TRANSCRIPTIONAL REGULATION DURING INNATE T CELL DEVELOPMENT

by

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DEDICATION

This thesis is dedicated to my mother, who taught me to believe in myself from an early age and who not only talked to me about the value of hard work and perseverance, but lived by these principles her daily life.

ABSTRACT

The broad-complex tramtrack and bric-à-brac zinc finger transcriptional regulator (BTB-ZF), promyelocytic leukemia zinc finger (PLZF), was recently shown to control the development of the characteristic innate T cell phenotype and effector functions of NKT cells. Interestingly, the ectopic expression of PLZF was shown to push conventional T cells into an activated state that seems to be proinflammatory. The factors that control the normal expression of PLZF in lymphocytes are unknown. In this study, we show that PLZF expression is not restricted to NKT cells, but is also expressed by and functionally defines a subset of γδ T cells. These cells express the Vγ1Vδ6.3 TCR and are referred to as γδ NKT cells based on their expression of PLZF and capacity to simultaneously produce IFN-γ and IL-4. Importantly, the frequency these PLZF-expressing γδ T cells is controlled by TCR signal strength and expression of inhibitor of differentiation 3 (Id3). In an effort to uncover the transcriptional network that controls the development of the γδ NKT lineage, we undertook a candidate approach to evaluate genes that function in the Id3 pathway. We studied single- and double gene-deficient mice to determine the interrelation of different transcription factors. These studies led to the discovery that a combined deficiency of Id3 and RORγt results in a dramatic alteration of thymopoiesis. In these mice, the frequency of γδ- versus αβ T cells was reversed. γδ T cells were found to be the dominant lymphocyte, representing ~60% of cells. Furthermore, nearly all of the γδ T cells were now found to express PLZF and belong to the γδ NKT cell lineage. This dramatic phenotype appears to be T cell-specific. Analysis of thymic progenitors revealed major alterations in DN2 – DN4 stages of development, particularly at the β-selection checkpoint. Our findings uncover a fundamental regulatory pathway that favors the development of γδ NKT cells, while restricting the maturation of progenitor T cells into the αβ lineage.

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LIST OF ABBREVIATIONS

APC: Antigen Presenting Cell

APL: Acute promyelocytic leukemia

B lymphocyte: Bone marrow lymphocyte

BM: Bone Marrow

BTB-POZ-ZF: Broad complex Tramtrak Bric-à-brac or Poxvirus Zinc Finger

CLP: Common Lymphoid Progenitor

DC: Dendritic Cell

DETC: Dendritic Epidermal T Cell

DN: Double negative

DP: Double positive

DKO: *RORc*$^{-/-}$*Id3*$^{-/-}$ Double Knockout

Eomes: Eomesodermin

ETP: Early Thymic Progenitor

FTOC: Fetal Thymic Organ Culture

HD: Helper-Deficient

HIV: Human Immunodeficiency Virus

i-IEL: Intestinal Intraepithelial Lymphocyte

Id3: Inhibitor of Differentiation gene 3

Ig: Immunoglobulin

IGF1: Insulin Growth Factor 1

IL: Interleukin

ITK: Inducible T cell Kinase

KGF: Keratinocyte Growth Factor

KO: Knock out (referring to gene-deleted mice)

LRF: Leukemia/lymphoma Related Factor, *ZBTB7a*

LSK cells: Lineage-negative, Sca-1^{+}c-Kit^{+}

LTi cell: Lymphoid Tissue Inducer cell

MHC: Major Histocompatibility Complex

NHEJ: Non-Homologous End Joining

NK cell: Nature Killer cell

NKG2D: Natural Killer Group 2 Member D

NKT cell: Natural Killer T cell

OP9-DL1: OP9-delta like-1

PLZF: Promyelocytic Leukemia Zinc Finger, *ZBTB16*

PMA: Phorbol 12-Myristate 13-Acetate

RAG: Recombination activating genes

RARα: Retinoic Acid Receptor-α

RORγt: Receptor-related Orphan nuclear Receptor-γt

RSS: Recombination Signal Sequence

s-IEL: Skin Intraepithelial Lymphocyte

SAP: SLAM-Associated Protein

SLAM: Signaling Lymphocytic Activation Molecule

SLP-76: Src homology 2-domain-containing Leukocyte Phosphoprotein of 76 kDa

Sox13: SRY-related mobility group box transcription factor 13

T lymphocyte: Thymus lymphocyte

TCR: T Cell Receptor

TEA promoter: T Early α promoter

TGF-β: Transforming Growth Factor- β

TIL: Tumor Infiltrating Lymphocyte

TLR: Toll-Like Receptor

ThPOK: T helper POK, *ZBTB7b*

V(D)J recombination: Variable to Distal to Joining regions of antigen receptor

WT: wild-type

XLP: X-linked Lymphoproliferative Syndrome

INTRODUCTION

GENERAL PRINCIPLES OF THE IMMUNE SYSTEM

The immune system is composed of a variety of specialized cell types, proteins and organs that protect the host from infection by microbial and viral pathogens[1]. The skin is the physical barrier that prevents pathogens from entering the body. Mucus membranes are moist linings that cover all body passages that are exposed to the outside and function to trap pathogens for elimination. Pathogens that successfully breach the skin, and/or avoid being trapped in mucus membranes, often colonize host tissues and cause damage to the organism. During infection, cells of the immune system are the principle safeguards that operate to provide host immunity by destroying foreign pathogens.

The concept of immunity from disease dates back to 5th century Greece where Thucydides, a historian and author, famously wrote about his observation that individuals who already contracted the plague and recovered became 'immune' or protected from the disease. Several efforts to achieve protective immunity by means of inoculation were attempted throughout history dating back to 8th century India[2]. In 1796, Edward Jenner inoculated an 8 year-old boy, James Phipps, with matter from a cowpox lesion on the hand of Sarah Nelms, a young dairymaid. Although the boy became ill after the first inoculation, he was inoculated a second time six weeks later without producing any sign of disease. Jenner was the first to demonstrate a safe and successful vaccine and, as a result, is widely celebrated as the 'father of immunology'. In 1908, German scientist Paul Ehrlich won the Nobel Prize for his 'side-chain theory', which proposed that special receptors or 'side-chains' on the surface of cells function by attaching to food molecules in order to absorb nutrients for the cell. Ehrlich suggested that the cell produced an endless number of these receptors, each with a unique structure, which

functioned as a ‘lock’ while their targets that required a matching structure behaved as the ‘key’ in order to enter the cell. Ehrlich later suggested that this ‘lock-and-key’ concept would bind antigens of infectious agents and block their entry into the cell. He recognized the cell could not possess an inordinate number of these side-chains on their surface and therefore must be secreted by the cell. Ehrlich’s side-chain theory was the first to describe the principles of antibody-antigen recognition, which served as the foundation for acquired immunity.

In the 1930’s Jens Bing proposed a connection between antibody production and plasma cells, which was later found to be true in cleverly designed experiments by a graduate student named Astrid Fagraeus. During the 1950’s and 60’s, the molecular basis for antibody production was unknown. Building on Ehrlich’s ‘lock-in-key’ theory, in 1955 Danish immunologist Niels Jerne published a paper that postulated that all animals had the inherent capacity to produce a large amount of a diverse set of antibodies against antigen through a ‘selective’ process that occurred when antigen transported ‘selected’ globulins to plasma cells, which would then make identical copies of the globulins presented to them[3]. Jerne’s natural selection theory, which suggested antigen selects a pre-existing antibody repertoire, won him the Nobel Prize in 1984. Frank Burnet expanded on and improved upon Jerne’s theory in a paper published in 1957 that argued antigen bound by specific receptors on surface of lymphocytes instructed, rather than selected, cells to proliferate and differentiate into clones that produced antibodies with antigen specficity[4]. Brunet’s ‘clonal selection theory’ posits that a selection process occurs whereby self-tolerant lymphocytes genetically committed to the production of a unique antibody respond to antigen through clonal expansion, some cells of which are maintained over time to protect during subsequent infection by the same antigen[4]. Burnet won the Nobel Prize in 1960 for discovering the principle of immunological tolerance. In 1962, a landmark study led by James L. Gowans proved that B

lymphocytes could mount both cellular and humoral immune responses to specific antigens.

After George Snell and Baruj Benacerraf (among others) discovered the major histocompatibility complex (MHC), Rolf Zinkernagel and Peter Doherty demonstrated that T lymphocytes respond to non-self antigen in the context of MHC molecules in a paper published in 1974. Snell, Dausset, and Benecerraf were awarded the Nobel Prize for their studies in histocompatibility in 1980. Zinkernagel and Doherty won the Nobel Prize for establishing the principle of MHC restriction in cell-mediated immune responses. In the early 1980's, three groups using monoclonal antibodies or T cell-specific serum, discovered heterodimeric T cell receptor proteins on the surface of T cell clones grown in culture[5-7]. A couple of years later, Davis and Tonegawa isolated cDNAs encoding distinct T cell receptor (TCRs) genes belonging to both the $\alpha\beta$- and $\gamma\delta$ lineages[8-12].

During the late 1980's, immunologists focused their attention toward determining the precise mechanism of T cell activation. However, this was a scientific endeavor that Bretscher and Cohn were trying to tackle back in 1968[13]. While the details of their model were ultimately incorrect, Bretscher and Cohn proposed a two-signal model for lymphocyte activation[13]. Not until 1987 was it shown that T cells required a second signal, or co-stimulation in order to achieve full activation[14]. One of the confounding issues, however, was how did T cells distinguish 'self' from 'non-self'.

In his opening essay at the Cold Spring Harbor Symposium on Quantitative Biology in 1989, Charles Janeway, Jr. introduced one of the most profound theoretical concepts that addressed the quandary of self-versus non-self recognition of the immune system. Janeway pointed out that hapten-based approaches for antibody production only occurred in the presence of adjuvants, such as killed bacteria that he famously described as, "the immunologists' dirty little secret"[15]. He recognized that primitive

species did not have the necessary components of acquired immunity (e.g., MHC, TCRs, etc), but that higher vertebrates did posses components of a primitive, or 'innate' immune system. Simply, Janeway proposed that lymphocyte activation is controlled through the activation via this ancient pathway[15]. He reasoned that germ-line encoded receptors he called 'pattern recognition receptors' were expressed by antigen presenting cells (APCs) to provide the necessary stimulus required to initiate lymphocyte activity. Furthermore, Janeway predicted that these receptors would have evolved to only detect certain features commonly found on foreign, but not endogenous antigens[15]. It was through these receptors that Janeway suggested innate immune cells could distinguish 'infectious non-self' and 'non-infectious' self. Janeway's provocative idea of innate immunity was realized in 1997 when Rulsan Medzhitov, a postdoctoral fellow in his lab isolated and characterized the human homologue to the *Drosophila* Toll receptor, Toll-like receptor-4 (TLR4), which when stimulated could activate cellular properties necessary to initiate adaptive immune responses[16]. Soon thereafter, Beutler and colleagues showed that TLR4 specifically recognized LPS (lipopolysaccharide), a major component of the outer membrane of Gram-negative bacteria[17]. Medzhitov and Janeway's discovery of TLRs and the role innate cells play in triggering adaptive immunity represented a paradigm shift in our understanding of immune cell function in all metazoans.

Innate Immunity: the shock and awe of the Marine unit of the immune system

Innate immune cells, which rapidly respond to infection without prior antigenic exposure, are often considered a 'first-line-of-defense' against pathogenic microorganisms[18]. These cells express a myriad of receptor families, however, the lack of diversity in each of these receptors restricts antigen recognition to general features

commonly shared among many pathogens (PAMPS; pathogen associated molecular patterns)[18]. Despite the inherent capacity of these cells to respond with fluid transience, they are short-lived cells and do not confer life-long immunity[18]. The primary function of innate immunity is to detect and respond to pathogens by producing pro-inflammatory cytokines and chemokines that cause fundamental changes within the microenvironment around infected tissues, and to mobilize other cells of the immune system. Myeloid-derived neutrophils, eosinophils, basophils, macrophages/monocytes, mast cells, dendritic cells (DCs), and lymphoid-derived natural killer (NK) cells altogether form the innate arm of the immune system. Innate cells including, but not limited, to macrophages, neutrophils, and DCs are archetypal examples of phagocytes that consume harmful pathogens, foreign particles, and dead or dying cells in the bloodstream and tissues. During the immune response, specialized phagocytes such as DCs function as professional antigen presenting cells (APCs) that display pathogenic peptides in the context of MHC I or II on the cell surface for T cell recognition[1,19]. Antigen presentation by APCs is essential for peripheral T cell activation. In addition to peptide:MHC molecules, T cell activation requires costimulation signals which are achieved through interaction with CD40 and B7-2 molecules expressed on the surface of APCs. Thus, innate cells have a fundamental role in the initiation of adaptive immune responses, which demonstrate a vital interdependence among various cells of the innate and adaptive immune system in order to clear infectious agents.

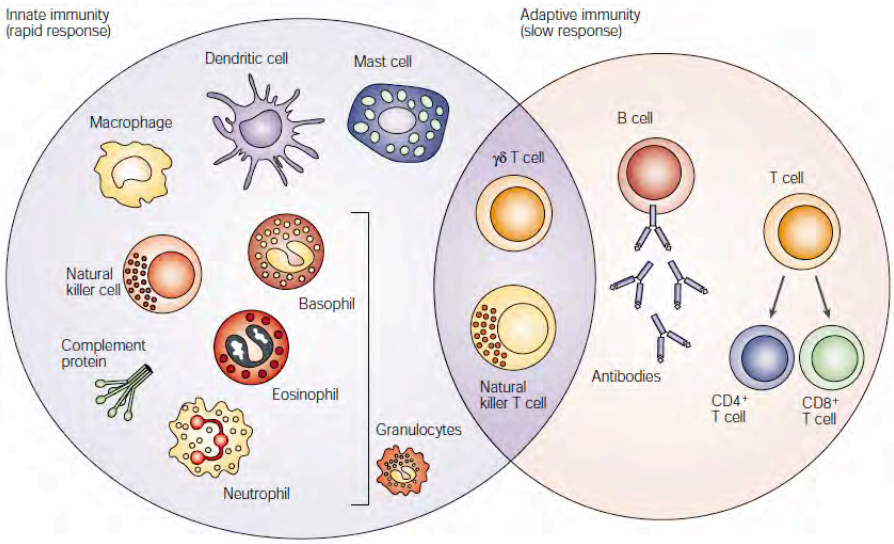
Adaptive Immunity: the precision and tactical prowess of the Navy SEALs of the immune system

Adaptive immunity evolved in jawed vertebrates (gnathostomes)[20,21]. In contrast to the innate immune system, cells of the adaptive immune system have developed a more versatile mode for the detection and elimination of pathogens. Through a process

of somatic recombination, highly specialized B (bone) and T (thymus) lymphocytes can generate almost a limitless number of antigen receptors from a small number of genes (Figure 1)[20,21]. Indeed, the theoretical B cell receptor repertoire is approximately 10^{11} while it is as high as 10^{15} for T cells[12,22]. Upon encountering antigen, naïve B and T cells take several days to weeks to differentiate into effector cells capable of participating in the immune response. Thus, the nature of adaptive immunity is highly specific but slow compared to the innate immune system. Once an infection is cleared, the bulk of the innate and adaptive immune cells die. However, a small but distinct population of lymphocytes will persist for the life of the host. This immunological memory provides a way for cells that have previously encountered antigen to rapidly respond to a secondary exposure to a particular pathogen[23]. Immunological memory is not only critical for long-term protective immunity, but also is the fundamental principle behind the development of vaccines against viral pathogens. Collectively, the highly specialized cells of the innate and adaptive immune system operate in an interdependent manner and have overlapping but distinct roles in controlling infection, allergy, cancer and metabolism[24].

Innate T cells: bridging innate and adaptive immunity

In contrast to the conventional T cell lineage, both natural killer T (NKT) and γδ T cells represent a minority of specialized T lymphocytes that exhibit several phenotypic and functional features/properties typically associated with cells of the innate immune system[25,26]. These 'innate-like' T cells express a restricted TCR repertoire, constitutively express activation markers such as CD44 and CD69, and rapidly produce a multitude of effector cytokines upon primary activation. Both NKT and γδ T cells have the capacity to lyse target cells, however, it is clear that their robust effector functions play a significant role in galvanizing a variety cell types during infection, allergy, autoimmunity, and



Dranoff, G. (2004) *Nat. Rev. Immunol.* 4, 11-22

Figure 1. The innate and adaptive immune system. Innate cells are comprised of mostly myeloid cells, except for NK cells that are from the lymphoid lineage. Innate cells rapidly respond to antigen, but do so through a restricted repertoire of receptors that recognize common structures commonly found among pathogens. Adaptive immune cells are of the lymphoid lineage that can rearrange antigen receptors to produce a nearly unlimited repertoire and form long-term memory cells. Adaptive immunity often takes days to weeks before full participation in the immune response. γδ- and NKT cells are innate T cells that are T lymphocytes that share many features with innate immune cells, including but not limited to Toll-like receptors, NK1.1, NKG2D, and produce a multitude of cytokines upon primary activation. Innate T cells are commonly thought to bridge the innate and adaptive immune response – figure adapted from G. Dranoff[27].

cancer[25,26]. Based on the phenotypic and functional properties of both NKT and γδ T cells, it is commonly perceived that these unique cells operate at the interface of the innate and adaptive immune system (Figure 1).

