin is sequestered in the cytoplasm and is targeted for degradation by the proteasome through the actions of an inhibitory destruction complex in the absence of WNT ligand

induced signaling. The destruction complex is composed of scaffolding and tumor suppressor proteins, Adenomatous Polyposis Coli (APC), Axis inhibition protein (AXIN) and the Serine/Threonine protein kinases, Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3β (GSK3β) (220). Phosphorylation of β-catenin at multiple residues by CK1 and GSK3β creates recognition sites for β-transducin repeat containing protein (βTRCP), leading to ubiquitination and proteasomal breakdown of β-catenin. In the absence of β-catenin in the nucleus, its binding partners, TCF and LEF, function as transcriptional repressors by recruiting transcription inhibitory proteins such as Groucho (221). At the cell membrane, WNT proteins bind to the Frizzled receptor and co-receptor Low-density Lipoprotein Receptor related protein 5 (LRP5) or LRP6 to activate the signaling cascade. In the presence of WNT, the Frizzled and LRP5/6 co-receptors form a complex with the kinases CK1 and GSK3β phosphorylate LRP5/6, thus creating docking site for AXIN. Subsequently, the activation of Dishevlled (DVL) destabilizes the destruction complex, leaving β-catenin free to translocate to the nucleus where it binds to TCF1/LEF1 and activates transcription (220).

TCF1 and LEF1 belong to the high mobility group (HMG) box TFs (222), (223). HMG box TFs function by their unique ability to bind to the minor groove of DNA where they induce a significant structural bend allowing unique juxtaposition of transcription regulatory complexes (224). WNT signaling is important at the DN to DP stage transition and DP to SP T cell transition as demonstrated in *Tcf1*$^{-/-}$ mice in which T cell development is impaired with a block in the transition of thymocytes from DN to DP stage (225). This block in T cell development was associated with defects in

differentiation, proliferation and survival of αβ lineage T cells. While TCF1 is expressed in only T cells, LEF1 is expressed in both T and B cells and are necessary for their normal development (226). LEF1 deficiency leads to neonatal fatality in mice due to its requirement for development of multiple organs. However, T cell development in these mutant fetuses was not as severely affected as in *Tcf1*$^{-/-}$ mice, suggesting that LEF1 and TCF1 have unique requirements (227). Importantly, the loss of both TCF1 and LEF1 resulted in the most severe block in T cell development, indicating that synergistic and overlapping TCF1/LEF1 functions drive T cell development (227).

As many as eight TCF1 isoforms exist in the murine thymus but only the overexpression of one of the dominant TCF1 isoform that contains the N-terminal β-catenin binding domain restored αβ T cell development in *Tcf1*$^{-/-}$ mice (228). This result suggested that TCF1 functions in the canonical WNT signaling pathway to regulate T cell development. However, it is possible that TCF1 also binds to proteins other than β-catenin using the same N-terminus and thus potentially transduces signals independent of β-catenin. Sen et al showed that β-catenin deficiency blocks transition of T cells from DN3 to DN4 stage (229). However, other studies failed to show the requirement for β-catenin in T cell development (230), (231). Further, it was thought that there exists redundancy between β-catenin and γ-catenin, both of which are expressed in the thymus and have been shown to compensate for each other in distinct cell types. But, this was refuted by two studies showing that *bcat*$^{-/-}$*gcat*$^{-/-}$ mice do not show significant impairment in T cell development (232), (233) while absolutely required for development of non-lymphoid tissues. A recent study also confirmed that T cell precursors are absolutely dependent on

TCF1 for differentiation by inducing critical TFs of T cells such as *Gata3* and *Bcl11b,* but modulations of β-catenin did not significantly impact TCF1 function (231). Thus, studies to date establish that while β-catenin is active during T cell differentiation (234), it appears not to be absolutely essential and that there may be other TCF1 interacting factors that can replace β-catenin for instituting T cell developmental programs.

TCF1 function in γδ T cell development has been similarly controversial. In *Tcf7*$^{-/-}$ mice, the number of γδ thymocytes had been shown to be largely unchanged (227) although the differentiation of gut γδ IELs is impaired (235). These results, along with limited and unconvincing assays using *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ fetuses (227), were interpreted as evidence supporting distinct effects of WNT signaling in αβ T versus γδ T cell development. The role of WNT signaling and TCF1 in γδ T cells was reassessed with the identification of the HMG box TF SOX13 that was shown to be specifically expressed in γδ T cells (134). SOX13 is the first TF identified that distinguished γδTCR^{+} thymocytes from αβ T cells. SOX13 was shown to directly interact with TCF1/LEF1 and it altered the expression of TCF1 target genes, suggesting that distinct modulation of WNT signaling and/or TCF1/LEF1 regulated T cell lineage commitment in the generation γδ and αβ T cells from precursors. Further, other SOX proteins were expressed in developmental stage and cell type-specific manner, pointing to a complex network of HMG TFs in generating T cell subtypes. For instance, SOX4 is highly expressed in all DN precursors, DP cells, and γδ T cell (134), (236). SOX4 has been shown to interact with LEF1 and β-catenin (237) and is a candidate TF participating in canonical WNT signaling in the immune system. *Sox4*$^{-/-}$ mice are embryonic lethal due to defects in heart valve formation (238),

and studies using fetal liver chimeras showed that the absence of SOX4 during hematopoiesis results in severe blocks in T cell differentiation (236). More detailed analysis of SOX4 function during T cell development is now possible with the generation of mice lacking SOX4 at different stages of T cell differentiation (unpublished).

The interactome of differentially expressed SOX proteins with TCF1/LEF1 will determine how these TFs perform distinct functions in a cell type-specific manner. The intertwining of the WNT pathway with Notch signaling presents further entanglement in the complex web of TF networks that control T cell developmental processes. Notch signaling dominantly commits hematopoietic progenitors to differentiate into T cell lineage and is required at multiple stages of T cell maturation (239). While the WNT-β-catenin pathway can upregulate Notch1 and Notch target genes *Hes1* (hairy and enhancer of split 1) and *Dtx1* (Deltex homologue1) (240), Notch, signaling in turn promotes the expression of *Tcf7* itself and TCF1-target gene *Gata3* in early DN progenitors (231). Of the four mammalian Notch receptors, only Notch 1 and Notch 2 are expressed in the thymus, along with Notch ligands Jagged1, Jagged2, Delta-like 1 and Delta-like 4. Notch is a single pass transmembrane receptor that is activated when its extracellular domain interacts with ligands of Delta and Serrated families. Ligand-receptor interactions cleave the intracellular domain of Notch, which enters the nucleus and affects gene transcription through its interaction with recombination signal binding protein Jk (RBP-Jk) (241). While the dependence of αβ lineage differentiation on Notch signals is clear, its precise role in γδ T cell development remains controversial due to conflicting results generated by different in vivo model systems. The overriding theme however, suggests that γδ T cells

become independent of Notch signals after TCR expression while αβ T cells continue to require Notch to reach the DP stage (242), (243). Hence, it is proposed that Notch is required in a stage specific manner for γδ/αβ T cell lineage commitment. The interconnected TCF1 and Notch signaling regulated gene networks in specifying αβ versus γδ T cell lineage commitment and αβ and γδ T cell subtypes and effectors are beginning to be uncovered, but important details of the networks remain poorly elucidated.

Classical morphogen signals and cytokines such as IL-7 and Kit ligand generate heterogeneous DN precursor cells that respond distinctly to other developmental cues (244), (245). For αβ T cells the TCR is the central determinant of their differentiation post DN3, TCR-mediated T cell selection processes give rise to mature CD4 SP and CD8 SP thymocytes. The naïve CD4 and CD8 T cells migrate to the periphery where they differentiate into different effector lineages upon encountering their cognate antigens and cytokine signals. In contrast to αβ T cells, γδ T cell development in the thymus entails effector function programming in distinct γδ cell subsets (Narayan et al.,in press). Thus, unlike αβ T cells, γδ T cells home to the peripheral tissues in a pre-differentiated effector state that can mount rapid innate-like responses to inflammatory settings. Similar to peripheral αβ T cells, γδ T cells in an adult thymus have been segregated into Th1-like (V1), NKT (V6) and Th17 (V2) effector subsets. Though V2 cells form a significant proportion of Tγδ17 cells, fetal thymus-derived V4 cells also contribute towards IL-17 production. Thus Tγδ17 cells can be further subdivided into V2 Tγδ17 and V4 Tγδ17 cells. The Tγδ17 cell lineage provides protection to the host in several infections as well

as has potential to initiate and enhance many organ specific autoimmune disorders. Extensive amount of data has been published describing the cytokines and TFs that regulate the differentiation of αβ T cells into Th17 effector cells. However, little is known about the thymic programming of γδ T cells into their effector lineages. A major goal of studies described in this chapter has been to identify pathways that regulate the development of Tγδ17 cells. While both Th17 and Tγδ17 cell differentiation is dependent on RORγt (71), Tγδ17 cells are not strictly reliant on TGFβ (246) and IL-6 (110) cytokines that generate Th17 cells. As discussed in Chapter I, Tγδ17 cells are present in normal numbers in the absence of STAT3 (180) and IRF4 (159), the key TFs for Th17 cell differentiation. To deduce distinct pathways involved in the production of innate effectors requires mapping gene networks controlling early T cell development. The programming events of γδ T effector differentiation are embedded within and coincident with their development and maturation processes in the thymus. Therefore, TFs belonging to Notch and WNT pathway are candidate regulators of Tγδ17 cell differentiation. Indeed, HES1, a transcriptional repressor and a direct Notch1 target, has been shown to be critical for the development of fetal V4 Tγδ17 cells and may also impact the generation of adult V2 Tγδ17 cells (180).

Initial analyses of adult *Sox13*$^{-/-}$ mice showed a selective depletion of CD44^{+} V2 cells in peripheral tissues. Further, it was shown that SKINT1, a ligand required for normal maturation of fetal V3 cells (dendritic epidermal T cells, DETCs, or skin IELs) (247) downmodulates the expression of *Sox13* and *Rorc* (248). In the absence of SKINT1 V3 cells aberrantly expressed IL-17, suggesting that SOX13, along with the IL-17 lineage

associated TF RORγt, polarizes the cells toward Tγδ17 cell differentiation and that the suppression of this gene circuit is necessary to generate alternate effector cell types. In this chapter, I present studies that demonstrate that for normal adult Tγδ17 cell differentiation SOX13 and its target genes are the central positive regulators, and that TCF1, a SOX13-interacting factor, is the primary negative regulator of IL-17 expression. Thus, innate Tγδ17 differentiation program is fundamentally distinct from that of adaptive Th17 cells that are primarily dictated by balances of regulatory and inflammatory cytokines induced during pathogen challenges.

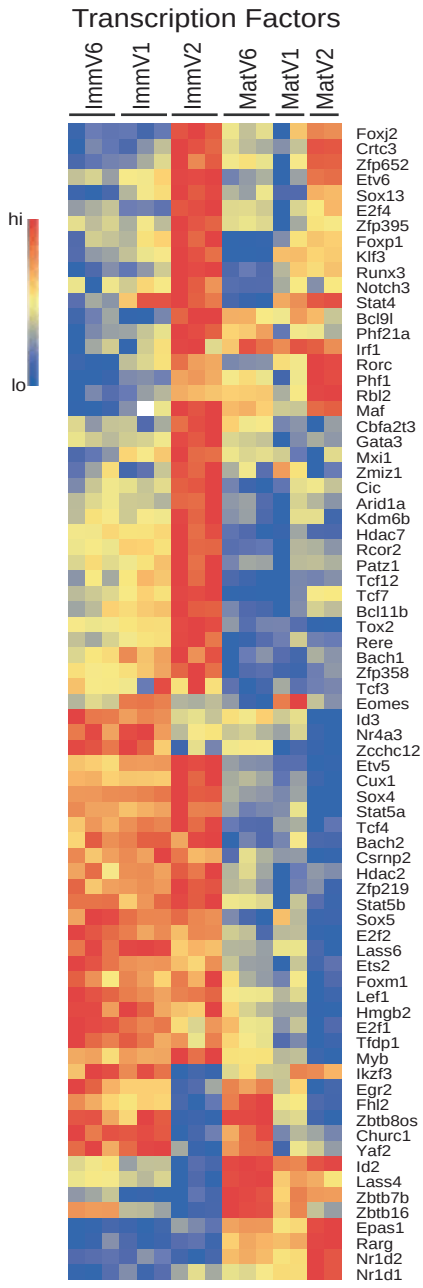
Results and discussion

Emerging γδ TCR thymocytes are composed of different lineages:

It had been previously assumed that all γδ T cells in the adult murine thymus originated from common thymic precursors and distinct TCR signaling dictated effector cell fate (123). However, recent work from our lab showed that the emergent immature γδ T cell subsets are embedded with distinct gene expression profiles (Narayan et al., in press). These analyses further showed that immature V2 γδ T cells are radically different from other immature γδ T cell subsets and this is not due to any difference in their cell cycle properties or susceptibility to death, which are similar between all immature adult γδ T cell subsets. One of the candidate features that propelled the distinct lineage commitment process in immature V2 cells is their relatively high expression of TFs. Among them, the most prominent TFs are the High Mobility Group (HMG) box family members, which are known to be critical regulators of cell lineage fate in multiple tissues (Fig. 3.1).

Figure 3.1. The expression of WNT-HMG TFs is enriched in the Immature V2 γδ T cells. Heat map showing the expression of TFs in immature and mature γδ T cell subsets. HMG box TF *Sox13* is highly expressed in the immature V2 cells as compared to other γδ subsets. Other HMG box TFs, *Tcf7, Tcf4, Tcf12, Tox2* also show higher expression in the immature V2 cells.

Figure 3.1


Transcription Factors
ImmV6
ImmV1
ImmV2
MatV6
MatV1
MatV2
hi
lo
Foxj2
Crtc3
Zfp652
Etv6
Sox13
E2f4
Zfp395
Foxp1
Klf3
Runx3
Notch3
Stat4
Bcl9l
Phf21a
Irf1
Rorc
Phf1
Rbl2
Maf
Cbfa2t3
Gata3
Mxi1
Zmiz1
Cic
Arid1a
Kdm6b
Hdac7
Rcor2
Patz1
Tcf12
Tcf7
Bcl11b
Tox2
Rere
Bach1
Zfp358
Tcf3
Eomes
Id3
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Etv5
Cux1
Sox4
Stat5a
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Stat5b
Sox5
E2f2
Lass6
Ets2
Foxm1
Lef1
Hmgb2
E2f1
Tfdp1
Myb
Ikzf3
Egr2
Fhl2
Zbtb8os
Churc1
Yaf2
Id2
Lass4
Zbtb7b
Zbtb16
Epas1
Rarg
Nr1d2
Nr1d1

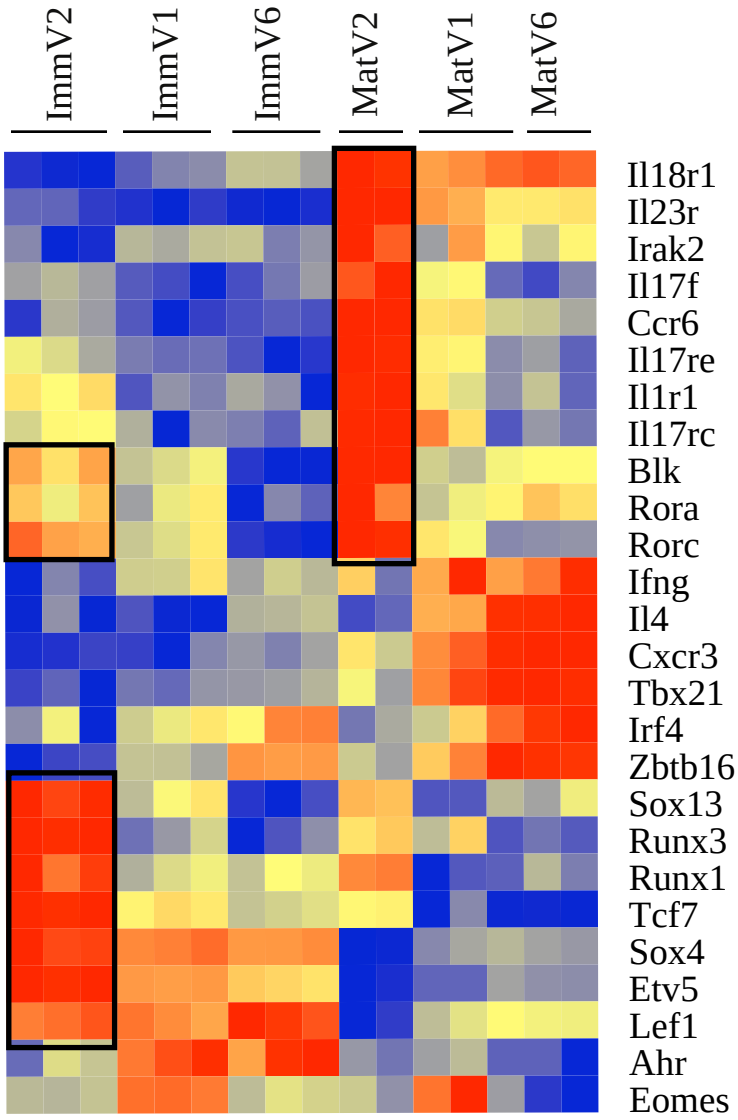
We also showed that maturation of γδ T cells subsets can be identified by their downregulation of CD24 (Heat Stable Antigen, HSA), which had been previously established as a maturation marker of αβ T cells. By the comparative gene expression analysis of TFs in immature and mature γδ T cell subsets, we showed that upon maturation, V2 cells downregulate the expression of subset-specific TFs. Furthermore, the maturation phase of V2 cells is characterized by a surge of expression of genes involved in the execution of specific effector functions, including genes that encode for chemokine receptors and cytokine receptors. For example, *Rorc*, *Rora*, *Ccr6*, *Il23r, Il1ra, Il17rc, Il17re* and *Il17f* are observed to be significantly and specifically upregulated in mature V2 cells as compared to other γδ T cell subsets (Fig. 3.2). Upon maturation, γδ T cell subsets migrate to their destined peripheral tissues where they function as memory like effector T cells. Thus, the initial burst of expression of TFs that distinguishes immature V2 cells is transient, but likely to be sufficient to generate Tγδ17-associated gene program by inducing heritable epigenetic modifications in maturing V2 cells. The high expression of HMG box TF in the immature stages of V2 cells indicated their involvement in programming their effector differentiation. Thus, we tested this hypothesis by determining the function of HMG box TFs SOX13, TCF1 and LEF1 in the Tγδ17 cell differentiation.

SOX13 programs development of adult V2 Tγδ17 cells:

While screening for genes that were differentially expressed between αβ and γδ thymocytes, we identified *Sox13* as a γδ lineage-specific gene that can act as the lineage

Figure 3.2. Effector functions of γδ cells are programmed in thymus. A heat map of relative expression of cytokine/chemokine receptors, cytokines and TFs is shown. Upon thymic maturation, V2 cells enter the effector-poised phase by abrupt and specific superinduction of cyokine receptor genes that are dedicated for IL-17 production and responsiveness: *Il23r* (the most induced gene, by ~100 fold, in $CD24^{lo}$ relative to $CD24^{hi}$), *Il17re* and *Il1r1* are the strongest induced genes in mature V2 cells. Receptors for modulators of IL-17 production are divergently expressed upon maturation. Among TFs, programming TFs of WNT-HMG family, *Sox13*, *Sox4*, *Tcf1*, *Lef1* undergo downregulation upon thymic maturation of V2 cells. TF associated with effector function, *Rorc* is expressed in immature V2 cells and its expression is strongest in mature V2 cells in comparison to other γδ subsets. Similarly, Sox13 target *Blk* exhibits intermediate expression in immature V2 cells and undergoes strong upregulation in mature V2 cells. Expression of cytokines and TFs associated with V1 cells- *Ifng*, *Eomes,* or V6 cells- *Il4* and *Plzf* are increased upon their respective maturation.

Figure 3.2


ImmV2
ImmV1
ImmV6
MatV2
MatV1
MatV6
Il18r1
Il23r
Irak2
Il17f
Ccr6
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Il17rc
Blk
Rora
Rorc
Ifng
Il4
Cxcr3
Tbx21
Irf4
Zbtb16
Sox13
Runx3
Runx1
Tcf7
Sox4
Etv5
Lef1
Ahr
Eomes

determining TF (134). Subsequent systematic global gene expression analyses of different γδ thymocyte subsets performed in conjunction with the Immunological Genome Project (Immgen, C. Benoist, Harvard) showed that while *Sox13* is expressed in all immature γδ subsets, its expression is highest in the immature V2 γδ T cells (Fig.3.1).

To study the function of SOX13 in adult γδ T cell development and function, *Sox13*$^{-/-}$ *(129/J)* mice, previously generated in our lab, were utilized. We observed a selective decrease in the frequency of V2 cells in the peripheral lymphoid organs of *Sox13*$^{-/-}$ mice (Fig.3.3). In addition, the remaining V2 cells in *Sox13*$^{-/-}$ mice expressed significantly lower amounts of TCR on their surface (Fig.3.3). Detailed analysis of *Sox13*$^{-/-}$ V2 cells illustrated that there is a complete loss of V2 Tγδ17 cells (characterized as CCR6^{+}IL-17A^{+}RORgt^{+}CD44^{hi} V2 cells) in the periphery of these mice. (Fig.3.4A, 3.5). Further assessment of *Sox13*$^{-/-}$ thymocytes revealed that CD24^{lo}RORγt^{+}CCR6^{+}CD27^{-} V2 thymocytes that are the earliest identifiable thymic Tγδ17 cells do not develop when SOX13 is missing (Fig.3.4B, 3.5). This defect is cell intrinsic as the Tγδ17 differentiation defect is observed specifically in *Sox13*$^{-/-}$ cells when mixed (WT: *Sox13*$^{-/-}$*)* BM chimeras were analyzed (K. Sylvia, unpublished). Together, these results demonstrated that SOX13 is necessary for thymic development of V2 Tγδ17 cells in a cell intrinsic manner.

We further investigated the mechanism through which SOX13 could program development of γδ effectors. A clue came from an earlier work from our lab that had identified *Blk* as a SOX13 target gene. Ectopic expression of *Sox13* in all developing T cell precursors in vivo led to the aberrant expression of *Blk* in DP thymocytes (134). A

Figure 3.3. SOX13 regulates the proportions of V2 γδ T cells. Decreased frequency of V2 cells is observed in the periphery of *Sox13*$^{-/-}$ mice. Among V2 cells, Vγ2 TCR^{hi} cells are absent in the *Sox13*$^{-/-}$ mice.

Figure 3.3

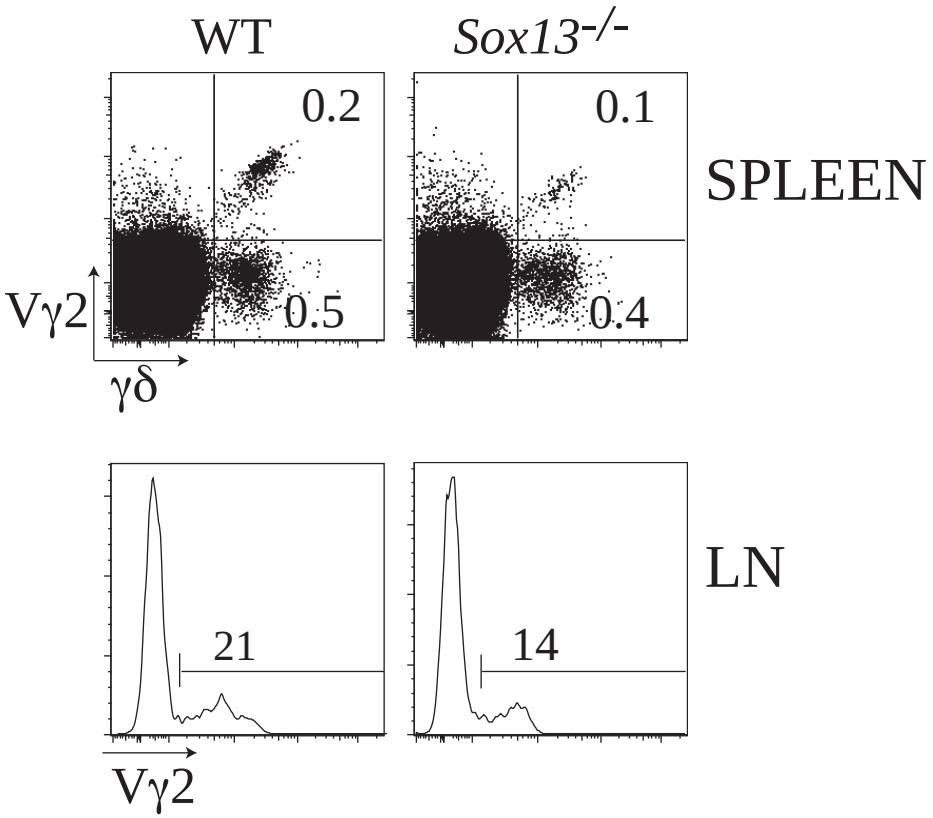


Figure 3.4. SOX13 is essential for V2 Tγδ17 generation. (A) V2 Tγδ17 cells are absent in the LNs of *Sox13*$^{-/-}$ mice. The differentiation of γδ effector T cells was examined by the analysis of intranuclear RORγt and EOMES and cell surface CCR6 and CD27 ex vivo, and the analysis of intracellular IL-17A and IFNγ after stimulation of LN T cells. (B) The defects in Tγδ17 generation originate in the thymus. Expression of the Tγδ17 markers as in (A) in mature (CD24^{lo}) Vγ2^{+} thymocytes of WT and *Sox13*$^{-/-}$ mice.

Figure 3.4

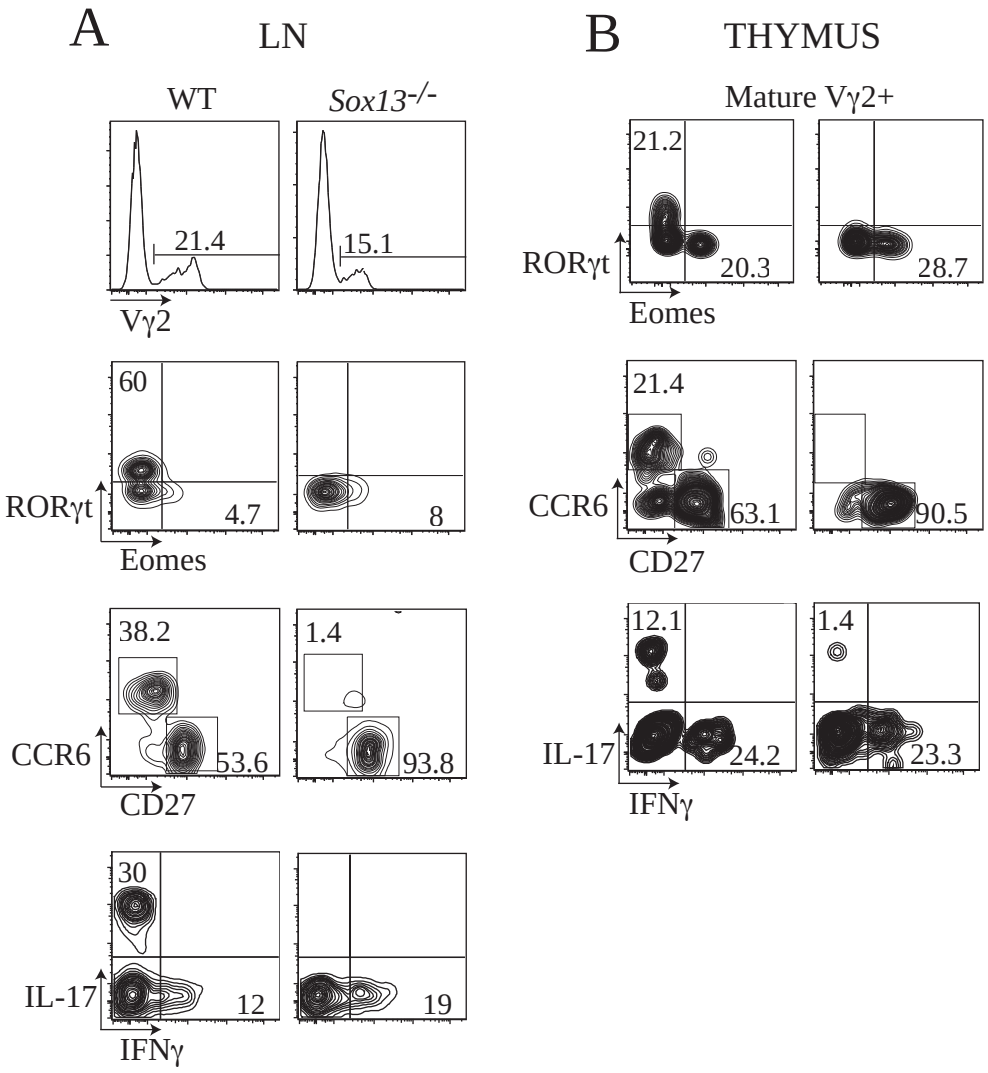
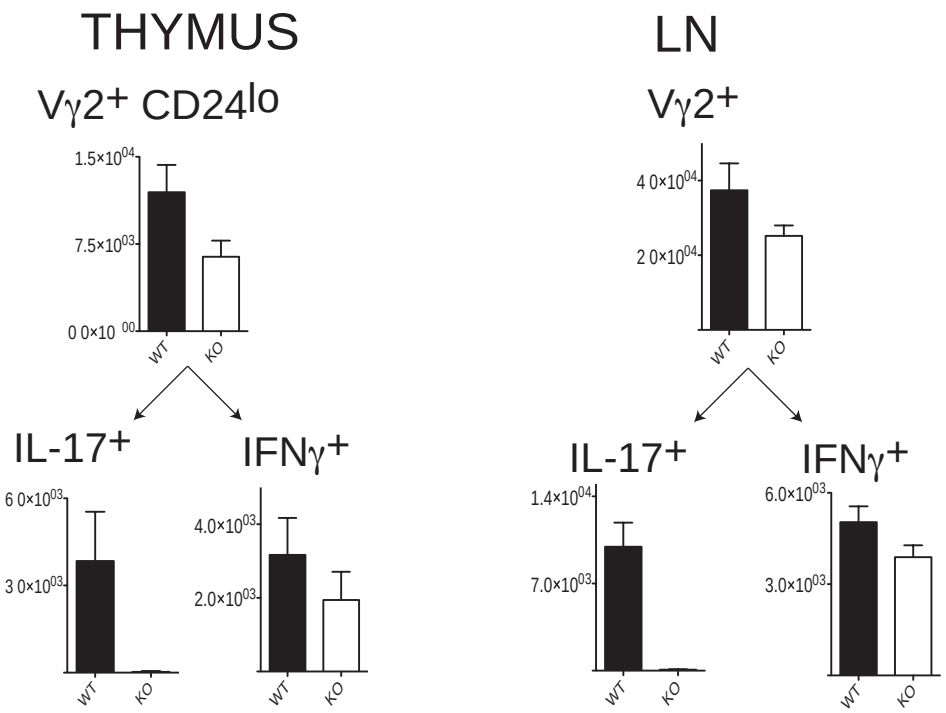

A
LN
WT
Sox13-/-
21.4
15.1
Vγ2
60
4.7
8
RORγt
Eomes
38.2
53.6
1.4
93.8
CCR6
CD27
30
12
19
IL-17
IFNγ
B
THYMUS
Mature Vγ2+
21.2
20.3
28.7
RORγt
Eomes
21.4
63.1
90.5
CCR6
CD27
12.1
24.2
1.4
23.3
IL-17
IFNγ

Figure 3.5. SOX13 positively regulates the development of V2 Tγδ17 cells. *Sox13*$^{-/-}$ mice do not generate IL-17^{+} V2 cells. Total number of mature V2 thymocytes is decreased ~2 fold in *Sox13*$^{-/-}$ mice with a complete block in the differentiation of IL-17 producers. The number of IFNγ producers is not altered significantly in *Sox13*$^{-/-}$ thymus or LNs.