ANTIGEN RECEPTOR REARRANGEMENT

Perhaps one of the most daunting undertakings in biological research during the early 1970's, before the advent of molecular cloning, was to illuminate the genetic mechanism behind antibody diversity. A theory by Dreyer and Bennett published in 1965 hypothesized that the generation of antigen receptors occurred through somatic DNA rearrangement[28]. However, at that time a fundamental dogma in biology held that genomic DNA remained unchanged throughout ontogeny. In 1976, Susumu Tonegawa challenged this dogma by showing that through restriction enzyme analysis of genomic DNA from mouse embryos and a mouse plasmacytoma cell line, large pieces of DNA between two flanking regions were missing in the plasmacytoma cells, but not in mouse embryos[29]. This was the first genetic evidence that cells do in fact undergo somatic rearrangement, which illuminated a mechanism by which enormous antibody diversity could be achieved from a single gene[29]. This seminal discovery by Tonegawa provided the basic template for V(D)J recombination at antigen receptor loci that won him the Noble Prize in 1987. While much progress has been made in understanding the basic mechanism of antigen receptor rearrangement, it is unclear what factors control V(D)J recombination and how many of these factors are similar or different between B and T cells.

V-to-the-D-to-the-J: antigen receptors and how lymphocytes make them

Ultimately, each lymphocyte expresses a unique surface receptor with specificity against a given antigen. B and T cells make up the two major classes of lymphocytes in the adaptive arm of the immune system[1,12]. Beginning at birth and throughout the life of the animal, B and T cells will randomly produce a myriad of rearranged immunoglobulins (Igs) and TCRs, respectively[8-12,22,29]. The generation of functional Igs and TCRs depends on extensive germline rearrangement of multiple somatic genes that normally exist in a non-functional state[29]. These antigen receptor genes are arranged in a 5'-3' configuration and consist of the variable (V), diversity (D), and joining (J) gene segements[12,22,29]. During antigen receptor rearrangement, the recombination activating gene (RAG) recombinases, which targets recombination signal sequences (RSSs) that exist at the boundaries of each V, D, and J gene segment, creates double strand breaks that are repaired by DNA repair enzymes in the non-homologous end joining (NHEJ) repair pathway[30-32]. Through V(D)J recombination, antigen receptors are assembled in each developing lymphocyte from gene segments that can range from a few kilobases to several megabases[33]. While the generation of antigen receptors equips B and T lymphocytes with the means to detect foreign antigens with enormous specificity, the precise mechanism that controls V(D)J recombination is unclear. Some pathways involved in spatial and temporal regulation of antigen receptor gene recombination have been identified. Notably, the interleukin (IL)-7/IL-7R pathway, for example, is required for the accessibility and recombination of TCRγ early in thymocyte development[34-38]. Biochemical and genetic evidence indicates IL-7 signaling activates STAT5, allowing its translocation into the nucleus for transcription of a variety of TCR-Vγ genes in mice[37,38].

Transcription factors are the gate-keepers of V(D)J recombination

The transcriptional activity of the E proteins E2A and HEB are indispensible for several aspects of T cell development, including V(D)J recombination[39-46]. E2A is also required for Ig rearrangement and is necessary in the early stages of B cell commitment[47,48]. Transcription through the TCR locus is required for access of RSSs by the RAG proteins to initiate V(D)J recombination[49-51]. Consequently, the introduction of mutations or deletion of promoter/enhancer elements from the antigen receptor locus causes a block in recombination[49-54]. A number of transcription factors have been implicated in the recruitment of chromatin remodeling complexes and recombination at various TCR loci, including E2A, HEB, c-Myb, CBF/PEBP2, Runx-1, and GATA-3[42,45,55-59]. Since accessibility of TCR genes requires changes in chromatin structure and the assembly of transcriptional complexes, clarification of the signaling pathways that control these activities should yield crucial details outlining specific transcriptional networks that control antigen receptor recombination in developing lymphocytes.

THYMOCYTE DEVELOPMENT

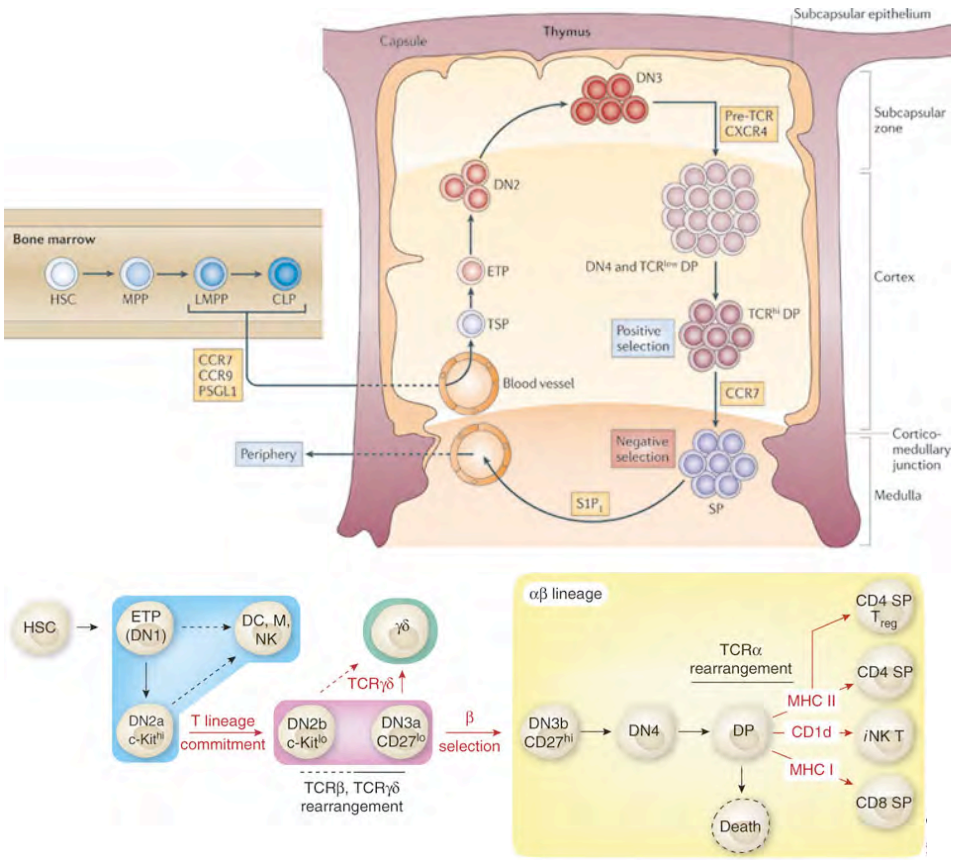
In mice, T cell precursors have been isolated from fetal livers as early day 11 during gestation. Progenitor T cells somehow 'know' how to find the thymus, which is a specialized organ dedicated to promoting the development of T cells. Once in the thymus, these progenitor cells receive signals that launch a complex network of regulatory circuits involving a large number of transcription factors that 'prime' these progenitors towards the T cell lineage. At a defined stage of development, these progenitors become irreversibly committed and receive additional signals that induce the rearrangement of $\alpha\beta$- or $\gamma\delta$ TCR genes, ultimately giving rise to two distinct T lymphocytes.

Conventional T cell commitment and selection

Multipotent progenitor T cells migrate from the bone marrow to the thymus and give rise to mature T cells that express an αβ- or γδ TCR. These early thymic progenitors (ETPs) do not express lineage markers typically found on mature cells, but do express several stem cells markers including c-Kit, CCR7, CCR9, and Sca-1[60-65]. Once ETPs colonize the thymus, they traverse through four general stages of T cell development that demarcate the transition of these immature uncommitted progenitors through T cell lineage commitment, and differentiation into mature functionally competent T cells[63-65]. These four stages of development precede the expression of CD4 and CD8 co-receptors and are termed double negative 1 – 4 (Figure 2)[63].

The most immature cells, ETPs or DN1 cells, are broadly identified by c-Kit^{hi}CD44^{+}CD25^{-}IL7R^{neg} expression[61,62,66-68]. While ETPs mostly give rise to T cells, these uncommitted cells retain the capacity to generate NK cells and myeloid cells such as DCs and thymic macrophages[69-73]. ETP/DN1 cells will migrate from the cortico-medullary junction to the cortex where they differentiate into DN2 cells[74]. DN2 cells rapidly upregulate IL-2 receptor-α (CD25) expression and can be subdivided into DN2a (c-Kit^{hi}CD44^{+}CD25^{+}IL7R^{hi}) and DN2b (c-Kit^{lo}CD44^{+}CD25^{+}IL7R^{hi}) based on the level of c-Kit expression[63,64,75-77]. Several lines of evidence indicate that DN2a cells remain uncommitted and can give rise to NK and myeloid cells similar to ETPs[69-73]. However, DN2b cells are committed to the T cell lineage, as these cells rapidly initiate TCR δ-, γ-, and β gene rearrangement, express high levels of Notch and become dependent on Notch signaling for survival and initiation of a T cell-specific gene expression program[78-84]. DN2b cells also express the highest levels of IL-7 receptor, which is absolutely necessary for TCRγ rearrangement and is also important survival[85,86]. At the DN3 stage of development, commitment to either the γδ- or αβ T cell lineage occurs upon

productive γ- or β-chain rearrangement. The DN3 stage of development can also be subdivided into two important checkpoints. DN3a cells (c-Kit$^{lo/neg}$CD44^{+}CD25$^{lo/neg}$IL7R^{lo}CD27^{-}) lack the tumor necrosis receptor CD27 and it appears that the bulk of γδ T cells, which by in large lack CD4 or CD8 co-receptors, are produced at this stage[78,87,88]. β-selected DN3a cells transition to DN3b as indicated by expression of CD27 (c-Kit$^{lo/neg}$CD44^{+}CD25$^{lo/neg}$IL7R^{lo}CD27^{+})[78,87]. The β-selection checkpoint ensures that a productive TCRβ chain pairs with pre-TCR (pre-TCRα; pTα paired with TCRβ/CD3 complex) on the cell surface, which results in a signaling cascade that leads to enormous proliferation and transition to DN4 (c-kit^{-}CD44^{-}CD25^{-}IL7R^{lo}CD27$^{+/lo}$)[78,82,84,87,89-91]. As DN3b cells evolve into DN4 cells, they move from the subscapular region back towardsthe cortex where they begin to express CD4 and CD8 co-receptors and develop into double positive (DP) thymocytes[92]. DP thymocytes that successfully rearrange TCRα will replace pre-TCRs with a clonotypic αβ TCR. Further, αβ T cell development requires productive interactions between the TCR and self-peptide:MHC (pMHC), primarily expressed on thymic stromal cells[93-99]. If appropriate signaling thresholds are generated from TCRs following interaction with self-peptide, then these cells undergo positive selection and differentiation into mature CD4 or CD8 single-positive T cells[97,99-104]. T cells that express TCRs that recognize pMHC I will downregulate CD4 and become cytotoxic CD8 T cells, while those that recognize pMHC II will downregulate CD8 and become helper CD4 T cells[97,99,101-105]. Signals emanating from TCR:pMHC interactions that are above, or below a particular threshold will die by negative selection or by neglect, respectively[97,100,106-108]. Between 95-98% of DP thymocytes will fail positive selection and die. This complex differentiation and selection process ensures that progenitor cells successfully produce a functional repertoire of self-tolerant T cells capable of combating foreign pathogens.



(A) Love, P.E., et al. (2011) *Nat. Rev. Immunol.* 11, 469-77, (B) Carpenter, A.C., et al. (2010) *Nat. Immunol.* 11, 666-73.

Figure 2. ETPs and the stages of thymocyte development. (A) Multipotent T progentior cell migration from the bloodstream into the thymus where ETPs undergo the ordered and tighly regulated multistage development program. Positive and negative selection of αβ T cells occurs in distinct regions of the thymus. γδ T cells appear through out the cortex and medulla, and do not appear to undergo a selection process. HSC, hematopoietic stem cell; LMPP, lymphoid multipotent progenitor; CLP, common lymphoid progenitor; TSP, thymic settling progenitor; $S1P_1$, sphignosin-1-phosphate receptor 1. (B) Detailed overview of T cell development. DN1 and DN2a cells are uncommitted progenitor cells that maintain lineage plasticity. T cell commitment occurs at DN2b. The bulk of γδ T cells are believed to develop from DN2b/DN3 stage of development. DN3b cells that under β-selection traverse toward the DP stage prior to maturing to CD4 and CD8 single-positive T cells or NKT cells. DN, $CD8^-CD4^-$; DP, $CD8^+CD4^+$ thymocyte; DC, dendritic cell; M, macrophage; T_{reg}, regulatory T cell; NKT, natural killer T cell – figures adapted from Love et al. and Carpenter et al.[65,109].

Unconventional pathways of selection for unconventional T cells

T cells can develop through an alternative pathway whereby ligands presented by some thymocytes can support development of other T cell populations (thymocyte-thymocyte (T-T) selection). For instance, NKT cell development requires interactions with CD1d expressed by DP thymocytes (for details see below)[110-112]. CD4 T cells can also be selected on DP thymocytes[113,114]. Many of these CD4 T cells are selected from DP cells also exhibited features of innate T cells, including PLZF[115]. These data are clearly demonstrate that T cells can develop on both thymocytes (T-T) and MHC II^+ epithelial cells, but that signals from T-T selection push some CD4 T cells into the innate T cell lineage[116].

γδ T CELLS

The discovery of γδ T cells was an accident. In 1984, Saito and coworkers were feverishly trying to clone the mouse TCRα gene through subtractive-cDNA hybridization, which is the technique that produced the TCRβ gene, but instead isolated something that resembled an immunoglobulin[9]. Unbeknownst to Saito and coworkers, what they isolated was not the TCRα gene, but a new TCR that was later named TCRγ. The isolation of a third type of TCR led to unyielding attempts to characterize the basic principles of αβ-versus γδ T cell development, and the role this new cell type plays during the immune response[9,10].

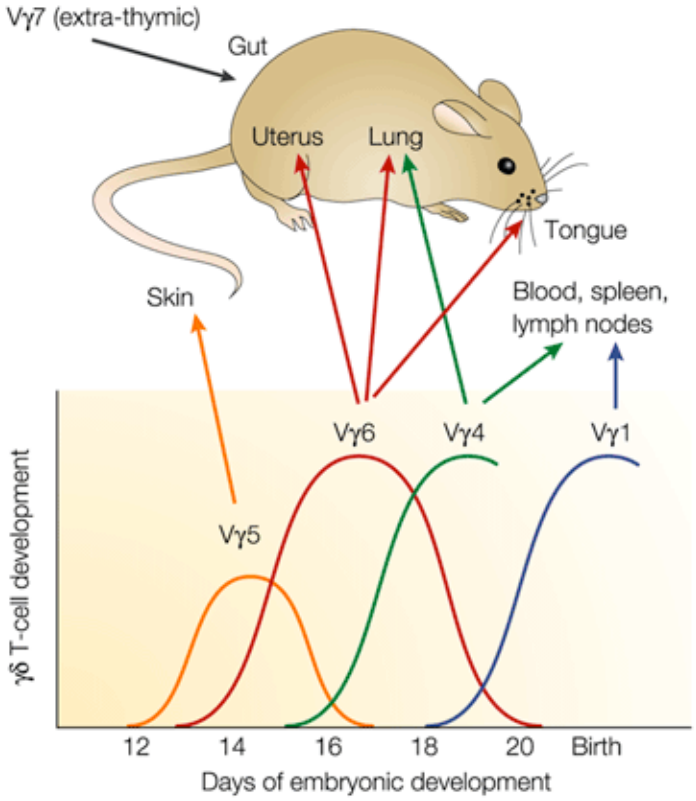
The ordered appearance of γδ T cells during ontogeny

The distinctive structure of the γδ TCR, which is able to detect antigen directly without the requirement for antigen presentation, suggests that these cells are more

ancient than αβ T cells. Phylogenetic analyses of the variable and constant region gene segments of TCRs places the evolutionary age of T cells within the same window as the RAGs at more than 450 million years ago, but indicate that γδ T cells arose slightly prior to αβ T cells[26,117]. In both mice and humans, γδ T cells develop in 'waves' at defined stages throughout fetal and neonatal development and are the first cells to rearrange and express TCRs during ontogeny (Figure 3)[118-123]. Recombination of certain Vγ:Vδ gene segments can be detected as early as E13.5 in mice, while Vδ2 to Dδ3 and Vγ1.8 or Vγ9 to Jγ1 rearrangements appear in the thymuses of 8.5- to 15-week old human embryos[118,124-129]. Comparative analysis of Vγ:Vδ usage in fetal and adult mice showed that Vγ5-and Vγ6-to-Vδ1 rearrangements occurred exclusively in the fetal thymus, while Vγ4, Vγ7, and Vγ1 rearrangements appeared in an overlapping fashion beginning prenatally and continuing throughout the lifespan of the adult[120,124-130]. Each wave of development produces cells that express specific Vγ:Vδ TCRs and migrate to specific tissues in a process that likely depends on explicit signals that shape the genetic program of these cells.