Figure 3.5

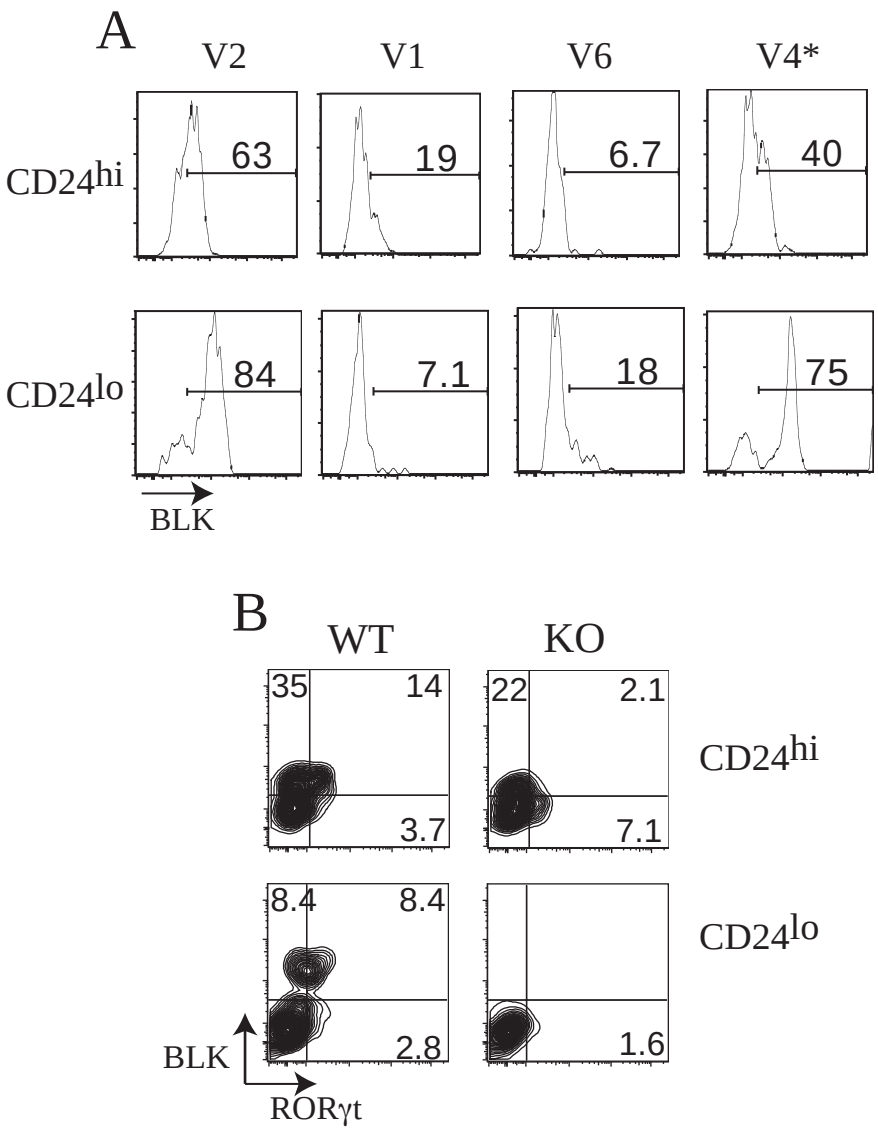

THYMUS
Vγ2+ CD24lo
1.5×10^04
7.5×10^03
0 0×10 00
WT
KO
IL-17+
6 0×10^03
3 0×10^03
WT
KO
IFNγ+
4.0×10^03
2.0×10^03
WT
KO
LN
Vγ2+
4 0×10^04
2 0×10^04
WT
KO
IL-17+
1.4×10^04
7.0×10^03
WT
KO
IFNγ+
6.0×10^03
3.0×10^03
WT
KO

recent study from the Hayes lab showed that BLK is highly expressed in Tγδ17 cells and that its loss affects the differentiation and/or survival of these cells (156). Our detailed analysis of BLK expression in γδ subsets showed that BLK is highly expressed in the immature V2 cells and some immature $V\gamma1^{-}V\gamma2^{-}$ cells (which contains V4 cells) (Fig.3.6A). In the mature γδ subsets, supporting the published study, we observed a strong BLK expression in all $ROR\gamma t^{+}$ γδ T cells (Fig.3.6A).

We next determined BLK expression in γδ T cells of *Sox13*$^{-/-}$ mice. In the thymus we observed a substantial reduction in the proportions of BLK^{+} cells as well as a significant decrease in the BLK expression in the immature V2 thymocytes of *Sox13*$^{-/-}$ mice (Fig.3.6B). Interestingly, by intracellular staining, we could observe the expression of BLK and RORγt in a fraction of WT immature V2 cells, which did not produce IL-17 at this stage. Approximately 20% of immature V2 cells co-expressed RORγt and BLK and these "double positive" cells were essentially absent in *Sox13*$^{-/-}$ immature thymocytes, while the frequency of $ROR\gamma t^{+}BLK^{-}$ immature V2 cells did not change significantly. These results, combined with the published data showing that SOX13 can induce *Blk* expression, indicated that SOX13 regulates BLK expression and the absence of sufficient BLK expression in developing $ROR\gamma t^{+}$ γδ thymocytes prevents their full maturation into Tγδ17 cells. The exact role of BLK in Tγδ17 cells is not known. Constitutive BLK activity in immature pro B cells has been shown to bypass the requirement for pre BCR signaling during differentiation in part by phosphorylation of other SRC kinases and Syk (249). Hence, it is possible that BLK could regulate the signaling threshold in γδ T cells. Tγδ17 and IFNγ producing γδ T cell subsets have been

Figure 3.6. SOX13 regulates BLK expression in the immature V2 cells. (A) BLK expression in immature and mature γδ T cell subsets. Intermediate expression of BLK is observed in immature V2 cells and it is not detected in most immature V1 and V6 γδ cells. BLK is also expressed in some immature V4 cells ($V1^{-}V2^{-}V6^{-}$) cells. Upon maturation V2 and V4 cells express high levels of BLK. (B) BLK and RORγt are co-expressed in a subset of immature V2 cells. The proportions of BLK expressing cells are reduced in the absence of SOX13, with a specific loss of $BLK^{+}ROR\gamma t^{+}$ immature and mature V2 cells.

Figure 3.6


A
V2
V1
V6
V4*
CD24hi
63
19
6.7
40
CD24lo
84
7.1
18
75
BLK
B
WT
KO
35
14
3.7
22
2.1
7.1
CD24hi
8.4
8.4
2.8
1.6
CD24lo
BLK
RORγt

suggested to undergo distinct signaling during selection and maturation in the thymus, with Tγδ17 cells requiring only basal signaling to mature (123). SOX13-regulated BLK in developing Tγδ17 cells may be a primary biochemical property distinguishing them from other γδ cell subsets, and conferring unique developmental signals necessary for full maturation of Tγδ17 cells.

Intriguingly, although the deficiency of SOX13 impaired V2 Tγδ17 cell generation, it did not affect the generation of fetal derived V4 Tγδ17 cells. However, we also observed that *Sox13* is highly expressed in the immature V4 cells. This indicated that SOX13 could be differentially important for IL-17 production from adult versus fetal-derived effector cells. To test this possibility, we analyzed the *Lck* promoter driven *Sox13* transgenic (Tg) mice that were previously generated in our lab (134). Complementary to *Sox13*$^{-/-}$ mice, we saw a marked increase in BLK expressing Vγ2^{+} and Vγ2^{-} γδ T cells in *Sox13* Tg^{+} animals (Fig. 3.7A). Moreover, we observed an increase in the frequency and numbers of V4 Tγδ17 (Vγ1^{-} Vγ2^{-}) cells in these mice (Fig. 3.7B). The effects of SOX13 overexpression are detrimental for the survival of V2 cells and their proportions are significantly reduced in these Tg mice (134). Thus, there was no substantial increase in mature V2 Tγδ17 cells (Fig. 3.7B). These results suggest that the quantitative effects of SOX13 affect the survival and development of V2 and V4 Tγδ17 cells differently. Although immature V2 and V4 cells are very similar in their gene expression patterns overall (Fig. 3.8), it is possible that trans acting factors differentially provided in fetal versus adult thymic tissues can alter a cell's dependence on the key TFs.

Figure 3.7. Increased V4 Tγδ17 cell differentiation in *Sox13* Tg^+ mice. (A) Increased proportions of BLK^+ γδ T cells are observed in the *Sox13*Tg^+ mice. BLK expression in immature ($CD24^{hi}$) and mature ($CD24^{lo}$) $V\gamma2^+$ and $V\gamma2^-$ γδ thymocytes of WT (C57BL/6) and *Sox13*Tg^+ mice, determined by intranuclear staining analysis. (B) The proportions of V2 cells are significantly reduced upon overexpression of SOX13. A substantial increase in the frequency of V4 Tγδ17 ($V\gamma1^-V\gamma2^-$) cells is observed in the *Sox13*Tg^+ mice. IL-17 and IFNγ production was measured after the stimulation of LN cells.

Figure 3.7

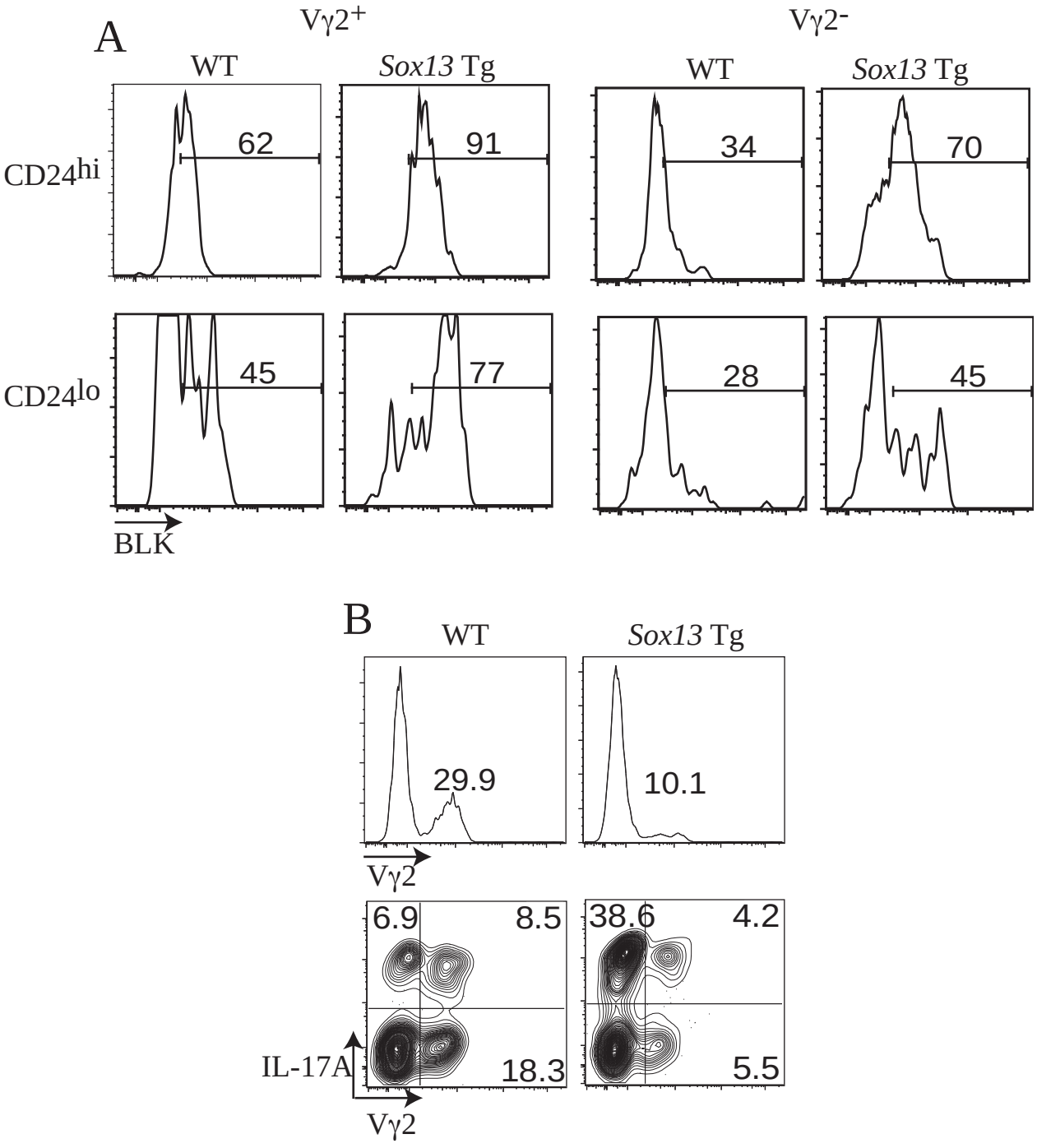
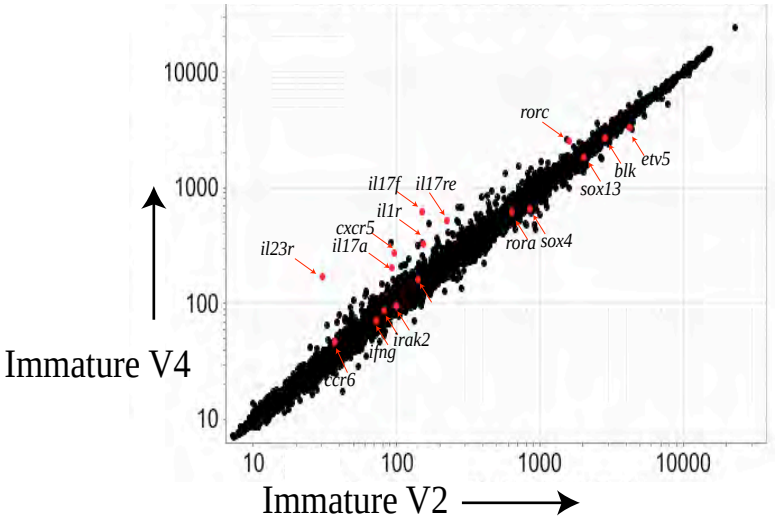

A
Vγ2+
Vγ2-
WT
Sox13 Tg
WT
Sox13 Tg
CD24hi
62
91
34
70
CD24lo
45
77
28
45
BLK
B
WT
Sox13 Tg
29.9
10.1
Vγ2
6.9
8.5
18.3
38.6
4.2
5.5
IL-17A
Vγ2

Figure 3.8. Fetal immature V4 and immature V2 cells are highly similar in their global gene expression. Scatter plots showing mean expression values for genes expressed in the immature V4 versus immature V2 cells. E17 thymocytes were sorted for the preparation of RNA. The red dots show Tγδ17 cell associated genes. A subtle increase in the expression of some of those markers is observed in the immature V4 cells compared to the immature V2 cells.

Figure 3.8


10000
1000
100
10
10
100
1000
10000
Immature V4
Immature V2
rorc
etv5
blk
sox13
il17f
il17re
il1r
cxcr5
il17a
il23r
rora
sox4
irak2
ifng
ccr6

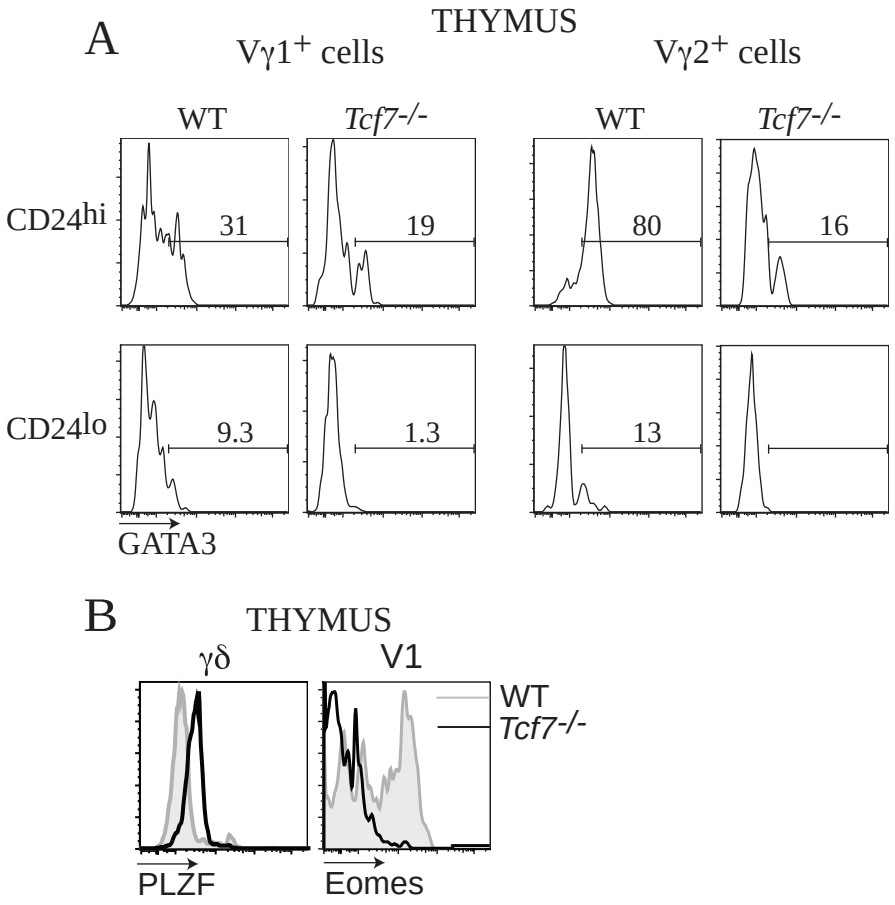
Overall, our results show that SOX13 and its target BLK are indispensable for the development of adult Tγδ17 cells. Further, SOX13 overexpression enhances the development of V4 Tγδ17 cells, but V4 cells are not absolutely dependent on SOX13 for development. Conversely, HES1, a target of Notch signaling, appears to be more critical for fetal versus adult Tγδ17 differentiation (180). Moreover, adult, but not fetal, LTi related effector ILCs are dependent on Notch2 for differentiation (250). These results show that distinct gene networks assume primacy in directing innate effector differentiation.