γδ T cell development: la cellule énigmatiques

In contrast to αβ T cells, γδ T cells represent a minor population (~1-5%) of adult murine and human lymphocytes[26,120,124]. While αβ T cells dominate the thymus and secondary lymphoid organs, γδ T cells are highly enriched in the mucosal tissues such as the tongue, lungs, and vaginal and intestinal epithelium[26,120,124]. Considerable progress has been made towards clarifying the general framework through which MHC-restricted T cells are selected and develop into mature immune effector cells. In sharp contrast, very little progress has been achieved over the past two decades that outlines the fundamental principles of γδ T cell development. One of the important challenges



Carding, S.R. and Egan, P.J. (2002) *Nat. Rev. Immunol.* 2, 336-45.

Figure 3. The ordered appearance of specific γδ T cell sublineages during mouse ontogeny. Vγ5 T cells (DETCs) are the first T cell to appear in the fetal thymus, which are followed by Vγ6 T cells. These two γδ T cell subsets develop between E13-E20. Both DETCs and Vγ6 T cells express invariant TCRs and home to specific intraepithelial tissues. Vγ4-, Vγ7-, and Vγ1 T cells predominate in the adult mouse – figure adapted from Carding et al.[123].

towards defining a framework that outlines the basic principles of γδ T cell development is the lack of any known self-antigen required for γδ lineage commitment.

Despite the limited diversity within the relatively restricted γδ TCR repertoire, early studies reported γδ T cell specificity toward classical and non-classical MHC moelcules[131-136]. The capacity to recognize cognate ligands suggested that TCR-ligand interactions might be required for γδ T cell development. Examples of both positive and negative selection of γδ T cells were demonstrated in MHC-deficient- and γδ TCR transgenic mice[136-142]. However, subsequent experiments aimed at clarifying the role of MHC ligands in γδ T cell development showed that γδ T cells do not require interaction with MHC molecules for development[143-145]. The notion that γδ T cells develop in the absence of TCR:MHC signaling pointed to an alternative model that postulated γδ T cells develop in a ligand-independent manner. Recently, however, evidence of a selecting ligand for Vγ5Vδ1 dendritic epidermal T cells (DETCs) has been identified in a specific mouse strain that harbors a truncating mutation in the transmembrane domain of the immunoglobulin superfamily gene *Skint-1*[146,147]. The lack of evidence for a direct TCR:Skint-1 interaction, however, questions whether this molecule is indeed required for the selection of DETCs might actually play a secondary role, possibly as a costimulatory molecule required for maintenance of DETCs in the skin epithelia.

THE THREE MODELS OF γδ versus αβ DEVELOPMENT

A long-standing challenge in the area of T cell development is defining the principles of αβ-versus γδ lineage commitment. Both lineages stem from a common progenitor, however, how these precursor cells 'decide' to become either a αβ- or γδ T cell is unclear. The lack of known selecting antigens required for γδ T cell development has made it exceptionally difficult to establish a fundamental model for αβ-versus γδ

lineage commitment. There is, however, experimental evidence that supports three distinct models of T cell lineage commitment. The instructive model postulates that lineage commitment is dependent on qualitative signals transmitted by the pre-TCR or γδ TCR to stimulate uncommitted progenitors to differentiate into αβ- or γδ T cells, respectively. On the other hand, the stochastic model argues that lineage commitment is pre-determined prior to TCR rearrangement. Apparent exceptions that occur in both interpretations had necessitated a revised model of T lineage commitment. The strength of signal model proposed that the intensity, or 'strength' of TCR signaling, controls lineage commitment regardless of TCR type. Furthermore, this view argued that lineage commitment is neither pre-determined, nor instructed. Instead, it proposes that precursor T cells have the flexibility to switch lineages if the magnitude of signaling does not match the TCR type. Below evidence is discussed that supports and contradicts each model of lineage commitment altogether illustrating the need for a fresh approach to an old problem.

The instructive model

The instructive model proposes that uncommitted thymic progenitors that express either a pre-TCR or a γδ TCR will be directed into the αβ- or γδ lineages, respectively. Therefore, according to this model, the TCR is the definitive signal that 'instructs' cells down one lineage or the other. Early on, however, it was evident that the TCR type does not instruct lineage commitment. For instance, the presence of rearranged TCRβ in γδ T cells contradicted the view upheld by a TCR-instructed lineage assignment model[148,149]. Several other studies also clearly demonstrated that the TCR type does not determine lineage fate. For example, mice that cannot express αβ TCRs (TCRβ$^{-/-}$; pTα$^{-/-}$TCRα$^{-/-}$) still possess a small DP population of cells that belong to the

αβ lineage[150-152]. These data indicate that the γδ TCR can support development of αβ DP cells *in vivo*. Remarkably, both αβ- and γδ TCRs can support the opposite lineage fate in various TCR Tg mouse lines[150-155]. Collectively, these data demonstrate that TCR alone cannot direct αβ- versus γδ lineage choice.

The stochastic model

The stochastic model argues that commitment to either lineage is pre-determined prior to TCR rearrangement, and that expression of a TCR type that matches the appropriate fate secures commitment to that lineage[85]. Thus, pre-committed γδ precursors that successfully produce a γδ TCR are 'rescued' from cell-death and proceeds toward a γδ lineage program. The same will occur in αβ precursors that express pre-TCRs. The foremost support for the stochastic model is based upon data showing that the intrathymic injection of DN2 cells expressing high amounts of IL-7 receptor into host mice gave rise to a disproportionally high number of γδ T cells[85]. In contrast, intrathymic injection of DN2 cells expressing low amounts of IL-7 receptor mostly produced αβ T cells. Therefore, these findings argued that commitment to either lineage occurred prior to TCR rearrangement. Further support for the stochastic model was based on the role of the SRY-related mobility group box transcription factor 13 (Sox13) as a putative regulator of αβ- versus γδ lineage commitment[158]. While the function of Sox13 is unclear, mice deficient in Sox13 have a ~50% decrease in the absolute numbers of fetal γδ T cells[158]. Overexpression of Sox13 resulted in a marked decrease in the frequency and absolute numbers of DP cells[158]. Surprisingly, there was no enhanced generation of γδ T cells in Sox13-Tg mice, although this might reflect low *Lck* promoter activity at DN2[158]. An enrichment of Sox13 was observed among in bulk WT DN2 thymocytes and detected in ~50% of single DN2 clones, a frequency that

correlated with the frequency at which DN2 cells can give rise to αβ- or γδ T cells *in vitro*[158]. Altogether, these data support a model where Sox13-expressing, IL-7 receptor high DN2 thymocytes preferentially give rise to γδ T cells[85,158].

There are several lines of evidence that contradict the stochastic model of development. Similar to the instructive model, the presence of in-frame TCRβ rearrangements can be clearly detected in γδ T cells[88,148,149,159-163]. The presence of rearranged TCRβ receptors in mature γδ T cells not only highlights the emergence from a common progenitor, but also clearly demonstrates that progenitor T cells are not pre-committed. IL-7 signaling is required for γδ T cell development, and based on the stochastic model, IL-7 receptor high DN2 cells express high levels of Sox13 and preferentially develop into γδ T cells. However, while Sox13 has been proposed to be the putative regulator of αβ- versus γδ lineage commitment, mice deficient in Sox13 have an incomplete block in γδ T cell development[158]. Furthermore, overexpression of Sox13 in developing thymocytes results in a modest decrease in the generation of DP and SP T cells[158]. Interestingly, Sox13 is not expressed by all mature γδ T cell subsets, indicating that Sox13 might not be necessary for all γδ sublineages[164].

The signal strength model

While early models contend that αβ- versus γδ T cell development is either instructive or stochastic, clear exceptions are apparent in both models. A third model of T cell development reasons that quantitative differences in the strength of TCR signaling determines lineage commitment[165,166]. The signal strength model, therefore, is a variation of the instructive model that builds in flexibility for the role that initial TCR rearrangement plays as a so-called 'irreversible' determinant in αβ- versus γδ lineage commitment. The pre-TCR is believed to be weak and potentiates the αβ T cell lineage while the relatively

strong signaling through the γδ TCR, on the other hand, is thought to secure the γδ T cell fate[165,166]. Using two distinct γδ TCR transgenic mice, where both the αβ- and γδ lineages develop, two studies essentially showed that decreasing the magnitude of γδ TCR signaling resulted in an increase in DP cells at the expense of γδ T cells, while increasing TCR signaling reduced the frequency of DP cells while obtaining modest gains in γδ T cells[165,166].

KN6 (Vγ4Vδ5) TCR transgenic mice recognize $T10^b$, a *B2m*-dependent non-classical MHC I molecule[135,142]. Haks and coworkers showed that by altering ligand availability ($KN6^+RAG^{-/-}\beta2m^{-/-}$), or reducing proximal TCR signaling ($KN6^+RAG^{-/-}Lck^{-/-}$), cells that normally would give rise to the γδ lineage were diverted into the αβ lineage, as there was a significant increase in the frequency and absolute numbers of DP cells[166]. Similar findings were observed in a study by Hayes and coworkers using mice transgenic for the G8 (Vγ4Vδ1) TCR, which also recognizes $T10^b$ [132,165]. In this study, $G8^+CD3\zeta^{-/-}$ (zeta-chain deficient mice), which fail to transmit downstream TCR signals, had a reduced the frequency of γδ TCR Tg cells[165]. The frequency of G8 Tg T cells was restored back to normal levels when the cells were reconstituted with a full length TCR-CD3ζ transgene[165]. CD5 is thought to be a negative regulator of TCR signaling[167]. Thus, the signal strength model predicted that loss of CD5 should increase or maintain high levels of TCR signaling in developing T cells. To test whether the loss of CD5 on $G8^+$ Tg T cells would decrease the development of αβ T cells, Hayes and coworkers generated $G8^+CD5^{+/-}$ and $G8^+CD5^{-/-}$ mice[165]. In both mouse lines, a decrease in the DP population was clear, with the most significant decreases in $G8^+CD5^{-/-}$ mice[165].

Both studies argued that TCR signaling induced high levels of ERK activity, which was proportionally higher in γδ TCR Tg cells compared to DP cells and appeared to be necessary for development of the γδ lineage[165,166]. Haks and coworkers went a step

further by arguing that strong signaling induced the Erk-Erg-Id3 pathway, and that Id3 was the critical regulator of αβ- versus γδ lineage commitment[166]. Erg 1-3 and Id3 mRNA were substantially higher in WT γδ T cells compared to WT DN3 and DN4 cells[166]. In fetal thymic organ culture (FTOC) assays, enforced expression of Erg 1 in $KN6^{+}RAG^{-/-}\beta2m^{-/-}$ thymocytes reduced development of αβ lineage development, as there was a reduction in the frequency of DP cells two days after culture[166]. This suggested that mimicking strong signals by Erg-1 overexpression redirected cells back to the γδ lineage[166]. Id3 is a direct target of Erg proteins and a natural inhibitor of E proteins[43,44]. Analysis of γδ TCR-positive cells from WT or Id3-deficient mice showed a reduction in the frequency of fetal γδ T cells from E16-E18[166]. Remarkably, the ability of enforced Erg-1 to enhance γδ lineage commitment was abolished in Id3-deficent γδ T cells[166]. Haks and coworkers argued that these data supported a model where the magnitude of TCR signals is proportional to Erg/Id3 induction, and that strong TCR signals and high Id3 activity promote γδ- versus αβ lineage commitment[166].

Recently, another study by Kreslavsky and coworkers applied cell fate analysis of non-Tg γδ T cells to clarify the role of TCR signaling in lineage commitment[164]. Here, FACS sorted DN3b cells (β-selected) that normally give rise to DP cells instead gave rise to DN cells in the presence of strong TCR stimulation[164]. In another experiment, different γδ T cell populations from $pT\alpha^{-/-}$ mice were grown on OP9-DL1 monolayers in the presence or absence of anti-TCRδ antibodies[164]. In the presence of anti-TCRδ antibody, $CD25^{+}$ and $CD25^{+}CD24^{hi}$ γδ T cells remained DN[164]. Importantly, these data demonstrate that surface γδ TCR expression does not irreversibly commit cells to the γδ lineage, as immature γδ precursors can adopt the αβ lineage fate. However, γδ lineage commitment appears irreversible once cells downregulate CD24 and reach maturity. Based on these findings, Kreslavsky and coworkers proposed that lineage commitment

occurs subsequently to TCR expression and that modulation of TCR signaling can influence commitment towards the opposite lineage, which directly challenges the stochastic model of T cell development.

Problems with strength of signal model and potential artifacts of experimental systems

There are several lines of evidence that challenge the conclusions affirmed by each model of T cell development. Clearly, manipulation of TCR signaling can influence αβ- versus γδ lineage commitment, which directly conflicts with the view that thymic progenitors are pre-committed to either the T lineage. The signal strength model resolves the inconsistencies of both the instructive and stochastic models of αβ- versus γδ lineage commitment. However, the conclusions based on the experimental approach that support the strength of signal model of T lineage commitment have many issues that must be addressed. First, both Hayes and Haks arrived at the same conclusion using two distinct γδ TCR transgenic mice that recognize non-classical MHC I molecules (T10/T22^{b})[165,166]. Haks and coworkers showed that the absence of ligand ($\beta 2m^{-/-}$), which is required for TCR signaling, resulted in a redirection into the αβ lineage as indicated by an increase in DP cells[166]. In addition, the induction of strong TCR signaling resulted in less DPs[166]. However, it was never shown by polymerase chain reaction (PCR) or by flow cytometry that these 'redirected' γδ T cells had either rearranged TCRβ genes or expressed surface TCRβ. In the absence of strong signals, the increase in DP cells was only measured by CD4 and CD8 co-receptor expression. However, it is clear that development of DP cells can occur in γδ TCR transgenic mice[156,157,165,166]. Whether these 'redirected' DP cells represent a *bona fide* αβ lineage, or reflect an abnormality observed in these TCR transgenic mice is unclear.

It is important to note that both KN6 and G8 Tg mice were initially used to study whether or not selection played a role in γδ T cell development[135,138,140,141,157]. Both KN6 and G8 Tg T cells, which recognizes $T10^b$, were thought to be positively selected by this molecule, as initial reports showed the absence of these cells when bred onto a $\beta 2m^{-/-}$ background[135,138,140,141,157]. On the other hand, G8 Tg T cells, which bind to $T22^b$ with extremely high affinity (>15 fold higher than KN6), were absent in both C57BL/6 (B6; $T10/T22^b$) and $\beta 2m^{-/-}$ mice, but not in BALB/c ($T10^b$)[132,134,135,138-141]. These data supported a model where γδ T cells undergo a ligand-mediated selection process during development. As mentioned previously, careful analysis of γδ TCR Tg mice demonstrated that neither positive nor negative selection is a requirement for γδ T cell development[143-145]. Furthermore, a recent study showed that in non-Tg mice, γδ T cells that recognize $T10/T22^b$ molecules were present in normal numbers in $\beta 2m^{-/-}$ mice by using newly developed T22 tetramers[145]. In addition, there was no indication of a skewing toward the αβ lineage[145]. Normal numbers of T22-specific γδ T cells were also found in MHC II-deficient mice, even in the presence or absence cyclosporin A treatment, which blocks positive selection of αβ T cells[145]. Collectively, Jensen and coworkers demonstrated that the absence of ligands does not preclude the development of the endogenous γδ T cell repertoire. The normal frequency of $T10/T22^b$-specific γδ T cells, and lack of enhanced αβ T cell development in the absence of ligand, directly contradicts the signal strength model proposed by Hayes and Haks and suggests that the data produced from these studies might be artifactual as a result of the use of TCR transgenes.