TCF1 is a negative regulator of Tγδ17 cell differentiation

TCF1 is expressed in all developing T cells and its role as a master regulator of αβ T cell development is well established. While αβ thymocyte number is reduced to 1-2% of normal in *Tcf7*$^{-/-}$ mice, the number of thymic γδ cells remained relatively unchanged in the absence of TCF1 (227). Interestingly, similar to *Sox13*, the expression of *Tcf7* was highest in the V2 cells. We had earlier shown that SOX13 can antagonize TCF1 function in γδ T cells. To determine if the relative activity of TCF1 impacts the development and function of γδ T cell subsets, we reassessed γδ T cell differentiation in *Tcf7*$^{-/-}$ mice. γδ T cell subsets in the thymus of *Tcf7*$^{-/-}$ mice exhibited a strikingly deregulated distribution and development. In particular, we observed that the effector programming of all γδ T cell subsets is extensively distorted in these mutant mice. For instance, GATA3 expression is significantly reduced in all immature adult γδ thymocyte subsets lacking TCF1 (Fig. 3.9A). This observation is consistent with the known function of TCF1 in

Figure 3.9. Deregulated γδ T cell effector differentiation in *Tcf7*$^{-/-}$ mice. (A) GATA3 expression in immature (CD24^{hi}) and mature (CD24^{lo}) Vγ1.1^{+} and Vγ2^{+} γδ thymocytes of WT (C57BL/6) and *Tcf7*$^{-/-}$ mice, determined by intranuclear staining analysis. (B) Expression of PLZF (left) in total γδ thymocytes and EOMES (right) in the splenic V1 gated cells of WT (grey filled) and *Tcf7*$^{-/-}$ (black line) mice

Figure 3.9


A
THYMUS
Vγ1+ cells
Vγ2+ cells
WT
Tcf7-/-
WT
Tcf7-/-
CD24hi
31
19
80
16
CD24lo
9.3
1.3
13
GATA3
B
THYMUS
γδ
V1
WT
Tcf7-/-
PLZF
Eomes

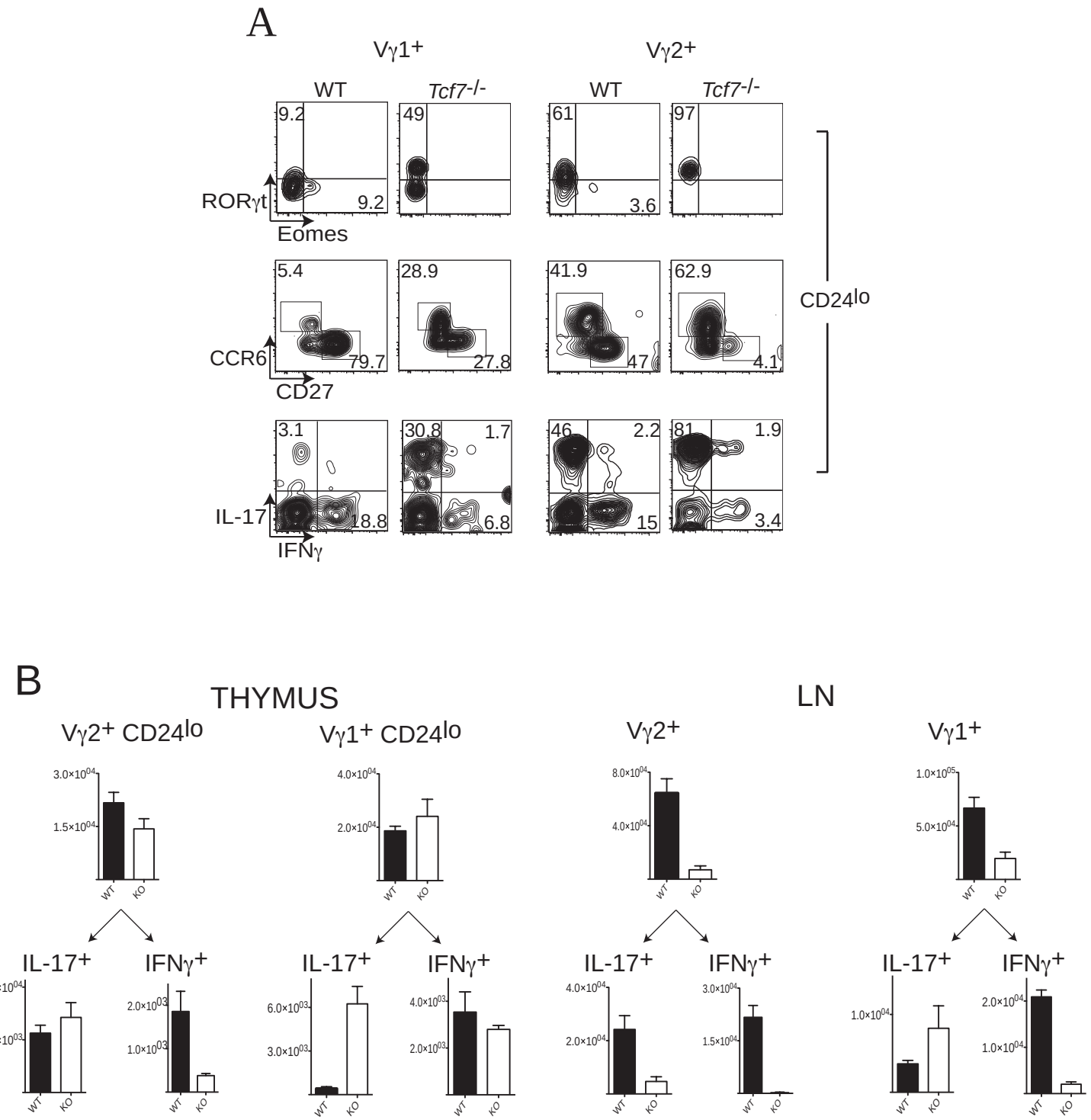
regulating GATA3 induction in the Th2 cell differentiation process and in T precursors (251), (231). The other uncommon feature among *Tcf7*$^{-/-}$ γδ T cell subsets is their higher expression of the TF PLZF (Fig.3.9B). PLZF expression is normally associated with V6 γδ cells (147). Interestingly, V6 cells are also present in increased proportions in these mice. Further, *Tcf7*$^{-/-}$ γδ T cells in the periphery are defective in the expression of EOMES and in their ability to produce IFNγ. The regulation of *Eomes* expression by TCF1 had been previously demonstrated in αβ lineage memory CD8 T cells (252) (Fig. 3.9B)

A major phenotype of *Tcf7*$^{-/-}$ γδ T cells is their dominant IL-17 production. We observed that the deficiency of *Tcf7* not only enhanced IL-17 production from V2 and V4 cells, but non-IL-17 producers such as V1 cells were capable of IL-17 production (Fig.3.10A, B). Overall, > 80% of all γδ T cells expressed IL-17, CCR6 and RORγt in the thymus and the periphery of these mice (Fig.3.10A). Importantly, we also observed that the absence of TCF1 in γδ T cells subsets reciprocally restricts differentiation to IFNγ^{+} γδ cells. One reason for this reduction could be the decreased expression of EOMES in the absence of *Tcf7*. These results are congruent with other recent studies that identified TCF1 as a negative regulator for IL-17 production in αβ Th17 cells, through its ability to bind and repress transcription from the *Il17a* promoter at the time of effector lineage programming (253).

The multiple defects in γδ T cell subset specification in *Tcf7*$^{-/-}$ mice demonstrate that gene networks in all γδ cell subsets are controlled by TCF1. Relatively high expression of both TCF1 and SOX13 in immature V2 cells (Fig.3.1) suggests that the

Figure 3.10. TCF1 negatively regulates Tγδ17 cell differentiation. (A) Enhanced generation of IL-17^{+} γδ effectors and a reciprocally diminished IFNγ^{+} cell differentiation and maintenance in *Tcf7*$^{-/-}$ mice. The differentiation of γδ effector T cells was examined by the analysis of intranuclear RORγt and EOMES and cell surface CCR6 and CD27 ex vivo, and the analysis of intracellular IL-17A and IFNγ after stimulation, in mature (CD24^{lo}) Vγ1^{+} and Vγ2^{+} cells isolated from isolated from thymus. (B) Numbers of mature γδ thymocyte and LN effectors were calculated. Among mature V2 thymocytes, the ratio of IL-17^{+}:IFNγ^{+} is skewed, with the biased ratio maintained in the LN, where a further loss of V2 cells is observed in *Tcf7*$^{-/-}$ mice. For mature Vγ1^{+} thymocytes there is a >10 fold increase in IL-17 producers, while the number of IFNγ^{+} cells is not altered. Overall, the numbers of γδ T cell subsets are dramatically reduced in the LN of *Tcf7*$^{-/-}$ mice. The peripheral Vγ1^{+} IFNγ producing cells are particularly dependent on TCF1. In contrast, there are more LN Vγ1^{+} cells making IL-17 than LN V2 cells in *Tcf7*$^{-/-}$ mice.

Figure 3.10


A
Vγ1+
Vγ2+
WT
Tcf7-/-
WT
Tcf7-/-
9.2
49
61
97
RORγt
9.2
3.6
Eomes
5.4
28.9
41.9
62.9
CCR6
79.7
27.8
47
4.1
CD27
CD24lo
3.1
30.8
1.7
46
2.2
81
1.9
IL-17
18.8
6.8
15
3.4
IFNγ
B
THYMUS
LN
Vγ2+ CD24lo
Vγ1+ CD24lo
Vγ2+
Vγ1+
3.0×10^04
1.5×10^04
4.0×10^04
2.0×10^04
8.0×10^04
4.0×10^04
1.0×10^05
5.0×10^04
WT
KO
IL-17+
IFNγ+
1.4×10^04
7.0×10^03
2.0×10^03
1.0×10^03
6.0×10^03
3.0×10^03
4.0×10^03
2.0×10^03
4.0×10^04
2.0×10^04
3.0×10^04
1.5×10^04
1.0×10^04
2.0×10^04
1.0×10^04

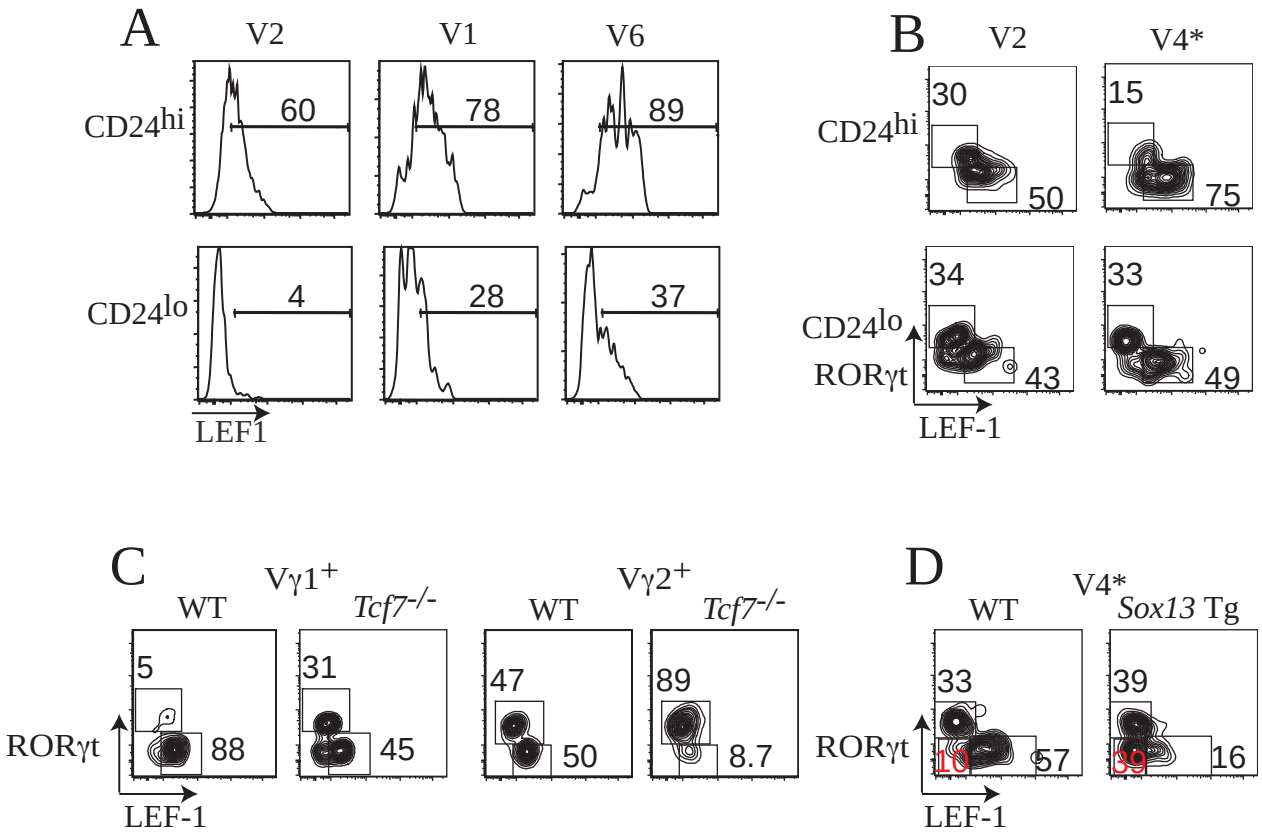
interaction between SOX13 and TCF1 is particularly critical for the development of Tγδ17 cells.

Mutually exclusive expression of LEF1 and RORγt

The HMG box TF LEF1 has been studied in conjunction with TCF1 for its independent and redundant functions in thymic T cell development. LEF1 and TCF1 act synergistically for the generation of αβ T cells, as the block in T cell development is most severe in *Tcf1*$^{-/-}$*Lef1*$^{-/-}$ double deficient mice. Similar to other HMG TFs, we observed *Lef1* to be highly expressed in immature γδ subsets (Fig. 3.1). Further, the flow-cytometric analysis illustrated relatively lower expression of LEF1 in immature and mature V2 cells as compared to the immature and mature V1 and V6 subsets, respectively (Fig. 3.11A). Interestingly, this expression pattern of *Lef1* in γδ T cell subsets is opposite to that of *Sox13* whose expression is highest in V2 cells and lowest in V6 cells. Indeed, we observed that LEF1 is not expressed in immature or mature RORγt^{+} V2 and V4 thymocytes. This mutually exclusive expression of LEF1 and RORγt (Fig.3.11B) is maintained in peripheral γδ T cells (Fig.3.11C). Further, there is a significant reduction in LEF1^{+} cells with a corresponding increase in RORγt^{+} cells among all γδ subsets in *Tcf7*$^{-/-}$ mice (Fig.3.11C). In *Sox13*Tg γδ T cells too, LEF1 expression is decreased overall (Fig.3.11D). Hence, it is possible that SOX13 interacts with LEF1 at the protein level (134) as well as inhibits *Lef1* transcription to promote Tγδ17 cell differentiation.

Figure 3.11. Mutually exclusive expression of LEF1 and RORγt in Tγδ17 cells. (A) LEF1 expression analyzed by intranuclear staining of thymocytes from C57BL/6 mice. LEF1 is highly expressed in immature γδ subsets with highest expression in V6 cells and lowest expression in V2 cells. (B) Intranuclear staining for RORγt and LEF1 in LN $V2^+$ (left) and $V4^+$ ($V1^-V2^-V6^-$) (right) T cells of WT mice shows mutually exclusive expression of the TFs. (C) RORγt and LEF1 expression in LN $V\gamma1.1^+$ (left) and $V\gamma2^+$ (right) T cells of WT and *Tcf7*$^{-/-}$ mice shows the loss of $LEF1^+$ γδ T cells when TCF1 is non-functional. (D) Similarly, RORγt and LEF1 expression in the LN of *Sox13*Tg^+ mice shows an increase in $ROR\gamma t^+$ V4 ($V1^-V2^-V6^-$) cells while $LEF1^+$ cells are significantly reduced.

Figure 3.11


A
V2
V1
V6
CD24hi
60
78
89
CD24lo
4
28
37
LEF1
B
V2
V4*
CD24hi
30
50
15
75
CD24lo
RORγt
34
43
33
49
LEF-1
C
Vγ1+
Vγ2+
WT
Tcf7-/-
WT
Tcf7-/-
RORγt
5
88
31
45
47
50
89
8.7
LEF-1
D
V4*
WT
Sox13 Tg
RORγt
33
10
57
39
39
16
LEF-1

Based on these results, we speculate that LEF1 may be a decisive repressor of IL-17 producing cells. A key difference between TCF1 and LEF1 expression pattern is that TCF1 is expressed in all γδ thymocytes, while apparently serving as a modulator of Il17 transcription, while LEF1 is only expressed in RORγt^{-} γδ cells. Preliminary results from our lab indicate that LEF1 is bound to a regulatory sequence associated with *Rorc* locus in cells that do not express RORγt, identifying LEF1 as a possible negative regulator of *Rorc* expression. Further verifications of LEF1 function await the generation of conditional *Lef1*-deficient mice. In the meantime, overexpression of LEF1-dominant negative (DN) protein in developing γδ T cells will help us further assess its importance in γδ T cell effector differentiation.

Distinct developmental origin of IL-17 producing V2 cells

Through our systematic analyses of γδ T cell subsets, we showed that developing immature thymic γδ subsets are marked with the gene expression profiles predictive of their eventual effector functions. Since γδ effectors are separable based on Vγ/Vδ TCR repertoire, it remains possible that distinct TCR signal strength of different γδ TCRs directs the γδ effector fate. Further, our earlier results had shown the SOX13 could induce *Tcr Vg2* transcription. Thus, it remained possible that the loss of V2 Tγδ17 cells in *Sox13*$^{-/-}$ mice is a result of impaired Vg2 TCR expression. Hence, to determine the role of γδTCR in determining the effector fate of V2 cells, we utilized mice that overexpress rearranged, functional *Tcrgv2* transgene under the endogenous *Vg2* promoter. Enforced Vγ2 TCR expression in all developing γδ thymocytes did not increase the differentiation

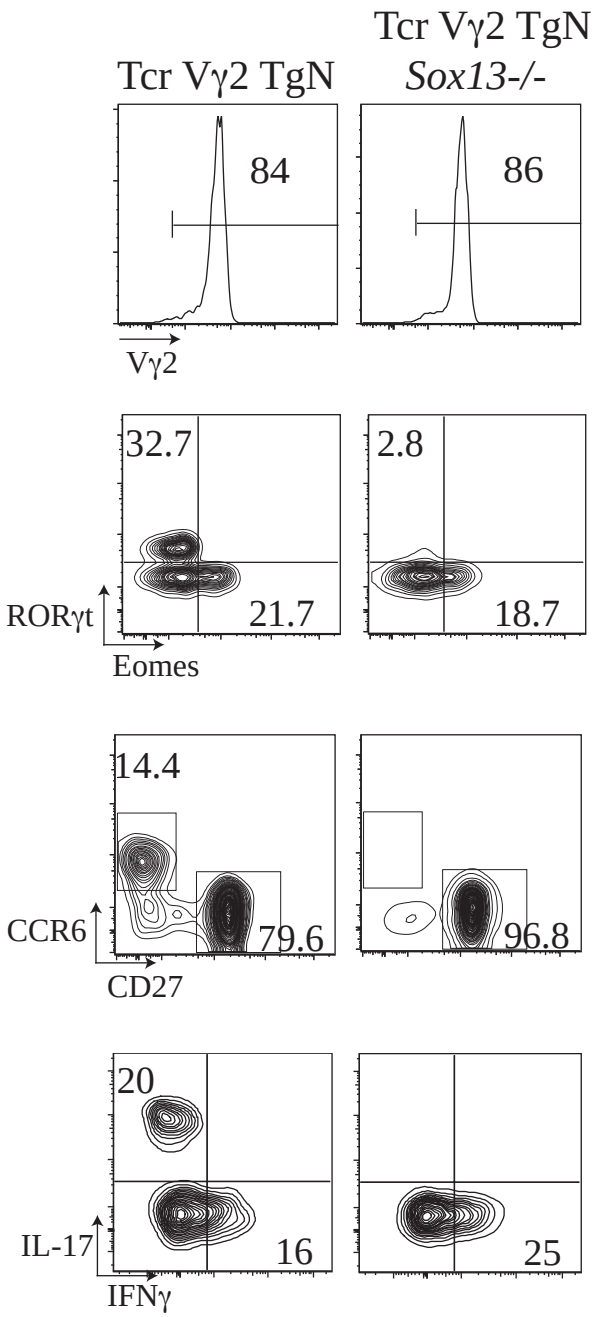
of V2 cells towards IL-17 or IFNγ expressing cells, which remained in the normal range in these mice (Fig.3.12), hence demonstrating that γδTCR type-specific signal by itself is not the primary determinant for γδ effector cell generation.

*Tcrgv2*Tg^{+} mice were also bred in the *Sox13*$^{-/-}$ background. If defective transcription of the *Vg2* gene is responsible for the loss of V2 cells in *Sox13*$^{-/-}$ mice, the provision of a rearranged *Vg2* gene should lead to normal production of V2 cells. Even when all γδ T cells expressed Vγ2 TCR in *Sox13*$^{-/-}$ mice, there was a complete absence of V2 Tγδ17 cells, indicating that the absence of SOX13-mediated promotion of *Tcr* gene transcription is not responsible for the block in Tγδ17 cell differentiation (Fig.3.12).

While not definitive, these results suggested that specific TCR signals are not likely to dictate γδ effector cell fate, at least for those programmed to produce IL-17. We then tested whether different T cell precursors possess distinct effector lineage potentials. The generation of γδ cell subsets is a developmentally ordered process. Hence, it was hypothesized that γδ cells arising early during step-wise progenitor maturation would possess a different effector fate than those developing from more differentiated precursors. To test this idea, we compared the developmental potential of the early c-Kit $^{+}$(CD117^{+}) progenitors (DN1 and DN2) with the late DN3 precursors, using the in vitro OP9-DL1 culture system. This in vitro culture system consists of the OP9 bone marrow stromal cell layer that ectopically expresses the Notch ligand delta-like 1 (OP9-DL1). T cell development into both αβ and γδ lineages is efficiently supported in this assay system, which recapitulates the in vivo thymic T cell development processes. We sorted and cultured c-Kit^{+} (DN1+DN2) and DN3 cells in the OP9-DL1 culture system for 5-12

Figure 3.12. TCR Vγ2 overexpression does not restore Tγδ17 cell development in *Sox13*$^{-/-}$ mice. The loss of V2 Tγδ17 cells cannot be prevented by the expression of a functional Vγ2-Jγ1-Cγ1 (*Tcrgv2*)$^{+}$ transgene in all developing thymocytes. LN cells from WT, *Sox13*$^{-/-}$, *Tcrgv2* transgenic (TgN, B6 x 129 F2) and *Tcrgv2*TgN*Sox13*$^{-/-}$ (B6 x 129 F2) mice were analyzed for the expression of intranuclear RORγt and EOMES, cell surface CCR6 and CD27 and intracellular IL-17A and IFNγ in gated LN V2 cells.

Figure 3.12


Tcr Vγ2 TgN
Tcr Vγ2 TgN
Sox13-/-
84
86
Vγ2
32.7
2.8
21.7
18.7
RORγt
Eomes
14.4
79.6
96.8
CCR6
CD27
20
16
25
IL-17
IFNγ

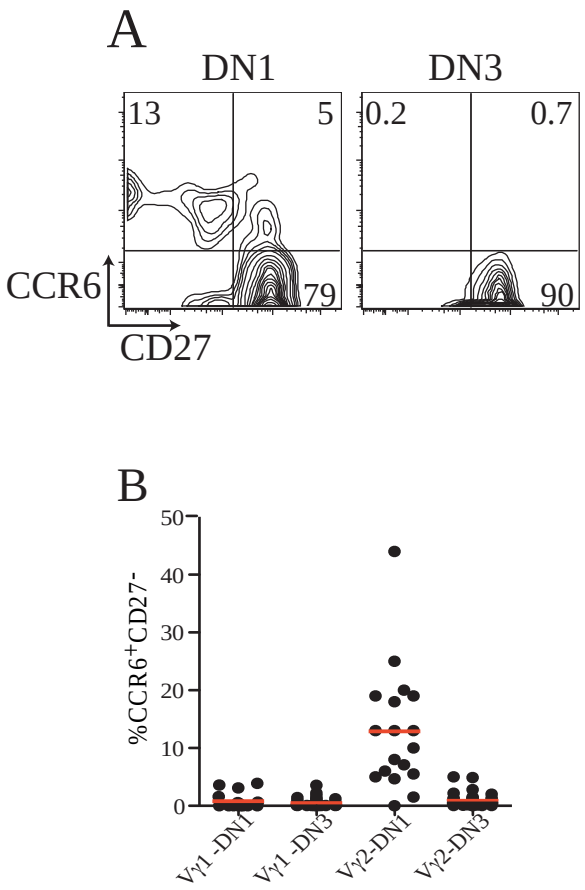
days. Subsequently we analyzed the effector potential of progeny V2 γδ T cells by their expression of CCR6 and CD27. These cell surface receptors faithfully mark the IL-17 and IFNγ producing effector cells respectively. $CCR6^{+}V2$ cells were generated predominantly from the early progenitors, but not the late precursors, which gave rise to only IFNγ expressing cells (Fig. 3.13A,B). These results favor the model that there exists a precursor cell intrinsic bias in γδ effector cell fate, with graded expression of *Sox13* (in 50% of single DN2 cells and very little in DN3 cells) (134), strongly influencing the cell fate decision process. Further, the established correlation of TCRγ/δ repertoire and effector function can then be accounted for by linking the temporally ordered rearrangement in V gene segments, with preferential earlier rearrangement of Vγ2-Cγ1, in $c\text{-}Kit^{+}$ precursors that are biased to produce Tγδ17 cells (254). A definitive demonstration of a developmental link among $c\text{-}Kit^{+}$ precursors, *Sox13* expression and Tγδ17 differentiation will necessitate tracking the developmental potential of $Sox13^{+}$ progenitors. A *Sox13* transcription reporter mouse model is currently in development in our lab.

Trans-conditioning through $TCRb^{+}$ cells does not limit the generation of Tγδ17 cells

Our results described so far illustrated that the intrinsic programming by the HMG-box TFs regulates the development of Tγδ17 cells. But, extrinsic cues originating from the thymic stroma and parenchymal cells as well as other thymocytes have also been proposed to be necessary for the normal maturation and acquistion of effector functions by γδ T cells. While WNT and Notch ligands provided in trans are highly relevant,

Figure 3.13. Distinct developmental origin of V2 Tγδ17 cells. (A) CCR6^{+} V2 cells develop exclusively from ckit^{+} thymic precursors. ckit^{+} (DN1 and DN2) and cKit^{-} DN3 subsets from *Tcrb*$^{-/-}$ mice were sorted and plated on OP9–DL1 monoayers at 1000-5000 cells per well respectively, in the presence of IL-7 and Flt3 ligands. CCR6 and CD27 expression on developing Vγ1^{+} and Vγ2^{+} γδ subsets was analyzed by flow-cytometry after separating the lymphocytes from stromal cells. (B) Frequency distribution of CCR6^{+} cells among Vγ1^{+} and Vγ2^{+} γδ cells, which developed from ckit^{+} (DN1+DN2) or DN3 precursor cells is shown. Around 18 DN1 wells and 30 DN3 wells were analyzed. Data are combined from 3 different experiments.

Figure 3.13

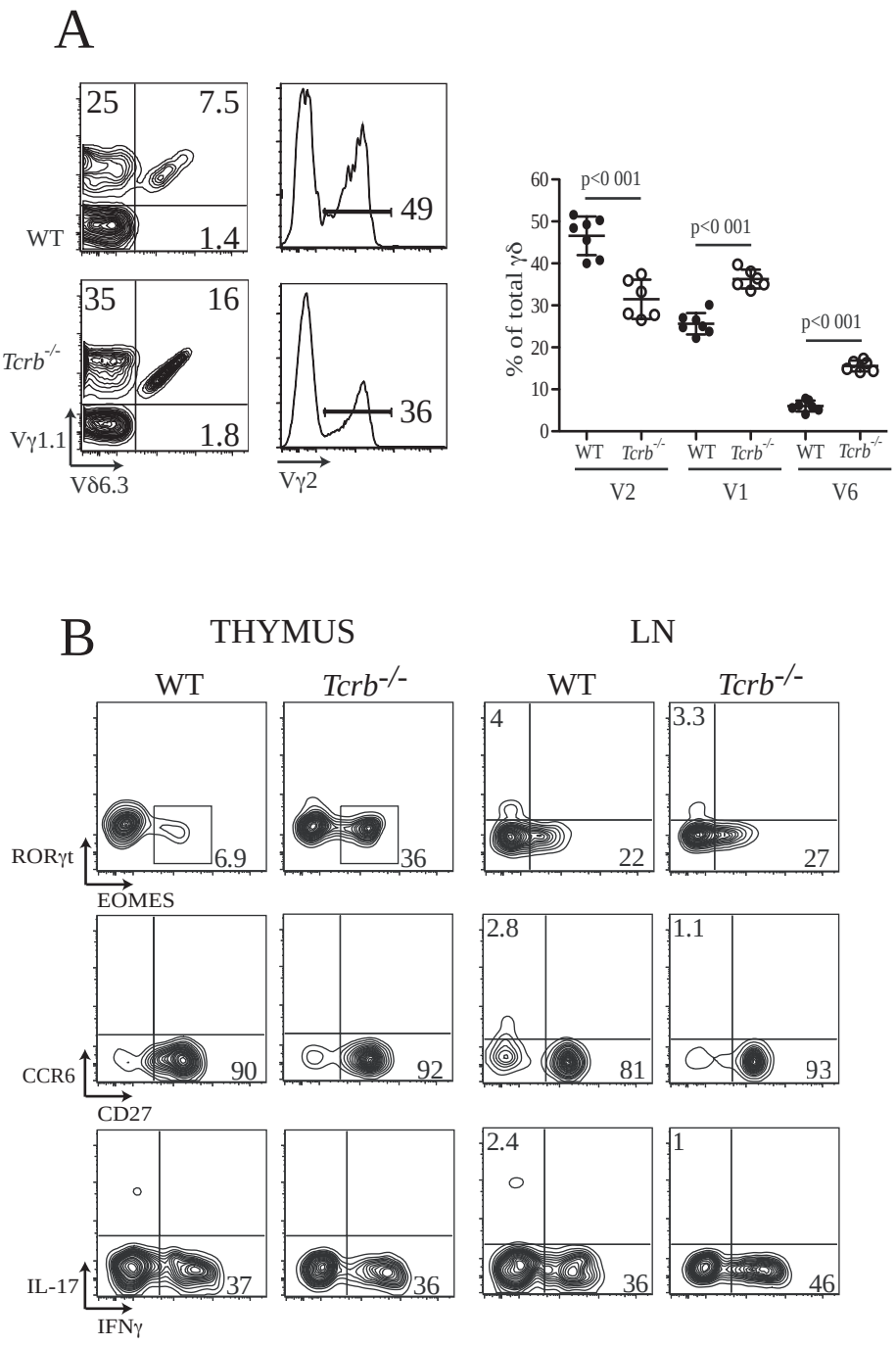

A
DN1
DN3
13
5
0.2
0.7
79
90
CCR6
CD27
B
%CCR6+CD27-
50
40
30
20
10
0
Vγ1-DN1
Vγ1-DN3
Vγ2-DN1
Vγ2-DN3

Hayday et al. also proposed that LTβ released from αβ DP thymocytes signals to γδ T cells and precursors to permit normal differentiation of γδ thymocytes into effectors. This cross lineage (αβ cells regulating γδ cells) trans conditioning has been a major theme in the field of γδ T cell development (255).

To determine the role of trans-condtioning in γδ T effector subset differentiation, we utilized *Tcrb*$^{-/-}$ mice which lack all DP cells. Initial studies that suggested the importance of αβ thymocytes for γδ cell differentiation emphasized that expression of a cluster of genes (the targets of trans conditioning) was silenced in γδ thymocytes from *Tcrb*$^{-/-}$ mice. We first noted that the proportions of different γδ subsets in *Tcrb*$^{-/-}$ mice was altered with a selective expansion of PLZF expressing V6 cells and a decrease in the frequency of V2 cells (Fig. 3.14A). Given that we now know that the gene expression profiles of γδ T cell subsets are highly distinct the differences in the relative proportions of γδ T cell subsets could potentially account for some of the differences in gene expression observed by the Hayday lab and others when only bulk γδ T cells from *Tcrb*$^{-/-}$ mice were compared against WT γδ T cells. When the gene expression profiles of each immature γδ cell subsets from *Tcrb*$^{-/-}$ mice were compared to their counterparts from WT mice, no overt differences in gene expression were observed (data not shown). Equally unexpectedly, the functional characterization of γδ T cells in these mice illustrated that the generation of IFNγ producing V1 cells was increased, not decreased (Fig. 3.14B). This was consistent with an increased expression of the EOMES in the V1 subset. (Fig.3.14B). Moreover, even V2 cells from *Tcrb*$^{-/-}$ mice misexpressed EOMES and were biased towards developing into IFNγ producers (Fig.3.15A,B). However, unlike an

Figure 3.14. Increased proportions of V1 IFNγ producing cells in the absence of trans-conditioning. (A left and right) Altered proportions of γδ subsets in *Tcrb-/-* mice, with an increase in V1 and V6 cells and a decrease in V2 cells. (B) An increase in the IFNγ producing V1 cells is observed in the *Tcrb*$^{-/-}$ mice. Differentiation of γδ effector T cells was examined by the expression analyses of intranuclear RORγt and EOMES, cell surface CCR6 and CD27 and intracellular IL-17A and IFNγ in mature (CD24^{lo}) Vγ1.1^{+} thymocytes and LN T cells. IL-17A and IFNγ production was measured after stimulation.