Inconsistencies were also apparent in the *in vitro* study using non-Tg T cells by Kresvlavsky and coworkers. Here the authors showed, as predicted by the signal strength model, the failure of DP cells to develop from DN3b cells in the presence of

strong TCR signals[164]. Interestingly, however, some of the cells from this experiment that remained DN (did not progress to DP) either expressed a αβ- or γδ TCR[164]. Also it was unclear whether DN3b cells receiving strong TCR signals were in fact failing to develop into DP cells not because of a presumed lineage switch, but rather a failure to survive in prolonged *in vitro* culture conditions. Conspicuously, progenitor cells that received strong signals using anti-TCRδ antibodies also failed to upregulate CD5[164]. To examine the fidelity of γδ T cell development in this system, *in vitro* OP9-DL1 derived γδ T cells were transferred into nude mice to examine their tissue distribution. Two weeks post-injection, nearly all of the transferred γδ T cells were found in the intestinal epithelium, but not in the spleen or lymph nodes[164]. The general absence of transferred cells in secondary lymphoid tissues might reflect a limitation of this *in vitro* strategy and questions whether this system faithfully characterizes γδ T cell development *in vivo*.

Apparent caveats in the experimental approaches used to elucidate αβ- versus γδ lineage commitment highlight important exceptions that exist in each model. Future experiments that aim to resolve these issues by using non-Tg T cell approaches, and by considering intrinsic developmental requirements of each γδ sublineage, should reveal important distinctions for normal γδ T cell development, and provide a revised model of αβ- versus γδ lineage commitment.

IMPRINTING OF γδ EFFECTOR FUNCTIONS IN THE THYMUS

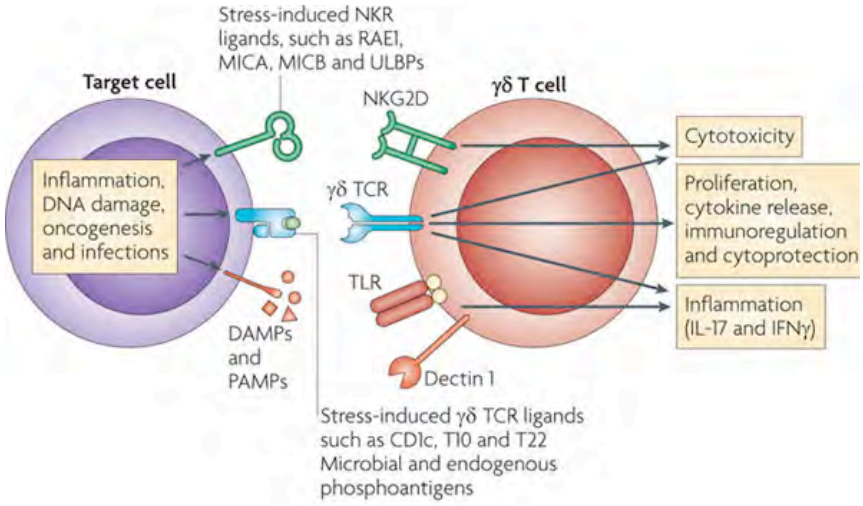
In contrast to conventional αβ T cell lineages, γδ T cells reach functional maturity before leaving the thymus. Notably, signals generated from TCR:MHC interactions have recently been shown to play an crucial role in the developmental programming of T10/T22-specific γδ T cells *in vivo*[145]. In the absence of cognate ligand, this subset of γδ T cells failed to produce IFN-γ and instead made IL-17[145]. The phenotype of *β2m-*

deficient γδ T cells was also altered as these cells failed to express CD122[145]. Thus, antigen-inexperienced γδ T cells adopt an alternate effector fate. This study provided the first evidence demonstrating a role for MHC ligands in programming the effector functions of γδ T cells. It is noteworthy that only the T10/T22-tetramer positive γδ T cell population was evaluated in this study. Therefore, it is unclear whether all γδ T cell subsets require TCR:MHC interactions for normal functionality. Notably, a recent study showed that production of lymphotoxin-β produced by RORγt$^+$ DP thymocytes was required to *trans-condition* expression of γδ lineage genes in γδ T cells[168]. However, only bulk γδ T cells were evaluated in this study, which again questions whether *trans-conditioning* affects the development of all γδ subsets.

Distinct γδ T cells sublineages and their broad coverage throughout host immunity

Although the precise role of γδ T cells unclear, there are several lines of evidence that indicate γδ T cells have important functions during both the early and late phases of the immune response (Figure 4)[131]. Upon activation, γδ T cells rapidly respond by producing large amounts of pro-inflammatory cytokines, which promotes both leukocyte recruitment to the site of infection and their differentiation into effector cells[131]. Once the infection is cleared, γδ T cells can downmodulate the immune response by producing immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and IL-10[131]. Importantly, γδ T cells can also detect stress ligands expressed on damaged or infected host tissues and target their elimination through the engagement of the death receptor Fas (CD95) and the release of cytolytic molecules such as perforin and granzyme[131]. After clearing infection or damaged tissues, γδ T cell promote wound-healing and tissue regeneration through production of epithelial growth factors including keratinocyte growth factor (KGF1), KGF2, insulin growth factor 1 (IGF1), TGF-β, and IL-

A



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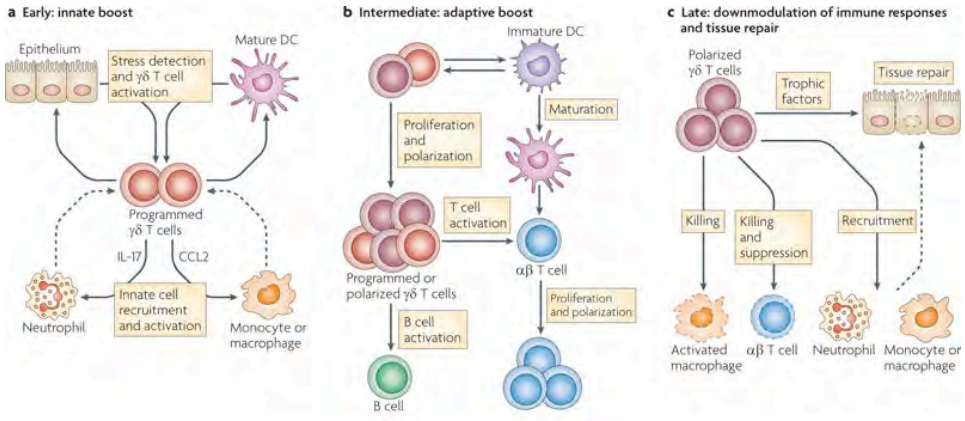

(A,B) Bonneville, M., et al (2010) *Nat. Rev. Immunol.* 10, 467-478.

Figure 4. γδ T cells and their specialized roles during the immune response. (A) γδ T cells can recognize a variety of antigens through receptors typically associated with other cell types in three distinct ways: ligand MHC Ib:TCR interaction, recognition of stress antigens through NKG2D, or detecting PAMPs (pattern-associated recognition receptors) recognized by TLRs (Toll-like receptors) such as Dectin-1. (B) γδ T cells initiating the immune response by promoting activation of other innate cells such as neutrophils and macrophages, direct or indirect activation of αβ T cells by γδ T cells through cytokine production or through DC differentiation, respectively. Producing Th2 cytokines and antibody class switching in B cells, suppressing the immune response through immunosuppressive cytokine production and/or cytolytic activity of activated/infected cells, tissue remodeling through production of growth factors. DC, dendritic cells – figure adapted from Bonneville et al.[131].

22[131,169]. In line with these observations, mice that lack γδ T cells have profound defects in wound-healing[169]. Analysis of a variety of solid tumors consistently revealed the presence of γδ T cells as a component of the tumor infiltrating lymphocyte (TILs) population, and that clones of these TILs can recognize and kill autologous tumors[170-172]. Furthermore, the absence of γδ T cells in mouse models of cancer resulted in a greater frequency of epithelial tumors and cutaneous malignancies[170]. Corollary immune responses are also found among the γδ T sublineages in humans[131]. Recent data implicate a role for γδ T cells in fighting human immunodeficiency virus (HIV), as patients that contained more activated Vγ9Vδ2 T cells also had more αβ CD4 T cells[173]. Interestingly, vaccination strategies and other therapies targeting γδ T cells against HIV, cancer and other diseases are currently under development[171-173].

Functional specialization among γδ T cell subsets within tissues

Specific γδ T cell subsets expressing distinct Vγ:Vδ TCRs migrate to and populate certain tissues and carry out unique functions often in a tissue-restricted manner. For example, DETCs and $V\gamma7^+$ T cells are the principle T lymphocytes in intraepithelial tissues of the skin (s-IEL) and gut (i-IEL), respectively[26,120]. These are the archetypal γδ T cell subsets, which exhibit several important functions including but not limited to cytolysis, tissue immunoregulation, and anti-tumor activities[26,131]. Both DETCs and $V\gamma7^+$ produce large amounts of the pro-inflammatory cytokines IL-2 and IFN-γ. Indeed, γδ T cells express several molecules typically associated with NK cells and can become activated independent of TCR engagement. For example, TCR independent activation of the natural killer group 2 member D (NKG2D) receptor on DETCs resulted in cytolytic responses sufficient for the elimination of epithelial tumors[131,170]. Intraepithelial Vγ6Vδ1 T cells are highly enriched in the mucosal layer of the vagina, tongue and lung, and when

activated almost exclusively produce IL-17[174]. In contrast, Vγ4$^+$ T cells are found in the blood and secondary lymphoid tissues and can produce both IL-17 and IFN-γ[174]. The pro-inflammatory cytokine IL-17 is critical for the activation and recruitment of neutrophils to sites of infection, and it is now appreciated that Th17 γδ T cells and not Th17 CD4 T cells, are responsible for the recruitment of neutrophils in the early stages of the immune response[174,175]. IL-4 production by γδ T cells is uncommon, however, Vγ1Vδ6.3 T cells, which are primarily found in the thymus, spleen and liver can rapidly produce Th-2 cytokines[176,177]. Notably, Vγ1Vδ6.3 T cells can produce IFN-γ and IL-4 simultaneously, a feature that is only shared by NKT cells[178,179]. In addition to the robust effector functions of Vγ1Vδ6.3 T cells, it has been shown in a mouse model of *Listeria monocytogenes* that these cells have the capacity to lyse infected macrophages[180].

Transcriptional programming of γδ T cell subset effector functions in the thymus

Naïve CD4 and CD8 T cells must go through activation-induced differentiation, followed by secondary activation by the same or a similar antigen prior to acquiring the capacity to produce effector cytokines. For example, naïve CD4 T cells can differentiate into Th1 (IFN-γ), Th2 (IL-4), Th17 (IL-17), and regulatory T cells (T_{regs}; IL-10) under certain cytokine conditions during TCR:ligand activation. Functional specialization of γδ T cells, on the other hand, occurs during development in the thymus. While the framework outlining the basic principles of the genetic programming of γδ T cells during development is in the early stages, it is clear that the acquisition of distinct effector functions are imprinted in cells that express a restricted TCR repertoire. IL-17 production by Vγ6Vδ1 and Vγ4$^+$ T cells is controlled by the transcription factor retinoic acid receptor-related orphan nuclear receptor-γt (RORγt)[174,175,181]. GATA-binding protein 3 (GATA-3) controls IL-4 production by Vγ1Vδ6.3 T cells[182,183]. Vγ1Vδ6.3 T cells do not produce IL-17

and predictably these cells do not express RORγt[178]. The absence of RORγt in Vγ1Vδ6.3 T cells suggests that these cells failed to receive the appropriate signals to launch the Th17 program during development. Most γδ T cell subsets have the capacity to produce IFN-γ, which is controlled by the T box transcription factors Tbet and eomesodermin (Eomes)[174,184-186]. It is noteworthy that most T cells only express Tbet or GATA-3, as studies have shown that these transcription factors specify Th1 or Th2 lineage commitment through reciprocal inhibition[184,185]. While it is generally accepted that γδ T cells do not require interactions with ligands for development (see above), elegant *in vivo* studies showed that γδ T cells are fated to produce IFN-γ instead produced IL-17 in absence of cognate ligands[145]. Based on this study, a new paradigm emerged that proposed TCR-ligand interactions in the thymus initiate lineage specific transcriptional 'circuit' necessary for specifying a particular effector program, and not for the development and selection of γδ T cells[145].

Examples of functional plasticity among certain γδ T cell subsets are evident in certain models of infection[131]. This plasticity appears thus far to be context-dependent, and might not be a universal property shared by all γδ T cell subsets. The innate-like features of γδ T cells are a consequence of genetic programming in the thymus, and it is clear that the nature of TCR signaling can influence the transcriptional program of γδ T cells. However, whether the TCR type predetermines the functional programming of each γδ T cell sublineage, or whether additional signals that either operate independently from or in conjunction with TCR signals are also necessary, are areas that require clarification. Importantly, how these signals are organized into distinct lineage-restricted gene modules should lead to the identification of novel regulatory circuits that control γδ T cell differentiation and function. The mapping of these regulatory circuits should also reveal how specific transcription factors are assembled into interconnected circuits that

allow some functional plasticity in specific γδ T cell subsets, or permit the concurrent expression of Tbet and GATA-3 in Vγ1Vδ6.3 T cells. The combination of biochemical and genetic approaches with high throughput genomic approaches will be necessary uncover the gene modules that endow γδ T cells with their innate-like functionality, and provide clues to their role in controlling disease.

NKT CELLS

NKT cells are another excellent example of the type of genetic programming that occurs during T cell development in the thymus. Similar to γδ T cells, the precise role of NKT cells during the immune response is unclear, but it is well documented that NKT cells have important roles in allergy, graft-versus-host disease, cancer, and autoimmunity[25]. The capacity to regulate such a broad range of immunity is surely due to the diverse panel of cytokines that NKT cells can produce. As with γδ T cells, NKT cells can express multiple lineage-specific transcription factors simultaneously. Much more is known about the requirements for development of NKT cells compared to γδ T cells. Notably, two recent studies identified an NKT cell-specific transcription factor, PLZF, which is critical for development and function[187,188].

NKT cell development

Early development of NKT cells appears to be identical to conventional CD4 and CD8 T cells. However, concurrent with positive selection, NKT cells are directed into a lineage that is clearly distinct from conventional T cells in several ways. In mice, NKT cells express a semi-invariant Vα14:Jα18 TCR, and NKT cells in humans express the related Vα24:Jα18α TCR[25]. Rather than interacting with pMHC molecules on thymic stromal cells, NKT cells are selected by lipid antigens presented on the MHC I-like

molecule CD1d expressed by DP thymocytes[110,189]. NKT cells are absent in CD1d-deficient mice[25,110]. The interaction with DP thymocytes is obligatory, as enforced expression of CD1d on thymic epithelial cells does not support NKT cell development[111,112]. The finding NKT cells fail to develop on CD1d-expressing thymic epithelial cells indicated that CD1d was necessary but not sufficient to mediate NKT cell selection. Indeed, additional signals provided by DP cells were likely to be necessary for commitment to the NKT cell lineage.

While seemingly dispensable for conventional T cell development, the Src kinase Fyn was found to be necessary for NKT cell development[190]. Fyn is a proximal TCR signaling molecule that phosphorylates tyrosine residues on a variety of targets in both the PI3-K and MAPK signaling pathways[190]. The precise mechanism of how Fyn controls NKT cell selection was not revealed until it was discovered that the SLAM (Signaling Lymphocytic Activation Molecule) family receptor adapter SAP (SLAM-associated protein) recruits Fyn in order to promote the signaling cascade required for NKT cell lineage commitment[191,192]. Both T and NK cells express SAP. Humans that harbor a mutation in *SH2D1A* (gene encoding SAP) develop a rare, but serious inherited immunodeficiency disease called X-linked lymphoproliferative (XLP) syndrome. NKT cells fail to develop in patients with XLP, and in SAP-deficient mice[191]. Recruitment of Fyn by SAP is dependent on arginine 78, as Fyn cannot bind SAP^{R78A} [192]. Obligatory signals generated by Fyn-SAP interactions indicated that one or more SLAM receptors participated in some aspect of NKT cell development.

Elegant studies using complex gene-deficient bone marrow chimera strategies revealed that homotypic interactions among the SLAM family receptors SLAMF1 (CD150) and SLAMF6 (Ly108) between DP thymocytes and immature NKT precursors were necessary for the expansion and differentiation of mature NKT cells[193]. Thus, the

SLAM/SAP/Fyn signaling pathway promotes a distinct pathway that is required for commitment to the NKT cell lineage.

TRANSCRIPTIONAL CIRCUITS THAT CONTROL T CELL DEVELOPMENT

There are at least five transcription factors that are required for specifying progenitors toward the T cell lineage during early development: PU.1, Ikaros, RUNX/CBFβ, E proteins (E2A and possibly HEB), and Bcl11b[40,43,46,64,194-199]. Mice deficient in GATA-3, Myb, or HEB exhibit a profound block in T cell development at the β-selection checkpoint[45,46,58,200]. ThPOK, Runx-3, and Mazr have critical roles in CD4 versus CD8 lineage commitment[201-203]. The gene expression profiles of many of these transcription factors oscillate throughout various stages of T cell development, which makes it challenging to study their specific roles at different stages by re-expression in transcription factor deficient mice. Currently, there is no known transcription factor required for directing γδ lineage commitment. As the role of TCR signaling in αβ- versus γδ T cell lineage commitment is unsubstantiated, a focus on transcription factor regulation of lineage commitment might illuminate a model that clarifies the genetic mechanism controlling αβ- versus γδ lineage choice.

E proteins and Id3 are critical regulators of T cell development

E2A, HEB, and E2-2 are the genes that encode E proteins, which are helix-loop-helix (HLH) transcription factors that bind E-box sites as homodimers or heterodimers to activate gene transcription[204]. Multiple isoforms of HEB and E2-2 exist through alternative splicing events, however, E2A encodes only two proteins E47 and E12[204]. E proteins are critical regulators of lymphocyte development (Figure 5)[40,43,204]. In E2A-

deficient mice, T cell development is partially blocked at the earliest DN stages and results in a substantial decrease in the overall cellularity in the thymus[43]. All E2A-deficient mice 3-6 months of age will develop aggressive lymphomas[40,204]. While the precise role of how Notch and E2A regulate T versus B cell lineage commitment is tenuous, it is clear that E2A is required for Notch expression in ETPs[205]. In contrast to B cells, both E2A and HEB (alternative HEB and not canonical HEB) function as heterodimers to regulate target genes in the initial stages of T lineage commitment[205]. In addition, E2A has important roles in both the DN to DP transition and in positive selection[40,41,43,44]. During the DN stages of T cell development, E2A expression is analogous to a gradient with peak expression at DN2 after which it progressively decreases until late DP to SP transition when levels drop considerably[204]. Beginning in early DN2 and throughout T cell development, E2A has a critical role in regulating TCR gene transcription and V(D)J recombination[39,42]. The ordered pattern of TCR-γ rearrangement during fetal and adult life offered a unique opportunity to study the molecular mechanism controlling TCR gene rearrangement. The earliest T cells to develop in mice are DETCs followed by Vγ6Vδ1 T cells, both of which develop exclusively in the fetal thymus[120,130]. Around the time of birth, a developmental 'switch' occurs at the TCR-γ locus and Vγ4, Vγ7, and Vγ1 TCRs become actively rearranged whereas Vγ5 and Vγ6 TCR rearrangements are effectively silenced[42]. This phenomenon is controlled by changes in chromatin structure and accessibility to certain TCR genes during ontogeny[42,51,130]. Therefore, analysis of the γδ T cell repertoire was evaluated in both fetal and adult thumuses of E2A-deficient mice[42]. The absence of E2A had no affect on Vγ5 or Vδ1 TCR rearrangement in E19 fetal thymocytes[42]. In adult mice, however, there was a marked reduction in Vγ4, Vδ4, and Vδ5 rearrangements, and a significant increase in Vγ5 and Vδ1 rearrangements[42]. These data suggest that the absence of E2A

alters programmed Vγ:Vδ rearrangement by regulating locus accessibility during development. In WT mice, E2A has a seemingly nonessential role in regulating Vγ5 and Vδ1 rearrangement in fetal thymocytes, but a vital role in Vγ4, Vδ4, and Vδ5 rearrangement in the adult thymus.

E2A regulates the β-selection checkpoint. Indeed, E2A controls RAG1/2 and pTα transcription and TCRβ rearrangement in developing thymoyctes[40,42,43,204]. A recent study examining the role of E2A in TCRβ rearrangement, found that mice lacking one or both copies of E47 showed profound alterations in chromatin structure that hindered accessibility to Vβ gene segements[39]. The overall loss of TCRβ rearrangements in $E47^{+/-}RAG2^{-/-}$ and $E47^{-/-}$ $RAG2^{-/-}$ mice indicate that E2A functions at TCR loci in a dose-dependent manner. At DN3, cells that fail to express a pre-TCR are arrested by E2A and fail the β-selection. Indeed, the role of E2A during β-selection was discovered when RAG1-deficient thymocytes progressed beyond DN3 and produced DP cells in the absence of E2A[40,41,43,44,204]. Successful pre-TCR signaling at DN3 triggers the induction of the ERK-Erg-Id3 pathway, which inhibits E2A activity, thereby allowing cells to transition from the DN to the DP stage of development[41,44,206]. E proteins also have a critical role during positive selection[44]. However, there is a higher frequency of CD4 and CD8 SP thymocytes in $E2A^{-/-}$ versus WT mice[204]. To test this hypothesis, both the HY TCR Tg mouse strain, which promotes selection of CD8 SP thymocytes and the AND TCR Tg mouse strain, which promotes CD4 SP selection, where bred onto a E2A-deficient background. Results from these studies indicated that the increases in CD4 and CD8 SP thymocytes were due to the absence of E2A and not a general increase in survival of SP cells[41]. These results indicate that E2A normally mitigates positive selection.

There are four Id proteins (1 – 4) in vertebrates. Id proteins are a class of HLH proteins that lack a DNA-binding domain and function as inhibitors of E proteins through E:Id heterodimers[204]. Id proteins have critical roles in lymphocyte development. In particular, the extent of Id3 expression and activity appears to be directly correlated with the magnitude of TCR signaling in developing T cells[44]. As a result, Id3 expression is highest among DN3 cells during β-selection[44]. Specifically, pre-TCR signaling induces Id3-mediated inhibition of E2A during the β-selection checkpoint, thereby reducing E2A activity below a particular threshold, allowing cells to progress beyond DN3 and toward the DP stage of development[41]. Notably, Id3 expression has been detected prior to TCR signaling[87]. Id3 expression in human $CD34^+$ progenitors resulted in enhanced NK cell development at the expense of αβ T cells, but not γδ T cells[207]. Thus, a delicate balance of E:Id protein activity must be achieved for normal T cell development. Id3 expression is highest among γδ T cells[87]. High levels of Id3 appeared to favor γδ T cell development, which suggested that Id3 might play a critical role in αβ-versus γδ lineage commitment. Collectively, these observations served as the foundation of the signal strength model that argues lineage commitment balances on the magnitude of TCR signaling and Id3 levels[165,166].

RORγt

The RAR-related orphan receptor gamma (RORγ)t is a thymic specific isoform of the *RORC* gene[208]. Several studies have shown that RORγt has important roles during T cell development and function[181,208-210]. Early reports indicate that RORγt expression is developmentally regulated, as it was mostly restricted to the DP thymocytes in wild-type mice[209,210]. Elevated levels of IL-7 signaling throughout the DN stages of T cell development were shown to suppress RORγt, until the downregulation of the IL-7

receptor at the DP stage[211]. There is a substantial loss of mature CD4 and CD8 SP T cells in RORγt-deficient mice[209,210]. Closer analysis revealed an accumulation of cells at the intermediate SP (ISP) stage, which suggested a role for RORγt at the DP to SP transition. The accumulation of ISP cells was shown to be a result of substantial cell death at DP *in vitro* and *in vivo*[210,212]. Cell death was rescued in RORγt-deficient mice that expressed a Bcl-X_L Tg[210]. This data suggested that RORγt was a positive regulator of Bcl-X_L in DP thymocytes.

An earlier study showed implicated RORγt in TCRα rearrangement through the regulation of the T early α promoter *in vitro*[213]. Mice lacking TEA have defects in recombination of proximal Jα segments[213]. DP thymocytes can undergo two rounds of TCRα rearrangement, which is proposed to provide a rescue mechanism for cells that fail to produce a functional TCR on the first attempt. DP thymocytes that fail to rearrange a productive TCRα chain on the second attempt cannot undergo positive selection and die by neglect. Therefore it was unclear whether RORγt only regulated DP thymocyte survival, or whether it played a role in controlling TCRα recombination. Analysis of Vα-Jα rearrangements in RORγt-deficient DP thymocytes showed a preferential rearrangement of proximal (5'), but not distal (3') gene segments. Interestingly, the 3' half of Jα was enriched in acetylated histone H3 among RORγt-deficient DP thymocytes compared with Eα-deficient mice, which suggested that the locus was accessible to the recombination machinery[212]. RORγt-deficient DP thymocytes exhibited a defect in 5' Vα rearrangement. RORγt$^{-/-}$Bcl-X_L^{TG} rescued the ability of RORγt$^{-/-}$ thymocytes to produce more distal 3' Jα segments[212]. Therefore, it was concluded that RORγt regulated the survival of DP thymocytes to extend a 'window' for cells to undergo secondary TCRα chain rearrangements.

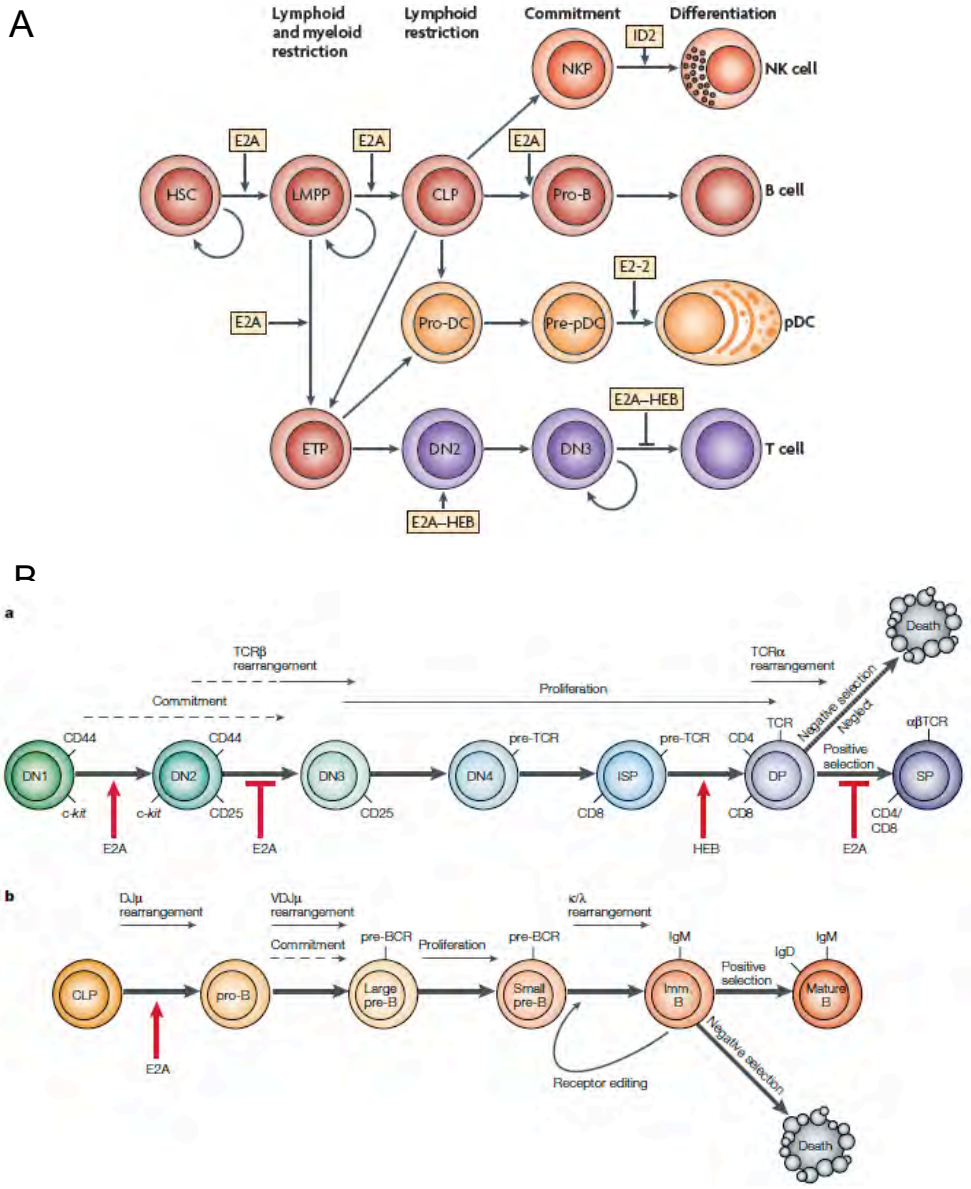

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Figure 5. E proteins and lymphocyte development. (A) E and Id proteins are critical at various stages of lymphocyte development. Curved arrows represent increased proliferation in the absence of E proteins. NK, natural killer cells; pDC, plasmacytoid dendritic cells; CLP, common lymphoid progenitor; DN, double negative, HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; NKP, NK cell progenitor. (B) Overview of T (top) and B (bottom) cell development. Red arrows indicate where E proteins function during T and B cell development. DP, $CD4^+CD8^+$ T cell; Imm. B, immature B cell; ISP, immature single-positive $CD8^+$ thymocyte; pre-TCR, pre-TCR antigen receptor; pre-BCR, pre-B cell antigen receptor; SP, $CD8^+$ or $CD4^+$ single-positive thymocyte; TCR, T cell receptor – figure adapted from Kee et al. and Engel et al[204,205].

A subsequent study demonstrated that pre-TCR signaling induced Erg-3 and Id3 activity at DN3, which inhibits E2A-dependent activation of RORγt[206]. Erg-3 promotes proliferation of β-selected thymocytes, where as the absence of pre-TCR signaling at DN4 causes release of E2A by Id3 resulting in RORγt and Bcl-X_L expression for TCRα rearrangement at DP stage. Interestingly, this study also indicated a role for RORγt in regulating RAG2 in DP thymocytes.

RORγt is necessary for lymphoid tissue inducer (LTi) cells which play a crucial role in the development of the lymph nodes and Peyer's patches[214]. Thus, these structures are missing in RORγt-deficient mice. Notably, RORγt is required for IL-17 production in both CD4 and γδ Th17 cells[174,175,181]. RORγt, therefore, defines the Th17 lineage program in both conventional and innate T lymphocytes. Defects in the T cell compartment of RORγt-deficient mice are apparent and result in susceptibility to certain types of bacterial and fungal infection, as well as impaired during allergic responses[174]. Altered RORγt activity might also promote autoimmune diseases and inflammation by Th17 cells.

BTB-ZF genes control fundamental aspects of immune cell development[a]

The BTB-POZ-ZF (broad complex, tramtrak, bric-à-brac (BTB) or poxvirus and zinc finger (POZ) are a family of transcription factors that are characterized by an N-terminal BTB domain that mediates protein-protein interactions combined with one or more C-terminal C_2H_2 *Krüppel*-type zinc finger DNA binding domains. This conserved family of transcriptional regulators controls a wide variety of biological process through the recruitment of several components of chromatin remodeling complexes[215]. While it

[a] *This section is reproduced in large part verbatim from reference 187, as required under the license granted by Macmillan Publishers Ltd: Nature Immunology, 2008.*

appears that BTB-ZF function primarily as repressors there is some evidence showing these transcription factors can activate gene expression. While the mechanism for gene regulation is not clear, these transcription factors function as homo- or heterodimers with different BTB-ZF family members.

Over the past several years, several BTB-ZF genes have been shown to control fundamental and non-redundant roles during immune cell differentiation and function. For example, LRF (Leukemia/lymphoma related factor; *Zbtb7a*) is expressed by several leukocyte populations, but is mostly restricted to cells of the B cell compartment[216]. Conditional deletion of LRF in mice results in a significant loss of mature B cells in the periphery and aberrant T cell development in the bone marrow[216]. The loss of LRF in lymphoid progenitors causes the activation of Notch signaling which initiates a T lineage gene expression program. Whether Notch is a direct target of LRF is unclear, however, this study indicates an important role for LRF in B-versus T lineage commitment in multipotent progenitor cells.

ThPOK (*Zbtb7b*), probably the most recognizable BTB-ZF gene, has clearly been shown to be necessary and sufficient for the differentiation of CD4 T cells[201]. During development, ThPOK expression is initially detected during the DP to SP transition in $CD4^{+}CD8^{-}$ cells that have recently been signaled for positive selection. These data indicate activation of ThPOK might be a consequence of TCR signals generated from interaction with MHC ligands. Stable expression of ThPOK in peripheral CD4 T cells suggests that ThPOK is necessary for the maintenance of the CD4 lineage program. Indeed, conditional depletion of ThPOK in mature CD4 T cells causes the spontaneous derepression of CD8[202]. The loss of CD4 T cells was apparent in targeted knockouts of ThPOK and in 'helper-deficient' (HD) mice, which harbor a point mutation in zinc finger domain of ThPOK[201]. Enforced expression of ThPOK in developing thymocytes causes a

redirection of the CD4 lineage into the CD8 lineage, even among MHC I-restricted T cells[201].