Figure 3.14

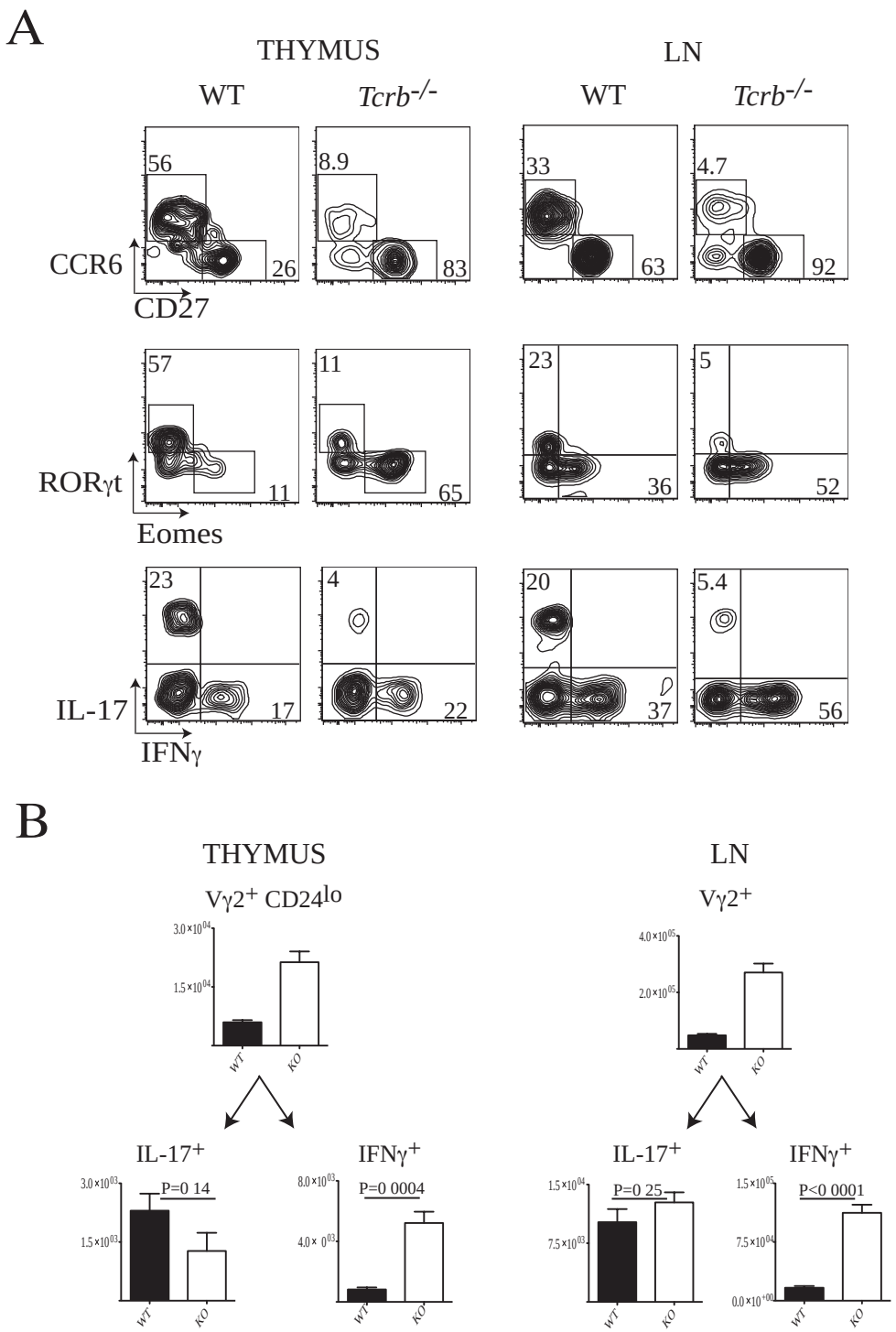

A
25
7.5
1.4
WT
49
35
16
1.8
Tcrb-/-
Vγ1.1
Vδ6.3
36
Vγ2
% of total γδ
p<0 001
p<0 001
p<0 001
WT
Tcrb-/-
V2
V1
V6
B
THYMUS
LN
WT
Tcrb-/-
RORγt
EOMES
6.9
36
4
22
3.3
27
CCR6
CD27
90
92
2.8
81
1.1
93
IL-17
IFNγ
37
36
2.4
36
1
46

increase in the proportion of IFNγ producing cells, we observed a significant reduction in the proportions of IL-17 producing V2 γδ T cells (Fig.3.15A). Consistent with the cytokine production pattern, we observed that the expression of RORγt was decreased in the *Tcrb*$^{-/-}$ mature V2 cells (Fig.3.15A). In fact, most of the genes necessary for IL-17 production were decreased in expression in mature V2 *Tcrb*$^{-/-}$ cells (data not shown).

Although the proportions of mature IL-17 producing cells were reduced in the thymus and the periphery of *Tcrb*$^{-/-}$ mice, the total numbers of IL-17^{+}V2 cells were not significantly altered relative to the WT mice (Fig.3.15B) (*Tcrb*$^{-/-}$ produce ~3 times the number of γδ T cells compared to WT mice). These results indicate that in the absence of αβ TCR thymocytes, no significant impairment in the generation of immature γδ thymocye subsets from precursors exists. Moreover, no significant block in the generation of γδ effector cells is observed in *Tcrb*$^{-/-}$ mice. There is a biased production of V2 cells that mature into IFNγ producers, while the size of IL-17 producing V2 cells is tightly maintained. Despite this relative normalcy, mature γδ thymocyte subsets appear to contain significant proportions of cells that do not fully differentiate. Whether this is a consequence of delayed or aborted differentiation is not established. Therefore, we conclude that the cross lineage trans conditioning is not a significant factor in generating distinct immature γδ cell subsets in the thymus. Rather, the development of Tγδ17 cells is intrinsically programmed by the HMG box family of TFs members, a process that appears to be independent of “instructive” TCR signaling and LTβ produced by αβ DP thymocytes.

Figure 3.15. Altered proportions of γδ effector cells in the absence of trans-conditioning. (A) Decreased proportions of IL-17^{+} V2 cells with a reciprocal increase in the frequency of IFNγ^{+} V2 cells is observed in *Tcrb*$^{-/-}$ mice. Differentiation of γδ effector T cells was examined by the analysis of intranuclear RORγt and EOMES and cell surface CCR6 and CD27 ex vivo, and the analysis of intracellular IL-17A and IFNγ after stimulation, in mature (CD24^{lo}) V2 thymocytes and LN T cells. (B) Total numbers of IL-17 producing V2 γδ T cells are similar in WT and *Tcrb*$^{-/-}$ mice. The number of IFNγ producers is significantly increased in the thymus and LNs of *Tcrb*$^{-/-}$ mice.

Figure 3.15


A
THYMUS
LN
WT
Tcrb-/-
WT
Tcrb-/-
56
8.9
33
4.7
26
83
63
92
CCR6
CD27
57
11
23
5
11
65
36
52
RORγt
Eomes
23
4
20
5.4
17
22
37
56
IL-17
IFNγ
B
THYMUS
LN
Vγ2+ CD24lo
Vγ2+
3.0×10^04
1.5×10^04
4.0×10^05
2.0×10^05
WT
KO
IL-17+
IFNγ+
IL-17+
IFNγ+
3.0×10^03
1.5×10^03
P=0 14
8.0×10^03
4.0× 0^03
P=0 0004
1.5×10^04
7.5×10^03
P=0 25
1.5×10^05
7.5×10^04
0.0×10^00
P<0 0001

Summary and future directions

Through extensive gene and protein expression analysis of γδ T cell subsets and by analyses of key mouse genetic models, we find that the HMG box TFs SOX13 and TCF1/LEF1 program the development of adult IL-17 Tγδ17 cells. We showed that SOX13 and TCF1 are positive and negative regulators of this pathway, respectively, and their previously shown interaction is likely to be the dominant parameter in Tγδ17 development. TCF1, a mediator of canonical WNT signaling pathway has been shown to function as a transcriptional repressor in the absence of β-catenin (256). It switches to an activator of transcritption in the presence of β-catenin. To understand the regulation of Tγδ17 cell differentiation downstream of TCF1 and SOX13, it will be important to define the relevance of WNT signaling for T effector cell differentiation. While Groucho has been identified as a key co-repressor associated with TCF1 in the absence of WNT signaling, very little is known about the mechanism of transcription suppression mediated by TCF1/LEF1 in T cells. Moreover, TCF1 can be an activator of transcription in the absence of WNT signaling, necessitating more detailed understanding of alternate transactivators that are present in TCF1/LEF1 complex.

We proposed that the absence of IL-17 producing cells in *Sox13*$^{-/-}$ mice is a consequence of the loss of SOX13-regulated BLK expression in immature V2 cells. How BLK signaling ensures normal Tγδ17 differentiation is unknown. BLK and SOX13 are expressed in early thymic progenitors, suggesting that signaling from BLK prior to TCR expression is potentially critical for Tγδ17 cell differentiation. Our future experiments will attempt to identify the unique BLK dependent biochemical properties of Tγδ17 cells.

It is important to note that there are likely other target genes of SOX13 whose function is absolutely required for Tγδ17 differentiation. Our lab has found that ETV5, a possible target gene of SOX13 highly expressed in immature V2 thymocytes, is also essential for Tγδ17 cell differentiation and/or maintence as mice lacking *Etv5* in T cells fail to generate Tγδ17 cells (Tal Shay et al. manuscript submitted). Thus, a network of genes downstream of SOX13 is required to promote Tγδ17 differentiation.

In support of the stochastic model of γδ T cell effector lineage development that predicts the existence of effector lineage biased precursors prior to TCR signaling we provided two main sets of data. First, we showed that when all immature γδ thymocytes express Vγ2 TCR the number of Tγδ17 cells generated was not altered compared to normal mice. Thus, specific TCR by itself cannot drive effector γδ cell subset differentiation. In other experiments performed in our lab we showed that the TEC kinase ITK critical for *Il17a* transcription downstream of TCR signaling in Th17 cells (73) is not required to generate Tγδ17 cells (data not shown). In addition, TCR signal activated IRF4 also necessary for Th17 cell differentiation by inducing *Rorc*, has been reported to be dispensable for Tγδ17 differentiation (159). Together, we suggest that while some form of TCR signaling is clearly necessary for γδ T cells to bypass developmental checkpoints, γδTCR signaling is unlikely to be the initial discriminator driving effector lineage diversification. Second, in support of the pre-programming model, we showed that V2 Tγδ17 cells arise exclusively from the early c-Kit^{+} progenitors, but not from the later T precursors in the OP9-DL1 culture system. While this result needs to be verified in an in vivo developmental assay, we propose that the development of different γδ effector cells

is governed by when and which *Tcrg* and *Tcrd* genes are functionally rearranged during T precursor cell differentiation. A temporal order that favors early *Vg2* gene rearrangement can account for the association of Vγ2 TCR with Tγδ17 effectors. It is useful to note that most of the TFs that constitute the immature V2 gene signature are expressed prior to TCR expression in c-Kit+ progenitors. There is no evidence to indicate that TCR signaling impacts *Sox13* or *Tcf7/Lef1* transcription (257), ruling out a potential direct link between TCR signaling and the TFs that are essential for Tγδ17 differentiation. As eluded earlier a definitive test of the SOX13-TCF1/LEF1 controlled Tγδ17 differentiation can occur with the successful generation of faithful *Sox13* reporter mice. Given that GALT LTi like IL-17 and IL-22 producing ILCs are generated without antigen receptor signaling we predict that variations in the gene network controlling Tγδ17 differentiation that is independent of TCR signaling are utilized to generate other innate effectors.

Materials and Methods

Mice: Sox13$^{-/-}$ mice maintained in 129/J background and *Lck-Cre Sox13*Tg^{+} mice in C57BL/6 background were generated previously in our lab (134). *Tcf7*$^{-/-}$ mice were a gift from Hans Clevers and *Tcrvg2* Tg^{+} mice were generated by Dr. Kang (258). *Tcrb*$^{-/-}$ and C57BL/6 mice (5-6 weeks old) were purchased from Jackson Labs. All experiments were approved by the UMMS IACUC (Worcester, MA).

Sample preparation for microarray analysis: Thymocytes were pooled from 5 week old C57BL/6 mice (Jackson Labs) and enriched for γδ T cells using Dynal depletion and MACS based selection. Depleted thymocytes were stained for cell surface markers and ~2-3 x 10^4 cells (>99% pure) were sorted directly into Trizol (Invitrogen) using a FACSAria cell sorter. For each population, independent triplicate samples were sorted, unless noted otherwise. RNA processing from sorted cells and microarray analysis using the Gene1.0 ST array (Affymetrix) was performed at the ImmGen processing center (SOP at ImmGen.org). The following abbreviations were used that correlate with the indicated ImmGen populations: ImmV2=immTγδ.vγ2^+.Th, ImmV1=immTγδ.vγ1^+vδ6^-.Th; ImmV6=immTγδ.vγ1^+vδ6^+.Th MatV2= matTγδ.vγ2^+.Th (sorted in duplicate); MatV1=matTγδ.vγ1^+vδ6^-Th, MatV6=matTγδ.vg1+vd6+.Th (sorted in duplicate);; Total γδ=Tγδ.Th; ImmV2.e17=immTγδ.vg2.e17.Th; ImmV3.e17=immTγδ.vg3.e17.Th; ImmV4.e17=immTγδ.vg4.e17.Th. Complete sorting details for each population can be found at Immgen.org. Approximate frequencies of TCRδ chains associated with sorted TCRγδ subsets are as follows: V2 (Vγ2^+, 50% Vδ4^+, 40% Vδ5^+, all Vδ6.3^-, ~45% of total γδ cells); V1 (Vγ1.1^+, diverse Vδs including 25% Vδ4^+, 15% Vδ5^+, others at lower frequencies, all Vδ6.3^-, 30% of total γδ cells); V6 (Vγ1.1^+, 100% Vd6.3^+, ~8% of total γδ cells); V5 (Vγ5^+, 40% Vδ5^+ and various others at lower frequencies, ~5% of total γδ cells); and fetal V3 and V4 thymocytes co-express Vδ1.

Data analysis and visualization: Data analysis was performed using GenePattern (Genepattern.org) analysis modules. Expression files were generated from raw

microarray CEL files using the ExpressionFileCreator module. Unannotated probe sets were removed and data were RMA normalized with quantile normalization and background correction. The ConsolidateProbeSets module (Scott Davis, Harvard Medical School, Boston, MA) was used to consolidate multiple probesets into a single mean probeset value for each gene. Identification of differentially regulated genes was performed using Multiplot. Unless otherwise indicated, genes were considered differentially regulated if they differed in expression by more than 2-fold, had a coefficient of variation (cv) among replicates of less than 0.5, had a p value of less than 0.05, and had a mean expression value (MEV) of greater than 120. Heatmaps were generated by hierarchical clustering (Hierarchical Clustering module) of data based on gene (row) and subset (column) using the Pearson correlation for distance measurement. Data were log transformed and clustered using pair-wise complete linkage. Data were row centered prior to visualization using the HeatmapViewer module. Data were log transformed, gene and subset normalized, and filtered for genes that had a MEV>120 prior to visualization. Pathway analysis was performed using Ingenuity software (Ingenuity.com) and by manual inspection. Some functional classifications were performed using AmiGO (Amigo.geneontology.org) and KEGG pathways (Genome.kp.kegg).

Flow cytometry: Antibodies (Ab) to the following cell surface markers and cytokines were purchased from BD Biosciences or eBioscience: CD3, CD4, CD8, CD25, CD44, cKit, Vγ2, Vδ6.3, CCR6, CD24, IL-7Rα, CD27, IL-17A, IFNγ, TNFα, streptavidin (SA)-

APC or -PE-Cy7. Vγ1.1 Ab was purified by Bio-XCell and biotinylated using the FluoReporter Mini-Biotin-XX Labeling Kit (Invitrogen). Intranuclear staining was performed using the FOXP3 staining kit (eBio) for the following Abs: BLK (Cell Signaling), EOMES (eBio), TCF1 and LEF1 (primary Abs- Cell Signaling, secondary Ab -Molecular probe), GATA3 (ebio), RORγt (eBio) and PLZF (Santa Cruz). All samples were acquired on a BD LSRII cytometer and data was analyzed using FlowJo (Treestar).

Ex vivo stimulation of lymphocytes: Freshly isolated thymic and peripheral LN cells were cultured ($2x10^6$/well) with PMA (10ng/ml) and Ionomycin (1μg/ml) for 4 hours at 37°C, with Golgi Stop and Golgi Plug (BD Biosciences) added after 1 hour, according to the manufacturer's protocols. After stimulation, cells were stained for cell surface markers and intracellular cytokine production using the Cytofix/Cytoperm kit (BD Biosciences).