PLZF (promyelocytic leukemia zinc finger gene; *Zbtb16*) was originally discovered in the chromosomal translocation t(11;17)(q23;q21) with retinoic acid receptor-α (RARα) in acute promyelocytic leukemia that yields the production of a reciprocal chimeric oncoprotein as a consequence of fusion between the zinc finger domain of PLZF and RARα[217,218]. In APL, mutant PLZF-RARα behaves as a dominant negative regulator of RARα by recruitment of chromatin modifiers that suppress transcription of RARα[217]. Aberrant transcriptional inhibition of RARα affects RARα-target genes involved in DNA repair, cell cycle and apoptosis[218]. PLZF is a critical regulator of male germ cell maintenance, as stem cells fail in their ability to self renew and proliferate in adult PLZF-deficient male mice[219]. PLZF is also required for proper axial and limb patterning. PLZF-deficient mice exhibit anterior-directed homeotic transformations throughout the axial skeleton and profound patterning defects in all limb structures[220]. PLZF regulates limb and skeletal patterning through growth inhibitory and pro-apoptotic pathways via regulation of Hox gene expression[220,221]. Recently, our lab identified a novel role of PLZF in the immune system by characterizing requirement of PLZF for development of the innate effector program of NKT cells[187,188].

PLZF controls the development of innate effector functions in NKT cells

Recognizing the increasing number of BTB-ZF proteins involved in the function of the immune system, we screened various thymocytes populations for the expression of several different family members. One, however, *Zbtb16*, which encodes the transcription factor PLZF[218], had higher expression in NKT cells than in conventional T cells. The expression of PLZF in NKT cells was apparent in thymocytes and also in

lymphocytes isolated from mouse livers. We used α-GalCer-CD1d tetramers to specifically identify NKT cells. Intracellular staining for PLZF in NKT cells was expression in variable amounts in thymic NKT cells and was moderately expressed in liver NKT cells. However, we did not find PLZF in conventional thymocytes or conventional liver T cells. The development of NKT cells is typically categorized into three stages, identified mainly by the cell surface upregulation of CD44, followed by NK1.1[222]. Nearly all cells at stage 1 had abundant expression of PLZF. Consistent with the finding that PLZF is down regulated in other cell types during differentiation, including $CD34^+$ human stem cells, we found that PLZF was downregulated in NKT cells in the more mature populations at stages 2 and 3. PLZF expression at stage 3 was equivalent to that in mature NKT cells of the liver.

To further examine the expression of PLZF in the lymphoid compartment, we examined B cells and NK cells by flow cytometry. We did not detect PLZF in either of these cell types. We also did not detect PLZF in eosinophils, neutrophils, or macrophages (data not shown). The development of the hematopoietic system was not overtly perturbed in PLZF-deficient mice. For example, we found similar percentages of all thymic subpopulations in the mutant and wild-type mice. The cell numbers of each population were proportionally lower, however, as thymi of PLZF-deficient mice were consistenly about 25% smaller. $DX5^+$ T cells, which represent mainly non-CD1d-restricted NKT cells[223], NK cells and γδ T cells were also present in wild-type numbers. Finally, we found no obvious differences in the B cell compartment (data not shown).

Although conventional T cell development was not perturbed in the absence of PLZF, the frequency of CD1d-tetramer-positive NKT cells in the PLZF-deficient thymus was about 10% as much as that of wild-type thymus. CD44, an activation marker for conventional T cells, is upregulated during development of all NKT cells in the thymus and is constitutively expressed by all mature NKT cells. Tetramer-positive NKT cells in

the thymi of PLZF-deficient mice, however, had not upregulated CD44. The requirement of PLZF was intrinsic to NKT cells, as the transfer of PLZF-deficient bone marrow in to irradiated C57BL/6 host mice resulted in the development of few $CD44^{lo}$ NKT cells. The finding of fewer NKT cells in the thymus of PLZF-deficient mice was not due to more apoptosis, as these cells were negative for annexin V staining.

In contrast to the many fewer PLZF-deficient NKT cells in the thymus and liver, PLZF-deficient mice actually had approximately two-fold more NKT cells in the lymph nodes than did wild-type mice. We also found NKT cells among the spleen cells of PLZF-deficient mice, although the frequency was lower than that of NKT cells in wild-type mice. PLZF-deficient NKT cells in the lymph node and spleen were $CD44^{lo}$ and mostly $CD69^{-}$. PLZF-deficient NKT cell populations in the lymph nodes also had a much higher ratio of $CD4^{+}$ cells to $CD4^{-}$ cells than did wild-type NKT cell populations.

Shortly after activation, NKT cells secrete copious amounts of various cytokines, including IL-4 and IFN-γ. To test NKT cell function, we sorted tetramer-positive NKT cells from the spleens and lymph nodes of wild-type and PLZF-deficient mice. We activated sorted cells with phorbol 12-myristate 13-acetate and ionomycin for a total of 5 hours and added brefeldin A for the final 4 hours of the culture. We then activated cells by intracellular staining, followed by flow cytometry. As expected, a large percentage of wild-type NKT cells produced cytokines in these conditions, with approximately half of the activated cells expressing both IL-4 and IFN-γ. Notably, far fewer PLZF-deficient NKT cells expressed the cytokines. Of particular note was the complete loss of the ability to simultaneously produce both IL-4 and IFN-γ. $CD4^{+}$ T cells from wild-type and PLZF-deficient mice produced some IFN-γ and little IL-4. To further analyze the functionality of PLZF-deficient NKT cells, we activated sorted cells with plate-bound anti-CD3 and soluble anti-CD28, allowed them to ‘rest’ for 5 d in fresh medium and then reactivated

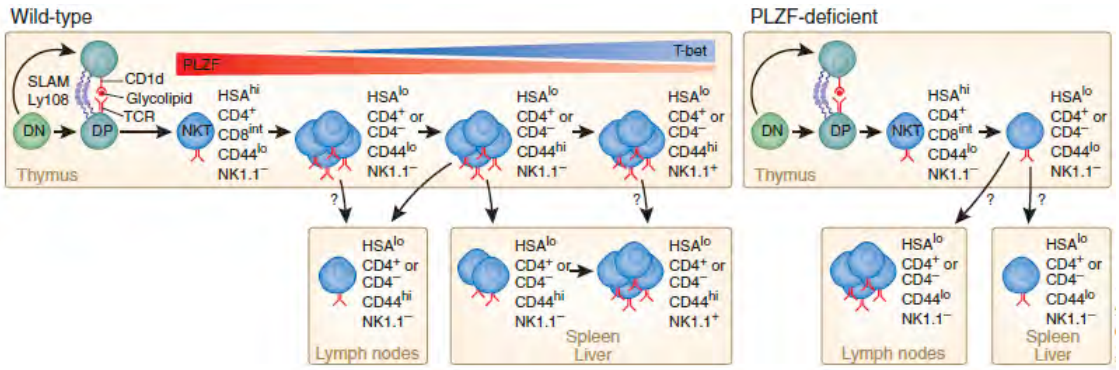
them in fresh medium. As a control and for comparison, we also collected wild-type and PLZF-deficient $CD4^+$ T cells and grew them in the same culture conditions. We analyzed a variety of cytokines in the supernatants of the primary and secondary activations. As suggested by the experiments reported above, PLZF-deficient NKT cells produced little IL-4 and IFN-γ after primary activation. The failure to produce cytokines was not a general defect of the cells, however, as after secondary activation, PLZF-deficient NKT cells had robust production of these two cytokines. The limited capacity of PLZF-deficient NKT cells to produce IL-4 and IFN-γ after primary activation was in contrast to results obtained with wild-type NKT cells. Wild-type NKT cells also produced more IL-4 and IFN-γ after secondary activation than did PLZF-deficient NKT cells. $CD4^+$ T cells were not affected by the loss of PLZF. Therefore, in the absence of PLZF, NKT cells still develop, but phenotypically and functionally are very similar to conventional naïve CD4 T cells.

Ectopic expression of PLZF in conventional T cells results in the spontaneous acquisition of memory/effector phenotypes and functions[224,225]. T cells in mice carrying a T cell-specific PLZF transgene were, like NKT cells, found to be nearly all $CD44^{hi}$ and $CD62L^{lo}$. These cells also were found to produce large amounts of several cytokines upon primary activation. Overall, PLZF expression appears to be necessary, sufficient and cell intrinsic for many of the salient features that characterize innate T cell function and phenotype (Figure 6).

Summary

Over the past 15 years, a large number of transcription factors have been implicated in several aspects of immune cell development of function. More recently, a number of studies have shown that multiple members of the BTB-ZF family of

transcription factors operate as critical regulators in lineage commitment and function. PLZF is necessary for the development of NKT cell effector functions. Since PLZF is interacts with various co-repressors and histone modifying enzymes, it is likely that PLZF exerts its function through changes in chromatin structure. The relative paucity of NKT cells makes studying the role of PLZF in chromatin regulation technically challenging. However, we are only now beginning to appreciate the role of BTB-ZF genes in broader context of the immune system. To date, Sox13 is the only known γδ T cell-specific transcription factor. Microarray results from our lab showed PLZF expression in γδ thymocytes in WT mice. Therefore, the work in this thesis aimed at evaluating the role of PLZF in γδ T cell development and function.



Gapin, L. (2008) *Nat. Immunol.* 9, 1009-11.

Figure 6. PLZF controls the differentiation and function of NKT cells. CD1d expressed on DP thymocytes is require for NKT cell selection (signal 1). Homotypic interactions with the SLAM family receptors LY108 and SLAMF1, also expressed on DP cells, are also required for NKT cell development (signal 2). Upon positive selection, immature NKT cells undergo a development program in a stage specific manner that can be defined into 4 distinct phases based on CD24, CD44, and NK1.1. PLZF expression is highest among immature NKT cells and progressively decreases as cells mature. Another transcription factor Tbet is required for the final stage of development. The expression Tbet inversely correlates with PLZF. Once in the periphery, NKT cells stably express intermediate to low levels of PLZF. *Right*, in the absence of PLZF, NKT cells fail to progress to later stages of development. PLZF-deficient NKT cells are nearly absent in the thymus and liver, but instead accumulate in the lymph nodes. In contrast to wild-type NKT cells, PLZF-deficient NKT cells produce cytokines upon secondary activation stimulus and fail to produce IFN-γ and IL-4 simultaneously. Thus PLZF-deficient NKT cells appear to resemble conventional CD4 T cells. PLZF, therefore, is an NKT-specific transcription factor required for the differentiation and function of NKT cells – figure adapted from L. Gapin[189].

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Fyn-, TCRβ-, and CD1d$^{-/-}$ mice were purchased from The Jackson Laboratory. ThPOK$^{GFP/+}$, ThPOK$^{GFP/-}$ mice were housed at the Skirball Institute, NYU, New York, NY, USA. ThPOK$^{-/-}$ were a kind gift from Dietmar Kappes (Fox Chase Cancer Center, Philadelphia, PA). SLP76^{Y145F} mice were a kind gift from Gary A. Koretzky (Abramson Family Cancer Research, (UPenn) Philadelphia, PA). Id3$^{-/-}$ (a kind gift from Dr. Robert Benezra, Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY, USA), RORc$^{-/-}$ (a kind gift from Dr. Dan R. Littman, Skirball Institute, NYU, New York, NY USA), RORc$^{-/-}$Id3$^{-/-}$, Lck-PLZF^{Tg}, and PLZF$^{-/-}$ mice were housed at MSKCC. For fate-mapping experiments, BAC transgenic mice expressing RORγt-Cre were crossed with mice containing a loxP-flanked eYFP reporter gene targeted to the *Rosa* locus (*Rosa26*-eYFP; a kind gift from Dr. Dan R. Littman) were housed at the Skirball Institute, NYU, New York, USA. For all experiments, mice between 4-10 weeks old were used (see Figure Legends for specific age used in each experiment). All animal work was done in compliance with MSKCC's Internal Animal Care and Use Committee and the guidelines of the Federal Office of Laboratory Animal Welfare. Animals were housed in the Research Animal Resources Center of MSKCC. Animal housing rooms were under temperature and humidity control, the mice were not subject to water or food restrictions, and bedding materials was placed in each cage. Four full-time veterinarians and six veterinarian technicians staff the facility. The veterinary staff is located on site, and a clinical veterinarian is available at all times. Animal care staff carried out routine husbandry procedures, including change cages, feeding and watering.

All mice were sacrificed prior to use. Euthanasia was conducted in accordance with the American Veterinary Medical Association Guidelines on Euthanasia. Briefly, mice were sacrificed by asphyxiation with CO_2 delivered into cages <5 pounds per square inch per second. Death of the animal was confirmed by lack of respiration and toe pinch. CO_2 euthanasia stations are inspected regularly by the Internal Animal Care and Use Committee personnel. Tissues were removed for experiments after confirmation of death.

Flow cytometry and cell sorting

Single-cell suspensions were incubated with normal mouse serum, unlabeled streptavidin, and Fc receptor-blocking antibody before stained at 4°C with specific monoclonal antibodies (mAb). The following mAb conjugates were purchased from eBioscience (San Diego, CA): anti-CD3ε (145-2C11), anti-γδ TCR (UC7-13D5; GL3), anti-TCRβ (H57-597), anti-CD4 (L3T4), anti-CD8α (53-6.7), anti-CD44 (IM7), anti-CD25 (PC61.5), anti-NK1.1 (PK136), anti-GR-1 (RB6-8C5), anti-CD11b (M1/70), anti-TER-119 (TER-119), anti-B220 (RA3-6B2), anti-CD27 (LG.7F9), anti-CD117 (2B8; ACK2), anti-CD34 (RAM34), anti-CD127 (A7R34), anti-CD16/32 (93), anti-IFN-γ (XMG1.2), anti-IL-4 (BVD6-24G2), anti-GATA-3 (TWAJ), anti-RORγt (AFKJS-9), anti-Eomes (Dan11mag). The following mAb conjugates were purchased from BD Biosciences (San Jose, CA): anti-CD3ε (500A2), anti-γδ TCR (GL3), anti-Vδ6.3 TCR (8F4H7B7), anti-Vγ4 (UC3-10AG), anti-Vδ4 (GL2), anti-TCRβ (H57-597), anti-CD4 (RM4-5, H129.19), anti-CD8α (53-6.7), anti-CD8β (53-5.8), anti-NK1.1 (PK136), anti-CD69 (H1.2F3), anti-CD49b (DX5), anti-CD11c (HL3), anti-CD11b (M1/70), anti-CD19, anti-Flt-3 (A2F10.1), anti-TNFα (MPG-XT22). The following mAb conjugates were purchased from Biolegend (San Diego, CA): anti-Vγ1 TCR (2.11), anti-Vγ5 TCR (536), anti-CD24 (M1/69), anti-Sca-1

(D7), anti-IL17A (TC11-18H10.1), anti-Tbet (4B10). For detection of biotinylated antibodies, the following secondary labeling reagents were also used: streptavidin-PeCy7 (BD Biosciences), -FITC (BD Biosciences), -PE (eBioscience), -APC-eFluor780 (eBioscience), -Qdot625 (A10196, Invitrogen). The anti-Vγ7 TCR (F2.67) was kindly provided by Pablo Pereira (Institut Pasteur, Paris, France). CD1d:PBS57 tetramers, used for some experiments, were obtained from the National Institutes of Health Tetramer Core Facility. The mouse anti-human PLZF (Mags-21F7) and anti-MHC II (212.A1) were generated by the Monoclonal Antibody Core Facility at MSKCC. For intracellular and intranuclear staining, cells were made permeable using the Cytofix/Cytoperm kit (BD Biosciences) and FOXP3 kit (eBioscience), respectively. In nonpermeabilized cell preparations, dead cells were excluded by incubating with the DNA-intercalating dye DAPI. For all experiments, cell doublets were excluded by comparing side-scatter width and -height and forward-scatter width and -height. Samples were collected on an LSR II flow cytometer (BD Biosciences), and data were analyzed using FloJo software (Tree Star, Ashland, OR). For cell sorting experiments, cells of interest were enriched using paramagnetic beads (Miltenyi Biotec) followed by magnetic-stand separation (Miltenyi Biotec). Enriched cells were double-sorted, first on yield and then purity (≥98% pure) on a FACSAria (BD Biosciences) or MoFlo (Beckman Coulter) flow cytometer by MSKCC's Flow Cytometry Core Facility.