Differentiation of γδ T cells from precursors: OP9-DL1 cells (a gift from Juan Carlos Zuniga-Pflucker, U. of Toronto) cells were plated in 96 well flat bottom plates prior to cell sorting. Thymocytes were pooled from ten (5 to 6 week old) *Tcrb*$^{-/-}$ mice. DN1 ($CD4^-CD8^-CD3^-CD25^-CD44^+cKit^+$), DN2 ($CD4^-CD8^-CD3^-CD25^+CD44^+cKit^+$) and DN3 ($CD4^-CD8^-CD3^-CD25^+CD44^-$) cells were sorted using a FACSAria onto confluent OP9-DL1 monolayer at 1000 and 5000 cells per well, respectively in 200μl of αMEM media containing 20% defined FBS (Gibco), PenStrep, 1ng/ml IL-7 (R&D Systems), and 5ng/ml Flt3L (R&D Systems). During culture, half the media was replaced every 3-4

days with fresh media containing cytokines. After 6 days of culture, cells were collected from wells and stained with Abs to analyze γδ T cell subset distribution and effector differentiation using flow cytometry. All data were acquired on a BD LSRII flow cytometer and analysis was performed using FlowJo (Treestar).

Statistical analysis: For the identification of differentially regulated genes, t-test p values were generated using Multiplot (Genepattern). Statistical analysis of flow cytometry data was performed using Prism (GraphPad Software). Data were tested for normality using F tests and then analyzed using unpaired two-tailed t-tests. Pathway analysis was performed using Ingenuity Pathway Analysis and statistical significance was determined using the program's built-in Fisher's exact test.

CHAPTER IV

GENERAL DISCUSSION

GENERAL DISCUSSION

The work presented in this thesis describes the distinct TF pathways that regulate the generation of Th17 and Tγδ17 cells. We focused on TFs that belong to two key morphogen pathways: SMAD2, a TGFβ signaling mediator, which we found to be essential for Th17 differentiation and SOX13-TCF1, WNT-HMG box TFs, which we showed as the central players for the thymic development of Tγδ17 cells.

Research in the past few years has shown that the unique combination of TGFβ with IL-6 is essential for the generation of proinflammatory Th17 cells (95). Importantly, TGFβ by itself can upregulate the expression of RORγt and FOXP3 in CD4 T cells and cells co-expressing both of these TFs have been visualized in the LP tissue sections of the murine small intestine (78). But, it is the presence of IL-6 that amplifies the proinflammatory IL-17 cell differentiation and simultaneously shuts down the programming towards the FOXP3 expressing regulatory T cell lineage. Furthermore, although RORγt is thought to be the central TF for IL-17 expression, it mediates the expression of *Il17a* and *Il17f* by not interacting at the promoter but at the CNS2 region of these genes (170). Unlike RORγt, STAT3 affects *Il17a* expression by directly binding to its promoter (98). Combined, these observations suggest that induction of *Il17* transcription requires parallel actions of both of these TFs, in complex with multiple chromatin modifiers and transcriptional activators, and mutual co-operation between TGFβ and IL-6 pathways.

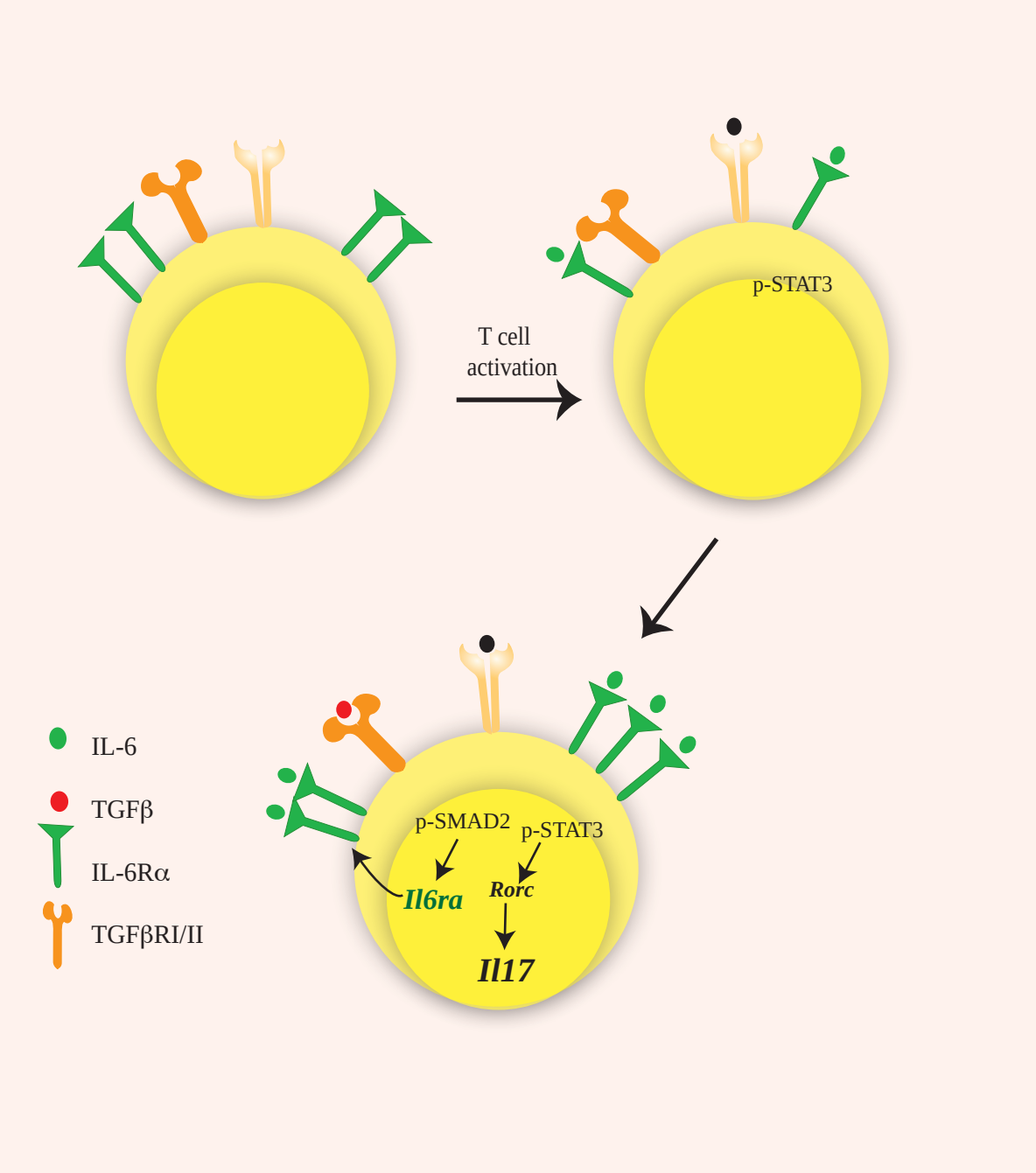
Intriguingly, activation of STAT3 occurs downstream of many other cytokines including IL-21 and IL-23. But, these cytokines cannot compensate for IL-6 in inducing Th17 cell differentiation. Thus, it seems that the kinetics and magnitude of STAT3 phosphorylation downstream of IL-6 is critical for inducing the expression of the Th17 cell cytokines and TFs. IL-6, however, restricts its own signaling by downregulating *Il6ra* expression and by upregulating inhibitory SOCS3 pathways (185), (93).

We showed that TGFβ signaling mediator SMAD2 is essential for maintaining STAT3 phosphorylation downstream of the IL-6 receptor pathway. *Smad2* CKO $CD4^+$ T cells do not efficiently differentiate into Th17 cells. Differentiation of SMAD2-deficient $CD4^+$ T cells was influenced by the concentrations of both TGFβ and IL-6 cytokines, suggesting a linked action of SMAD2 on both of these pathways. Our work illustrated that activated *Smad2* CKO CD4 T cells showed diminished expression of *Il6ra* as compared to their WT counterparts. Further, a reduction in STAT3 phosphorylation was observed when *Smad2* deficient CD4 T cells were stimulated with IL-6. Thus, we propose that lack of sustained STAT3 phosphorylation in the absence of SMAD2 affects T cell differentiation towards Th17 cell lineage (Fig. 4.1).

TGFβ affects diverse gene transcription pathways. Though we showed that *Smad2* deficient T cells have altered IL-6 signaling and that could be a potential reason for their reduced IL-17 production, SMAD2 could potentially affect Th17 cell differentiation by other mechanisms. The importance of SMAD2 in regulating Th17 cell differentiation has been supported by research from two other laboratories (259), (208). Chen Dong et al. showed that SMAD2 could interact with RORγt and enhance the

Figure 4.1. SMAD2 enhances STAT3 signaling to increase Th17 cell differentiation. A naïve T cell expresses high levels of IL-6R, which undergoes downregulation upon TCR activation and by autocrine IL-6 signaling. TGFβ in a SMAD2 dependent manner increases *Il6ra* expression and maintains STAT3 phosphorylation to maintain the positive loop of Th17 cell generation.

Figure 4.1


T cell
activation
p-STAT3
IL-6
TGFβ
IL-6Rα
TGFβRI/II
p-SMAD2
p-STAT3
Il6ra
Rorc
Il17

transcriptional function of RORγt. This is one possible mechanism by which SMAD2 increases Th17 cell differentiation, but further experimentation is required to show if this synergistic interaction happens at the *Il17* gene locus. The work from Yoshimura et al. (208) also showed a drastic reduction in Th17 cells in a different line of *Smad2* CKO mice generated in their laboratory. The CD4 T cells from these mice were extremely biased to produce IL-2 and IFNγ, hence they suggested that SMAD2 is essential to suppress Th1 differentiation and thereby indirectly promotes Th17 differentiation. CD4 T cells isolated from *Smad2* CKO mice maintained in our colony produce normal levels of IFNγ ex vivo and in Th1 differentiation conditions. This indicates that the increased Th1 skewing might not be the dominant mechanism for the decreased Th17 cell proportions in *Smad2* deficient mice. Cumulatively, studies so far have clearly illustrated the importance of SMAD2 in the differentiation of Th17 cells, but the underlying mechanisms of function are varied, likely reflecting diverse pathways under the control of TGFβ-SMAD2 signaling.

In addition to TGFβ, IL-1β and IL-6 together can also drive IL-17 expression in CD4 T cells. Initial studies showed that in IL-1β and IL-6 driven Th17 cell induction cultures, blocking TGFβ signaling abrogated IL-17 expression (79). This suggested that TGFβ released at low levels from T cells or TGFβ present in the serum in the culture media synergizes with IL-1β and IL-6 to support IL-17 expression. A recent study however challenged these observations and suggested that IL-1β with IL-6 and IL-23 can induce IL-17 transcription independent of TGFβ (80). These conflicting observations add to the complexity of Th17 cell differentiation pathway. Most cells secrete some

TGFβ in homeostatic or inflammatory settings. Hence, it is difficult to prove that in vivo Th17 cell differentiation occurs in the absence of TGFβ. However, IL-1β is probably equally important for inducing and enhancing Th17 cell differentiation, especially in an inflammatory environment. Therefore, further research is necessary to show if the Th17 cells present in the LP are distinct from than the ones induced in autoimmune disorders.

Similar to Th17 cells, IL-1R activation also stimulates IL-17 production from Tγδ17 cells (121). Tγδ17 cells in fact express many of the Th17 cell markers like IL-23R, TLR2, CCR6 and RORγt (3). However, unlike Th17 cells, the development of Tγδ17 cells occurs in the thymus where they acquire the expression of Th17 lineage markers. Upon migration to the peripheral tissues, the stimulation of their cytokine receptors IL-1R and IL-23R, without TCR engagement, leads to rapid IL-17 production. Thus, during the earliest inflammatory setting Tγδ17 cells are an innate source of IL-17.

Although we showed that SMAD2 is required for Th17 cell differentiation it is not essential for Tγδ17 cell differentiation. Furthermore, development of Tγδ17 cells does not require TFs IRF4 (TCR activated) and STAT3 (IL-6 activated) demonstrating that distinct TF networks guide the development of innate and adaptive IL-17 producing cells (180), (159). In contrast to *Smad2* CKO, proportions of Tγδ17 cells were decreased in *Smad3*$^{-/-}$ and *Tgfb*$^{-/-}$ mice suggesting that TGFβ could affect the generation or survival of Tγδ17 cells via a mechanism distinct from Th17 cells (110). However, both these models are associated with inflammatory disorders, raising the possibility that the decrease in Tγδ17 cells is environmentally driven.

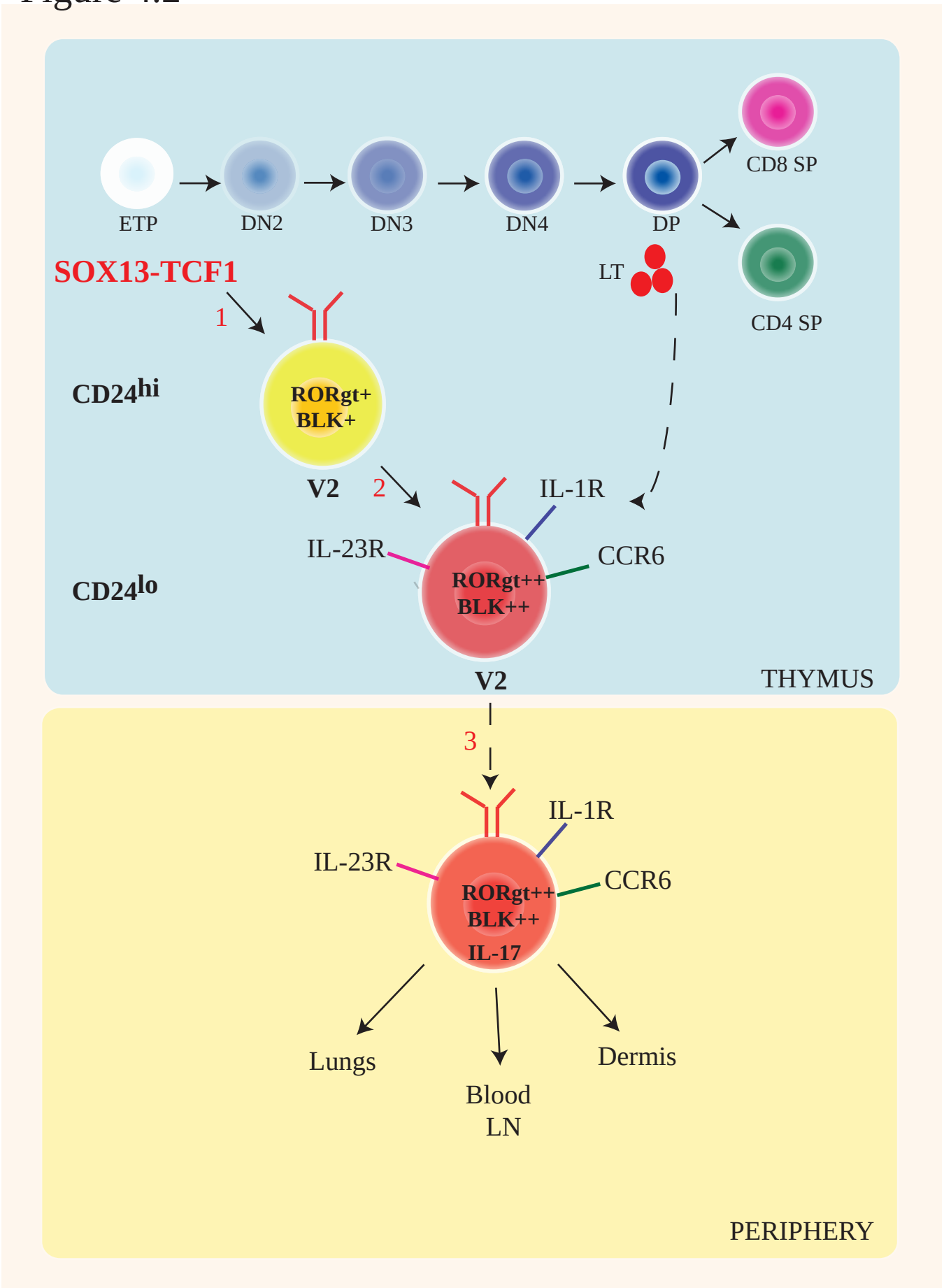
Instead of the components of TCR and cytokine receptor signaling HMG TF SOX13 programs the thymic development of Tγδ17 cells. Among all γδ subsets, V2 cells are the major IL-17 producing γδ T cells that are constantly produced by the adult murine thymus. SOX13 is expressed in all γδ subsets but its expression is highest in the immature V2 cells. Whether the quantity of SOX13 affects γδ effector cell differentiation remains to be determined. To answer this important question, we are generating *Sox13* reporter mice with the dual ECFP reporter-Cre knocked in to *Sox13* locus. These mice would potentially allow us to sort cells expressing different levels of Sox13 at the precursor level and track their differentiation in vitro (the OP9-DL1 stromal culture system) or in vivo (intrathymic injection assays). The ex vivo analysis of Tγδ17 cell markers (BLK and RORγt) in these discrete populations will test whether the intensity of SOX13 expression correlates with Tγδ17 programming.