In vitro T cell activation

Splenocytes were activated for 5 hours with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (500 ng/ml); brefeldin A (3μg/ml; eBioscience) was added for the final 4 hours of the incubation. Activation experiments that did not include anti-PLZF were fixed and made permeable with Cytofix/Cytoperm kit (BD Biosciences) followed by

intracellular staining for IFN-γ, IL-4, TNFα, and IL-17. For cytokine secretion assays, sorted γδ T cells were activated with plate-bound anti-CD3 (10μg/ml) in round-bottom 96 well plates (complete media) for 72 hrs. Secreted cytokines were detected with cytokine bead arrays (BD bioscience).

In vivo proliferation and cell cycle analysis

Analysis of *in vivo* proliferating lymphocytes were performed using the FITC BrDU flow kit (BD Biosciences) according to the manufacturer's instructions. Mice were injected i.p. with a single dose of BrDU (1 mg); 16-18 hours later, mice were sacrificed, cells were stained for surface markers, and the percentage of BrDU-positive cells was identified by flow cytometry. DAPI (10μg/ml) was added to cells to determine DNA content.

Immunohistochemistry

Serial sections 6μm in thickness were cut and prepared by standard methods. Sections were stained with biotin-conjugated anti-DEC205 and FITC-conjugated UEA-1, followed by streptavidin-PE.

Sample preparation for microarray gene expression profiling

RNA was harvested from frozen cell pellets of double FACS sorted Vγ1Vδ6.3 T cells from thymuses of WT, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and *RORc*$^{-/-}$*Id3*$^{-/-}$ mice in three independent experiments. RNA isolation and processing for microarray hybridization to MOE-430A2.0 GeneChip (Affymetrix) was done by the Genomics Core Laboratory at MSKCC. For quantitative real-time PCR experiments, RNA was isolated from double FACS sorted DN

thymocytes with the RNeasy Micro kit (QIAGEN). RNA was reverse-transcribed into cDNA with the Superscript III First Strand Synthesis kit (Invitrogen).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA) software. All samples were analyzed using unpaired, two-tailed Student *t* test.

RESULTS

PLZF regulates the function of NKT-like γδ T cells – J Immunol 2010[b]

Introduction

Multipotent progenitor T cells migrate from the bone marrow to the thymus and give rise to mature T cells that express an αβ or γδ TCR. Despite their emergence from a common T cell progenitor, γδ T cells are fundamentally distinct from conventional (αβ) T cells. αβ and γδ T cells transit through a multistage, differentiation program in the thymus. αβ TCR-expressing thymocytes go through a $CD4^+CD8^+$ double-positive stage prior to maturing into CD4 single-positive or CD8 single-positive T cells. In contrast, studies showed that γδ T cells emerge directly from double-negative cells, and the mature cells generally do not express CD4 or CD8 co-receptors[26,87]. αβ T cell development requires interactions between the TCR and self-peptide:MHC, primarly on the surface of stromal cells, although some hematopoietic cells can also support T cell development[114]. In contrast, γδ T cells do not seem to require positive selection for full maturation, except for skin-resident dendritic epidermal T cells.

In mice, γδ T cells appear in 'waves' at defined stages throughout fetal and neonatal development. Each wave of development produces cells that migrate to specific tissues in a process that likely depends on explicit signals that shape the genetic program of these cells[120,123,130]. It is clear that γδ T cells that express different TCRs are enriched in certain tissues and that these cells have specific roles, ranging from immunosurveillance to tissue homeostasis[26,123,169]. Several studies showed that γδ T cells are early responders in various models of infectious disease and carry out important roles in autoimmunity and tumor surveillance, although they constitute a relatively minor population (1-5%) of total lymphocytes[123].

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Unlike naïve conventional T cells, γδ T cells typically exhibit an 'activated' phenotype and rapidly respond to Ags[170,226-228]. Upon activation, γδ T cells largely produce IFN-γ, which modulates innate- and adaptive-specific immune responses during infection[123,184,229]. Recent reports showed that γδ T cells are also an important source of IL-17, another pro-inflammatory cytokine that is important for the recruitment of neutrophils to areas of inflammation[145,230,231]. Notably, some γδ T cells also secrete Th2 cytokines *in vitro* and *in vivo*. Originally identified as Th1.1^{dull} γδ T cells, studies revealed that these IL-4-producing γδ T cells express a Vγ1Vδ6.3 TCR. This unique subset of γδ T cell is CD44^{hi}CD69^{+}CD62L^{lo}, and nearly half express NK1.1 and CD4[177,228]. Thus, based on several phenotypic and functional characteristics, Vγ1Vδ6.3 (γδ) T cells, like invariant NKT (NKT cells), are categorized as 'innate-like' T cells and are believed to operate at the interface of the innate and adaptive immune response.

A central aim in understanding the role of γδ T cells during the immune response is identifying the molecular signals that govern their differentiation and function. Recently, we and other investigators showed that promyelocytic leukemia zinc finger (PLZF; encoded by *ZBTB16*) is necessary for the development of NKT cell effector functions[187,188]. PLZF belongs to the broad-complex and tramtrack bric-à-brac (BTB)-poxvirus-zinc finger (ZF) family of transcription factors, which have essential roles in various biology processes, including germ cell maintenance, specificity of neuromuscular connections, and axial limb development[215,219,221]. A number of BTB-ZF proteins have been identified that control fundamental aspects of immune cell development. For example, LRF directs B- versus T cell fate, whereas ThPOK is a critical factor for CD4 lineage commitment[216,232-234]. Given the innate-like T cell features shared by γδ and NKT cells, we investigated whether PLZF plays a role in the differentiation and function of γδ T cells. We found that a specific subset of γδ T cells, defined by expression of the

Vγ1Vδ6.3 TCR, expressed the innate T cell determinant PLZF and that PLZF was required for the function of these cells. Finally, recent studies indicated that strong TCR signaling is required for γδ T cell development[165,166]. Paradoxically, reduced TCR signal strength resulted in a dramatic expansion in PLZF-expressing Vg1Vδ6.3 T cells. Inhibitor of differentiation gene 3 (Id3), a molecular target of TCR signaling pathways, controls the development of PLZF-expressing Vγ1Vδ6.3 T cells. Our data supports a model in which reduced TCR signals alter Id3 activity and directs cells into the PLZF-expressing Vγ1Vδ6.3 T lineage.

Results

PLZF expression is limited to certain subsets of γδ T cells

Intracellular FACS staining revealed that PLZF was expressed in 10-18% of γδ T cells from the thymus, spleen, liver and lymph nodes (Figure 7). PLZF was highly expressed by Vγ1-and Vδ6.3 T cells, but not by Vγ4- or Vδ4-expressing T cells (Figure 8). Consistent with previous reports, we found that the majority of Vδ6.3 T cells were paired with Vγ1 (Figure 9). The bulk of PLZF expression was restricted to these Vγ1Vδ6.3 T cells (Figure 9), although PLZF expression was also detected in a small percentage of Vγ1-only and Vδ6.3-only cells and some γδ T cells that expressed neither chain. Furthermore, PLZF was not expressed in all Vγ1Vδ6.3 T cells. In contrast to NKT cells, the percentage of Vγ1Vδ6.3 T cells was not significantly altered in the absence of PLZF (Figure 9). There was a decrease in the absolute numbers of γδ T cells in the thymus and spleen (Figure 10). This reduction was attributable to a general decrease in the size

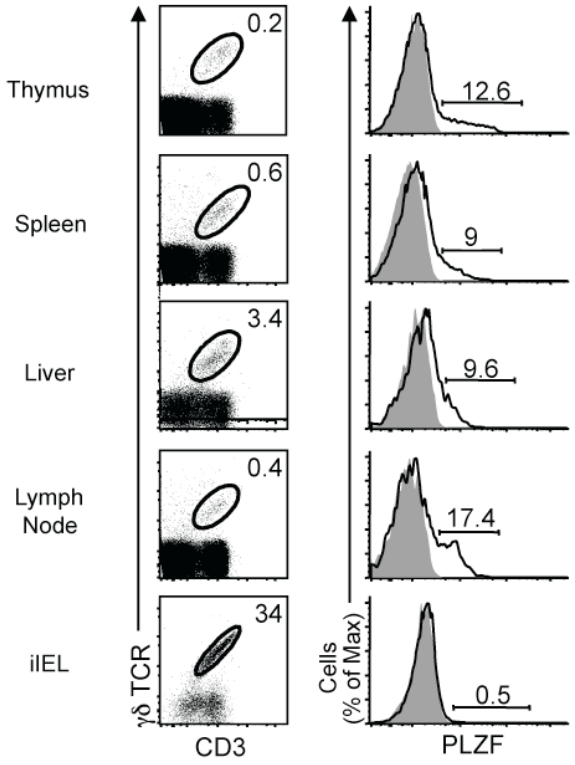

Figure 7. PLZF expression in γδ T cells from various tissues. Lymphocytes harvested from various primary and secondary lymphoid tissues, as well as from the IEL compartment stained with antibodies to detect bulk γδ T cells in wild-type (left; black line on histogram) and PLZF-deficient (right; filled histogram) mice. Data are representative of more than 6 independent experiments.

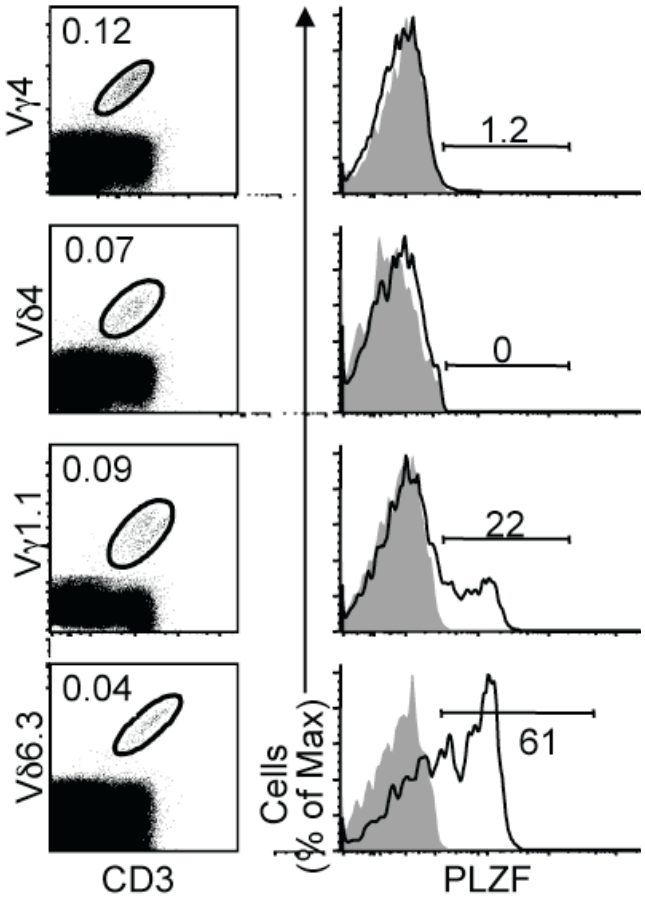

Figure 8. PLZF expression among γδ T cell subsets in the thymus. Wild-type and PLZF-deficient thymuses were stained with γδ TCR and one other γ- or δ-chain antibody to determine if PLZF expression was uniform among all γδ sublineages, or if the expression was restricted to a distinct subtype(s). Wild-type thymocytes (left; black line right) and PLZF-deficient thymocytes (right; filled histogram). Data are representative of at least 6 independent experiments.

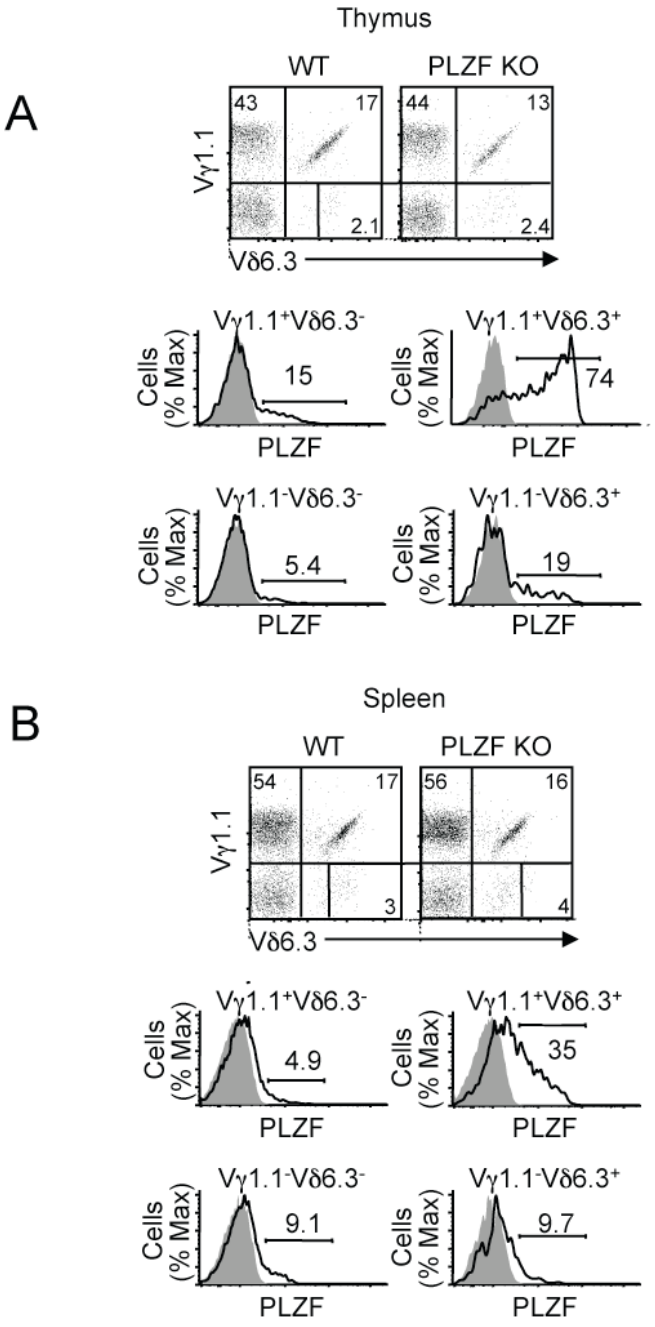

Figure 9. The majority of PLZF is expressed by $V\gamma1^{+}V\delta6.3^{+}$ T cells. (A) PLZF expression was ~70% of wild-type thymocytes (black line) and the pattern of expression indicated a heterogenous population of cells. In the spleen, (B) PLZF expression was found in ~25% of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells. All γδ T cell populations express some level of PLZF

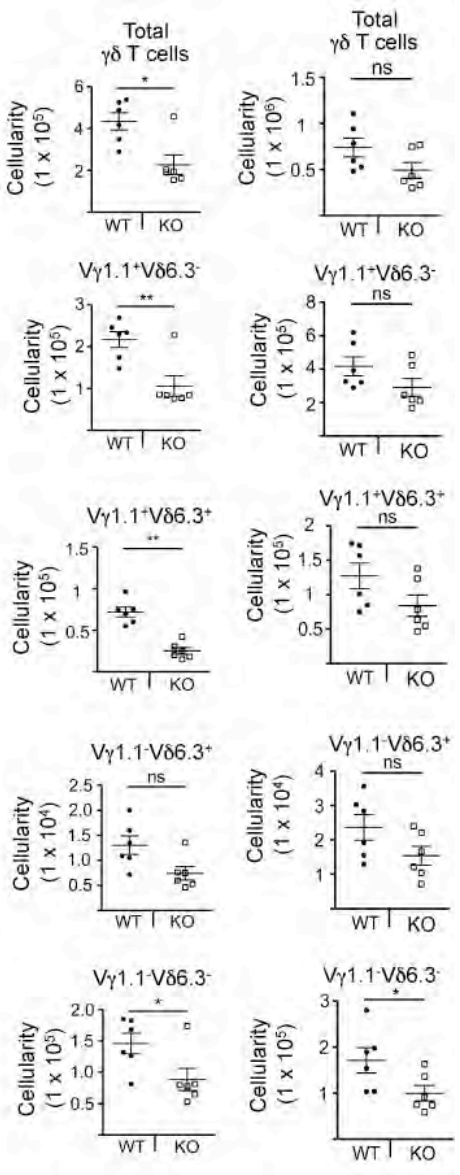

Figure 10. Absolute numbers of total γδ T cells and γδ sublineages in wild-type and PLZF-deficient thymocytes and splenocytes. Data are representative of six (wild-type n = 6; PLZF KO n = 6) independent experiments. Each symbol represents an individual mouse. $^{*}P = 0.05$; $^{**}P < 0.05$.

of the thymus and spleen, as previously reported[187]. PLZF expression was largely restricted to Vγ1Vδ6.3 T cells in the spleen and liver, but similar to NKT cells, the expression was reduced (Figure 9). However, Vγ1Vδ6.3 iIELs did not express PLZF (Figure 7). Overall, the data suggest tat there are essentially two different lineages of Vγ1Vδ6.3 γδ T cells: some that express PLZF and some that do not. This observation is consistent with the finding that Vγ1Vδ6.3 T cells developed in PLZF-deficient mice, which is in sharp contrast to the ~50 fold reduction seen for NKT cells.