To determine SOX13 regulated gene networks specifying the Tγδ17 fate we showed that the expression of known Tγδ17 cell associated signaling molecule BLK is substantially reduced in the *Sox13* deficient V2 cells. Moreover, we observed a significant loss of RORγt^{+}BLK^{+} cells immature V2 cells in the absence of SOX13. A previous study in our lab showed that ectopic *Sox13* expression in developing αβ T cells leads to *Blk* expression (134). Subsequently, Tγδ17 cell numbers were found to be severely reduced in the *Blk*$^{-/-}$ mice (156). Thus, SOX13 controls Tγδ17 cell development by regulating BLK expression (Fig.4.2). Interestingly, both SOX13 and BLK are expressed at intermediate levels in thymic DN1, DN2 and DN3 precursor cells, raising

Figure 4.2. Tγδ17 cell development is programmed in different stages in the thymus

Early thymic c-Kit$^+$ precursors that potentially express SOX13 and BLK give rise to immature V2 γδ cells that are biased to develop into Tγδ17 cells. At the immature stage itself the V2 cells express intermediate levels of RORγt and BLK. V2 cell maturation characterized by CD24 downregulation, is associated with an increase in the expression of intranuclear RORγt and BLK, chemokine receptor CCR6 and cytokine receptors for IL-1β and IL-23 among other markers. These mature, programmed cells subsequently migrate from the thymus and home to specific tissues in the periphery.

Figure 4.2


ETP
DN2
DN3
DN4
DP
CD8 SP
CD4 SP
LT
SOX13-TCF1
1
CD24hi
RORgt+
BLK+
V2
2
IL-1R
IL-23R
CCR6
CD24lo
RORgt++
BLK++
V2
THYMUS
3
IL-1R
IL-23R
CCR6
RORgt++
BLK++
IL-17
Lungs
Blood
LN
Dermis
PERIPHERY

the possibility that distinct signaling properties of Tγδ17 cells are set prior to TCR expression.

Recent experiments performed in our lab (K. Narayan) showed that the early cKit$^+$ (DN1+DN2) thymic precursors are biased to give rise to Tγδ17 cells in the in vitro OP9-DL1 cultures. Studies from other laboratories have also noted distinct γδ versus αβ thymocyte generative capacities of early vs. late T cell precursors (130). This cell intrinsic, developmental stage-specific bias in effector differentiation correlates with the higher expression of SOX13 and BLK in cKit$^+$ DN subsets, suggesting that SOX13 induction of BLK may be a key effector lineage commitment event. This hypothesis will be tested using the *Sox13* reporter mice described previously. Though we have identified that the regulation of BLK by SOX13 could affect the generation of Tγδ17 cells, the underlying BLK function in γδ T cells is not well understood. BLK in association with other Src family kinases, Lyn and Fyn, helps in the transition of pro-B cells to pre-B cells (260). Moreover, constitutive activity of BLK can bypass the requirement of BCR signaling (249). This suggests that its high expression in γδ T cells could potentially serve TCR independent regulation of antigenically naïve Tγδ17 cells. Identifying upstream activators of BLK and unique biochemical contributions of BLK to Tγδ17 differentiation will be essential for complete mapping of SOX13 regulated gene networks.

Given that many HMG TFs other than SOX13, TCF1 and LEF1 are expressed in developing T cells it was hypothesized that cell type-specific networks of these TFs constitute the primary determinant of cell lineage fate and function. For Tγδ17 differentiation SOX4 has been identified as the second essential HMG TF (N. Malhotra,

data not shown). Unlike SOX13, SOX4 expression is not restricted to γδ T cells and it is highly expressed in DN thymocytes, αβ lineage committed DP cells as well as in all γδ T cells. Our preliminary observations suggest that SOX4 affects the expression of *Rorc* by binding to its promoter (O. Cho). Further, the expression of RORγt is highly reduced in V2 cells of *Sox4* CKO mice (*CD2-Cre-Sox4*$^{fl/fl}$). Importantly, no significant impairment in the generation of Th17 cells was observed in *Sox4* CKO mice. Thus, SOX4 is likely be the primary regulator of *Rorc* expression for innate Tγδ17 cell programming. We propose that SOX4 and SOX13 act in a parallel manner, each with its own gene network, to program the development of Tγδ17 cells.

The effector functions of γδ T cells segregate with their germline encoded Vγ genes. The basis for this association is unclear. To address this issue, we bred *TcrVg2* transgenic mice in the WT or *Sox13* deficient background. We observed that the expression of the Vγ2 TCR by all γδ T cells did not lead to an increased generation of Tγδ17 cells, illustrating that the Vγ2 TCR-specific signals do not direct Tγδ17 cell differentiation. SOX13 was shown to enhance *TcrVg2* transcription and it was possible that the block in Tγδ17 differentiation in *Sox13*$^{-/-}$ mice was caused by a decrease in *Vg2* TCR expression. Tγδ17 cell development was not restored in the *TcrVg2* transgenic *Sox13*$^{-/-}$ mice, ruling out the possibility that altered TCR expression per se contributes to the loss of Tγδ17 development in the absence of SOX13. For Th17 generation TCR signal induced ITK-NFAT and IRF4 control *Il17* and *Rorc* transcription, respectively. Neither IRF4 nor ITK is necessary for Tγδ17 generation. Together, this data suggests that conventional TCR signaling is unlikely to specify Tγδ17 cell fate. Given our data that

Tγδ17 cell differentiation is strongly biased to be initiated from the earliest thymic progenitors we favor the model that the association of germline encoded γδTCR repertoire and effector function arises from developmental specific biases on the onset of specific *Tcrg* and *Tcrd* gene rearrangements. For instance, relatively early productive rearrangements of Vγ2 vs. Vγ1.1 locus would endow Tγδ17-biased progenitors with Vγ2 TCR, whereas a late assembly of Vγ1.1 TCR in more T cell lineage committed late precursors (DN3) would limit the production of $V\gamma1.1^{+}T\gamma\delta17$ cells. This developmentally staged rearrangement is built on the temporally ordered Vγ gene segment activation for rearrangement during fetal γδ T cell production. Thus, two developmental processes are proposed to establish the γδTCR repertoire-dependent γδ effector subset production in the thymus: cell intrinsic developmental stage dependent biases in effector lineage differentiation coupled to asynchronous V gene rearrangement in the *Tcrg/d* loci. Additional differentiation checkpoints such as SOX13 induced BLK or generic γδTCR signaling itself are predicted to further guide proper stepwise differentiation of effector-biased immature γδ thymocyte subsets.

Additional complexities in developmental requirements of Tγδ17 cells arise from the observation that fetal-derived Tγδ17 cells are programmed distinctly. Similar to V2 Tγδ17 cells, which originate in late fetal stages (E17 onwards) and continue to develop in adult thymus, V4 γδ T cells that predominantly develop in the fetal thymus (E15 onwards) also contain dedicated IL-17 producing subsets. Further, V4 cells persist in low numbers in the thymus and periphery throughout adulthood. By gene expression analysis, we observed that fetal V2 and V4 γδ T cells are virtually identical at their immature

developmental stages. However, while the absence of *Sox4* or *Sox13* resulted in a complete abrogation of development of V2 Tγδ17 cells, the V4 Tγδ17 cells analyzed in the adult mouse thymus and peripheral LNs of these gene mutant mice remained largely unaffected. Instead, HES1, a transcriptional repressor and Notch target gene has been shown to be important for fetal V4 Tγδ17, but not for IFNγ producing γδ effector, cell development (180). HES1 appears to also be playing a role in adult V2 Tγδ17 cell differentiation and/or maintenance. HES1 has been shown to be necessary for DN1 and DN2 differentiation but is dispensable for later maturation processes controlled by Notch (261). Notch signaling has been shown to induce TCF1 for T cell lineage specification and it is possible that HES1 is required in this event and in regulating other modulators of WNT signaling and/or TCF1 function (231). Notch and WNT pathways cross-regulate the expression of their target genes and *Hes1* expression can also be promoted by WNT signaling (240), which adds to the complexity of the developmental design of effector γδ T cells.

One of the reasons for this disparate effector programming could be that distinct hematopoietic precursors give rise to V2 and V4 γδ T cells. V3 and V4 γδ T cells constitute the first wave of fetal thymic γδ T cells. The precursors of these cells are considered to be fetal liver hematopoietic stem cells (HSCs) (141). Moreover, the development of these γδ T cells cannot be supported by adult thymic microenvironment or by repopulating adult thymic progenitors into fetal thymic lobes. V2 Tγδ17 cells originate from HSCs in the bone marrow.

We demonstrated that the SOX13 regulated positive loop for the generation of Tγδ17 cells is counterbalanced by TCF1 and LEF1. In Th17 cultures TCF1 has been shown to inhibit the expression of *Il17a* by binding to its promoter (253). Previously, we have shown that SOX13 interacts with TCF1 and LEF1 and can inhibit their transcriptional activities (134). We propose that a proper balance of TCF1/LEF1 with SOX13 is necessary for the generation of Tγδ17 cells. Despite being a repressor for IL-17 producing V2 cells, TCF1 is highly expressed in all immature V2 cells suggesting it is important for other facets of γδ T cell development. Consistent with this adult *Tcf7*$^{-/-}$ mice generate γδ thymocytes with deregulated γδTCR repertoire, with aberrant development of normally fetal restricted V3 cells and overt production of V6 cells. Overexpression of *Sox13* in *Tcf7*$^{-/-}$ mice leads to a complete block in γδ thymocyte differentiation, supporting a central function of SOX13 modulated TCF1 in γδ T cell development (K. Sylvia, unpublished data). Also, a recent publication showed that TCF1 is necessary for the maintenance of memory Th17 cells (262). Whether the strong expression of TCF1 in Tγδ17 cells helps in maintaining their terminally differentiated state is not known.

Importantly, TCF1 expression is not limited to T cells, as we found it to be highly expressed in the developing LTi cells and LTi-related RORγt^{+} ILCs generated in the gut and spleen that are capable of IL-17 and IL-22 production (N. Malhotra, data not shown). These ILCs originate from the fetal liver or BM derived CLPs (Lin^{-}Sca1^{lo}cKit^{int}IL-7R^{+}) in a Notch2-dependent manner and are critical for maintaining mucosal epithelial homeostasis and integrity during infections (250). The early LTi and ILC precursors

express α4β7 integrin and they subsequently upregulate the expression of RORγt (165). Our data shows that TCF1 is co-expressed in $\alpha4\beta7^{+}$RORγt^{-} and $\alpha4\beta7^{+}$RORγt^{+} ILC precursors but not by $\alpha4\beta7^{-}$RORγt^{+} mature ILCs, suggesting its requirement at early stages of ILC differentiation. In support of this possibility, we observed a drastic reduction in the proportions and numbers of $\alpha4\beta7^{+}$ ILC precursors in *Tcf7*$^{-/-}$ mice. This suggests that TCF1 is required for the development and/or maintenance of ILCs. Interestingly, the remaining ILCs in *Tcf7*$^{-/-}$ mice had a significantly higher expression of RORγt. Moreover, upon stimulation with the TLR2 ligand- Zymosan, these cells showed a much stronger capacity to secrete IL-17. While peripheral ILCs do not express SOX13, they express other HMG TFs SOX4 and TOX (Kang J and M. Coles, York University, personal communication), which are also expressed in the immature V2 thymocytes. *Tox*$^{-/-}$ mice have defective LTi development (263). We are currently generating appropriate *Sox4* CKO mice to assess its functions in ILC differentiation. Collectively, our results suggest that TCF1 is important not only for the development of T cells and T effectors, but also for and ILCs in peripheral tissues that do not express antigen receptors akin to T and B cells. Furthermore, we propose that TCF1 acts as a suppressor of IL-17 production in all lymphoid cells. Although there are multiple known inhibitors for Th17 cell differentiation, TCF1 is so far the only suppressor for IL-17 production from innate lymphocytes. Also, unlike *Tcf7*$^{-/-}$ CD4 T cells, which show an increased bias to convert to Th17 cells only when cultured with IL-17 inducing cytokines, the γδ T cells and the peripheral ILCs from *Tcf7*$^{-/-}$ mice show an extremely potent IL-17 production from subsets that normally do not produce IL-17, suggesting more prominent function of TCF1

in ILC effector programming. Given the central importance of HMG TF network in innate γδ effector cell programming we propose that a similar network, with distinct but related members, is responsible for programming LTi-related RORγt^{+} ILCs. If proven to be true, it will provide an answer to how the repertoire of innate effector function is established prior to foreign antigen recognition would be obtainable.

So far I have focused on cell intrinsic genetic programs that specify innate effector function. Given that γδ T cell development occurs in the presence of multiple interacting cell types, it is likely that the thymic environment plays an important role. The development of effector γδ T cells has also been proposed to be affected by the thymic microenvironment and intrathymic cross-lineage trans-conditioning. Hayday's lab proposed that Lymphotoxin (LT) released from αβ DP thymocytes activates the LTβR expressed on γδ T cells (255). This activation was suggested to be important for IFNγ production by γδ T cells. Further, recent studies concluded that LTβR signaling activated RelA and RelB in the classical NF-kB pathway are also essential for the development and/or maintenance of Tγδ17 (159). Using *Tcrb*$^{-/-}$ mice, which lack the DP cells and LT source from the cells, we and others (264) showed that the development of Tγδ17 cells is not affected in the absence of trans-conditioning but there is an increase in the IL-4 and IFNγ producing V6 cells and IFNγ producing V1 and V2 cells. Further, the proportions of γδ subsets are altered in the *Tcrb*$^{-/-}$ mice with an increase in the V6 cells and a reduction in the V2 cell compartment. Overall, we showed that global gene expression profiles of immature γδ cell subsets in *Tcrb*$^{-/-}$ mice are not different from their counterparts in WT mice and all functional γδ subsets can be generated in the absence of αβ cells. This result

is in conflict with data published from Hayday's and Silva-Santos labs. One reason for these different observations could be that their results were based on the analysis of total γδ cells, not γδ cell subsets (255), (151).

Thus, we propose that DP thymocytes do not impact γδ effector subset differentiation. While growth factors such as LT might be essential for maintaining the numbers of Tγδ17 cells their roles in the development are not firmly established. In addition, the nature of stromal cells and defined thymic niches required for different γδ effector subset differentiation is unknown, leaving open multiple research areas for further exploration

Il17 and *Rorc* genes are highly conserved in ontogeny. IL-17 homologs have been cloned from the nematode *Caenorhabditis elegans* and the mollusk *Crassostrea gigas* (265). Similarly RORγt has been cloned from vertebrates including rodents, primates and even zebra fish *Danio rerio* (266). The conservation of this cytokine and TF during evolution suggests they are indispensable for maintaining mucosal homeostasis and for development of the immune system. The pre-programmed innate γδ T cells and ILCs safeguard the epithelial lining of mucosal areas such as reproductive organs, oral cavity, respiratory tracts and gut associated lymphoid tissues. Not only do these cells and cytokines (IL-17 and IL-22) defend against pathogenic microbes, but also it is likely that they sample the microbiota to select the commensals and symbionts that form the intestinal milieu. The selected commensals would further help in the induction of IL-17 producing adaptive cells such as the microbe SFB, which helps in the differentiation of Th17 cells in the LP.

Thus, the cooperative network of innate and adaptive IL-17 producing lymphocytes shapes the development of immune system and maintenance of homeostasis at the mucosal sites. The supervision of the generation, functioning and maintenance of these cells is largely an output of the TFs of TGFβ and WNT morphogenic signaling pathways. Although all IL-17 producing cells are invariably linked to destructive inflammatory responses towards self or non-self, another perspective on their constant presence from fetal stages to adulthood is the potential existence of "physiological inflammation" that potentially requires release of IL-17 family cytokines. This low level inflammation might drive the pro-active innate γδ cells and ILCs and could help in their self-renewal. At the same time these innate and adaptive cells would keep the commensal association of the mucosal and gut microbiota in check, which if uncontrolled has the ability to invade and acquire pathogenic status as observed in immunodeficient hosts. Finally, although RORγt is recognized as an indispensable conserved TF in all IL-17 secreting lymphoid lineages, distinct morphogen pathways act in parallel or upstream of RORγt to control the IL-17 production from innate and adaptive lymphocyte populations. This division of labor employing distinct cell types for IL-17 production and their discrete regulation could be an outcome of the time of generation of these cell types: early emergence of the innate-like Tγδ17 cells and the ILCs vs. late origin of gut-flora dependent Th17 cells. Further, there is a requirement to yield epigenetically stable IL-17 producing innate cells versus plastic adaptive Th17 cells more tuned to inflammatory milieu and this branching in their distinct regulation could ensure a protection plan where at all times, some IL-17 is made availa

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