PLZF-negative Vγ1Vδ6.3 T cells are phenotypically and functionally distinct

It was reported that up to half of Vδ6.3 T cells express NK1.1[228]. Consistent with this finding, ~40-50% of the Vγ1Vδ6.3 T cells in WT mice expressed NK1.1; however, we reliably found a higher proportion of NK1.1 expression among PLZF-negative Vγ1Vδ6.3 T cells compared with PLZF-positive Vγ1.V6.3 T cells (Figure 11A). PLZF-expressing Vγ1Vδ6.3 T cells had sharply reduced levels of the adhesion molecule L-selectin (CD62L) compared with WT PLZF-negative, and PLZF-deficient cells. CD44 expression was clearly reduced on PLZF-deficient Vγ1Vδ6.3 T cells, but there was only a subtle change when WT PLZF-positive and –negative Vγ1Vδ6.3 T cells were compared. The expression of NK1.1, CD69, CD122, DX5, and NKG2D was not substantially different among these populations (Figure 11A and data not shown). We did not observe any noteworthy change in the phenotype of the non-Vγ1Vδ6.3 γδ T cell subsets. Interestingly, PLZF-positive Vγ1Vδ6.3 thymocytes are largely $CD24^{low}$ compared with PLZF-negative Vγ1Vδ6.3 thymocytes. This is also consistent with NKT cells, which are all $CD24^{low}$ in the thymus[235]. Notably, nearly all $CD4^+$ γδ T cells, but none of the $CD8^+$ γδ T cells, expressed PLZF (Figure 11B).

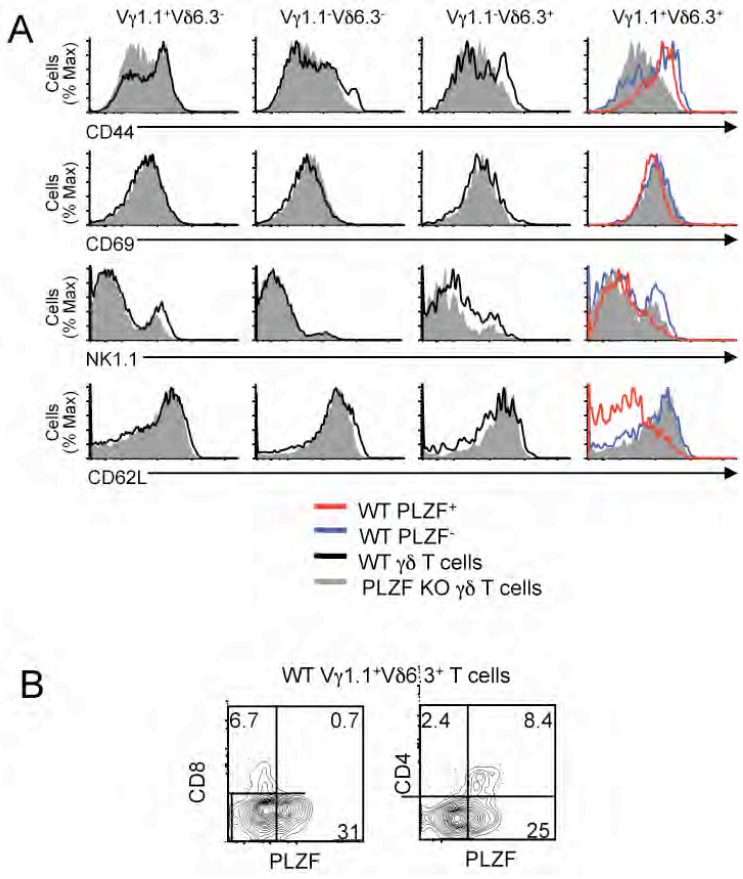

Figure 11. Phenotype of PLZF-positive and - negative $V\gamma1^{+}V\delta6.3^{+}$ T cells. (A) Expression of CD44, CD69, NK1.1, and CD62L by wild-type PLZF-positive (red), PLZF-negative (blue or black), and PLZF-deficient (gray) γδ thymocytes. (B) CD4 and CD8 expression on WT $V\gamma1.1^{+}V\delta6.3^{+}$ T cells compared with intracellular staining for PLZF.

We found a functional disparity between PLZF-expressing and PLZF-negative Vδ6.3 T cells in WT mice (Figure 12A). Sorted Vδ6.3 T cells from the spleens of WT mice were stimulated with PMA and ionomycin, followed by intracellular cytokine staining. More that 25% of the activated PLZF-expressing Vδ6.3 T cells produced IL-4 compared with ~1% of the PLZF-negative cells. Furthermore, similar to NKT cells[187], many PLZF-expressing γδ T cells simultaneously produced IL-4 and IFN-γ. Of particular interest, high levels of PLZF expression correlated with the ability of the γδ T cells to produce IL-4 (Figure 12A).

Next, we activated sorted Vδ6.3$^+$ and Vδ6.3$^-$ splenocytes from WT and PLZF-deficient mice with plate-bound anti-CD3 (Figure 12B). PLZF-deficient γδ T cells produced almost undetectable levels of IFN-γ and IL-4 compared with WT cells. Interestingly, expression of the CC chemokines MIP-1α and RANTES also was lost in the PLZF-deficient γδ T cells. These molecules are potent chemoattractants for macrophages and other immune cells in microbial and viral infections[236]. Using these experimental conditions, we did not detect obvious changes in the expression of IL-1, -10, -13, and -17 or TNF-α (data not shown).

It was shown that '*trans-conditioning*' of γδ T cells by αβ TCR-expressing thymocytes is necessary to induce the express of genes that regulate the differentiation and function of γδ T cells[168,237]. However, PLZF expression in Vγ1Vδ6.3 T cells is normal in TCRβ-deficient mice and, therefore, does not seem to require *trans*-conditiong for expression. Finally, the expression of PLZF and frequency of Vγ1.Vδ6.3 T cells is not significantly altered in CD1d-deficient mice, suggesting the absence of CD1d molecules and/or NKT cells has no significant affect on the development of these cells. Interestingly, transgene-mediated ectopic expression of PLZF does not alter the

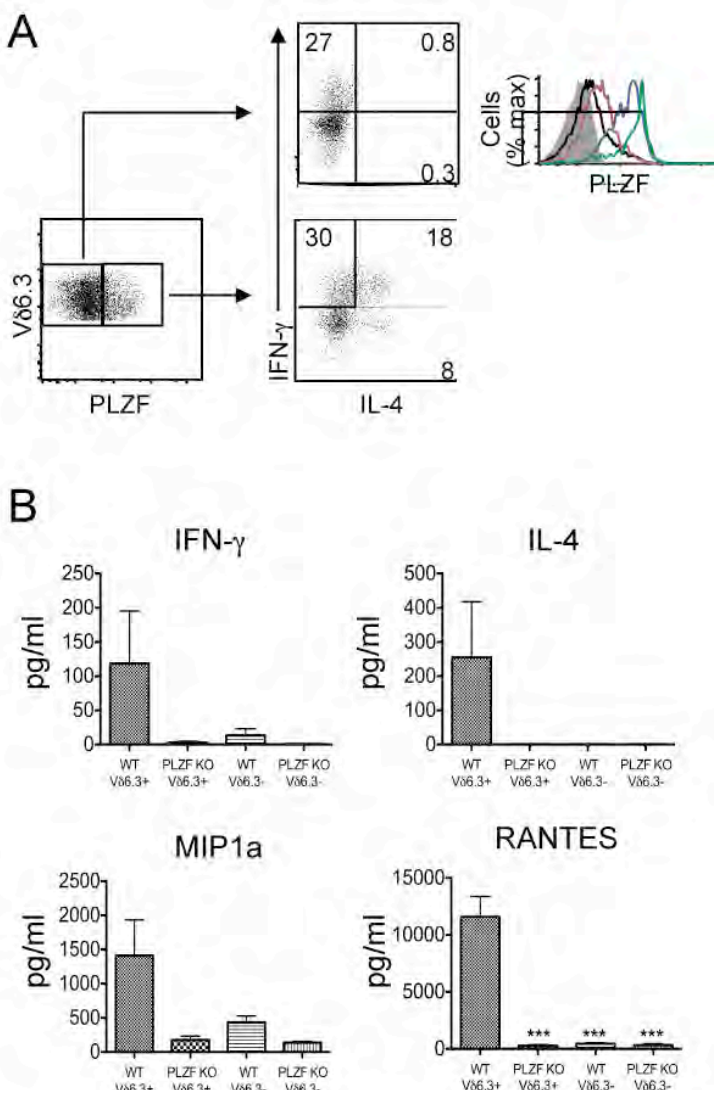

Figure 12. Functional analysis of wild-type and PLZF-deficient $V\gamma1^{+}V\delta6.3^{+}$ T splenocytes. (A) Intracellular staining for IFN-γ, IL-4, and PLZF in Vδ6.3 T cells following activation. Numbers indicate the percentage of cells in each quadrant. (B) Cytokine analysis of supernatants from wild-type and PLZF-deficient splenocytes with plate-bound anti-CD3 for 3d. Error bars represent the SD. ****P= 0.05.* Data are representative of five independent experiments.

phenotype of function of γδ T cells. It is noteworthy that endogenous levels of PLZF in WT Vγ1Vδ6.3 T cells was several fold higher compared with transgenic mice.

ThPOK expression in γδ T cells

In CD4 T cells, the BTB-ZF transcription factor ThPOK is required to maintain Th effector functions[202,233,234]. Interestingly, NKT cells some of which express CD4, were also shown to express ThPOK (T. Egawa and E.S. Alonzo, unpublished observations). Therefore, the finding that some of the PLZF-expressing Vγ1Vδ6.3 T cells also expressed CD4 raised the possibility that these cells also expressed ThPOK (Figure 11B). Therefore, tissues from two ThPOK-reporter mice were analyzed. One line had a copy of ThPOK replaced with a cDNA encoding a GFP ($ThPOK^{GFP/WT}$). The second line had the GFP allele and a null allele of ThPOK ($ThPOK^{GFP/-}$); therefore, it was deficient for the expression of ThPOK.

GFP expression was detected in ~25% of the γδ T cells in the thymus and nearly 45% of the γδ T cells in the spleen (Figure 13A). Other than in Vγ1Vδ6.3 T cells, the level of ThPOK expression in γδ T cells was far lower than in αβ TCR CD4 T cells (Figure 13A). Staining of the ThPOK reporter mice for PLZF showed that in Vγ1Vδ6.3 T cells, all of the ThPOK high-expressing cells expressed PLZF (Figure 13B). Interestingly, PLZF expression levels seemed to be reduced in ThPOK-deficient Vγ1Vδ6.3 T cells (Figure 13B). The decreased PLZF levels correlate with a loss of IL-4 expression but an increase in IFN-γ expression following activation of the Vγ1Vδ6.3 T cells. However, the absence of ThPOK did not significantly alter the frequency of Vγ1Vd6.3 T cells (data not shown).

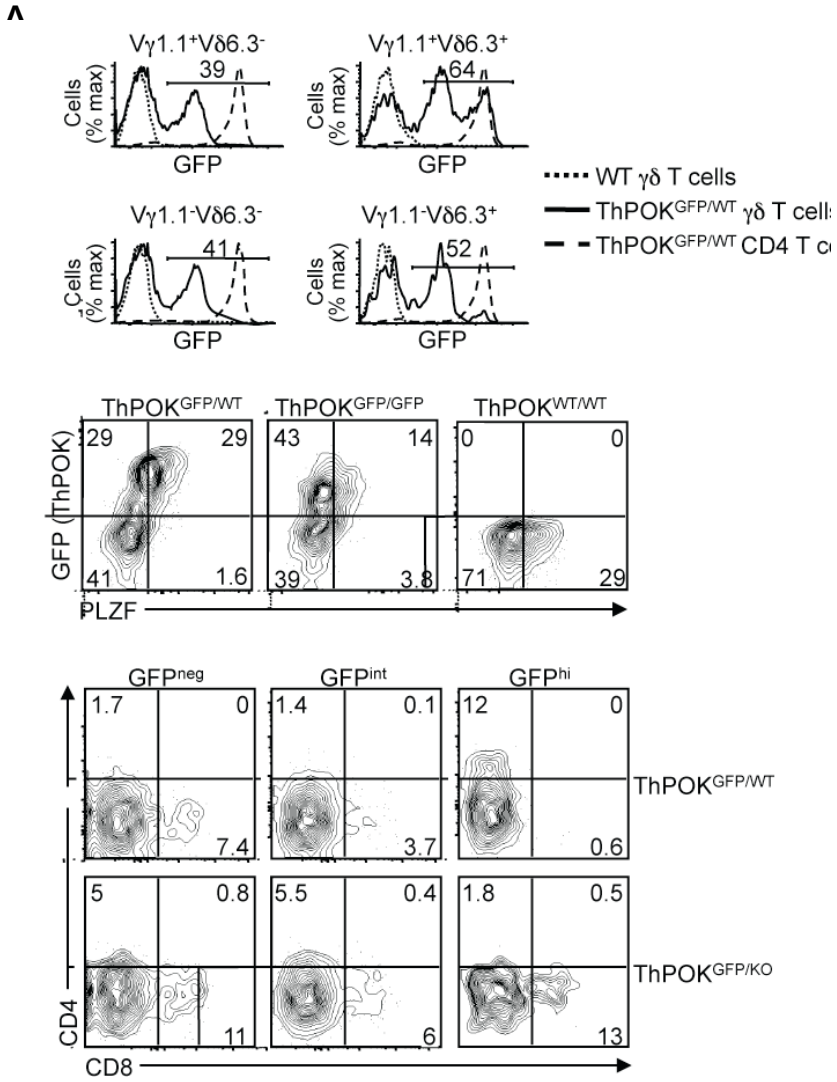

Figure 13. ThPOK expression in γδ T cell subsets. (A) ThPOK GFP reporter expression in spleens of γδ cells and CD4 T cells. Wild-type and ThPOK$^{GFP/WT}$. Numbers in quadrants indicate percentage of γδ T cells that express ThPOK$^{GFP/-}$. (B) GFP (ThPOK) and PLZF expression in spleen cells from ThPOK$^{GFP/+}$ and ThPOK$^{GFP/GFP}$ mice. (C) Expression of CD4 and CD8 in Vγ1.1^{+}Vδ6.3^{+} splenocytes from ThPOK$^{GFP/WT}$ and ThPOK$^{GFP/KO}$ reporter mice.

A distinct population of $CD4^+$ cells was found exclusively among the GFP^{hi} Vγ1Vδ6.3 T cells from $ThPOK^{GFP/WT}$ mice (Figure 13C). Interestingly, a large percentage of GFP^{hi} Vγ1Vδ6.3 T cells from $ThPOK^{GFP/KO}$ mice failed to express CD4. In the absence of functional ThPOK, some of the GFP^{hi} Vγ1Vδ6.3 T cells were essentially negative for the expression of CD4 or CD8 in both mouse lines.

Altered development and PLZF expression in signaling lymphocyte activated molecule-associated protein-deficient Vγ1Vδ6.3 T cells

NKT cell development is severely impaired in Fyn-[190] and signaling lymphocyte activation molecule (SLAM)-associated protein (SAP)-deficient mice[191]. Therefore, Fyn- and SAP-deficient mice were studied to determine whether these molecules were also necessary for the development of PLZF-expressing γδ T cells. Thymocytes and splenocytes from SAP- and Fyn-deficient mice were stained with Abs against Vγ1 and Vδ6.3 γδ T cell development was not overtly altered in either mouse (data not shown). The loss of Fyn correlated with an increased frequency of Vγ1Vδ6.3 T cells (+22%) in the thymus but a decreased frequency (−23%) in the spleen (Figure 14A). In SAP-deficient mice, there was a dramatic loss of Vγ1Vδ6.3 T cells. In the thymus and the spleen, the frequency decreased 61% and 36%, respectively, compared with age-matched WT mice (Figure 14). Furthermore, there was a substantial decrease in PLZF expression in the Vγ1Vδ6.3 T cells from SAP-deficient thymuses and spleens (Figure 15A). However, PLZF expression levels in Fyn-deficient Vγ1Vδ6.3 T cells were comparable to WT levels. The transgenic expression of PLZF in SAP-deficient mice did not restore the frequency of Vγ1Vδ6.3 T cells to WT levels (Figure 15). Finally, although SAP deficiency clearly impacted the development of the PLZF-expressing γδ T cells, there were not significant alterations in the expression of SLAMF1 (CD150), 2B4

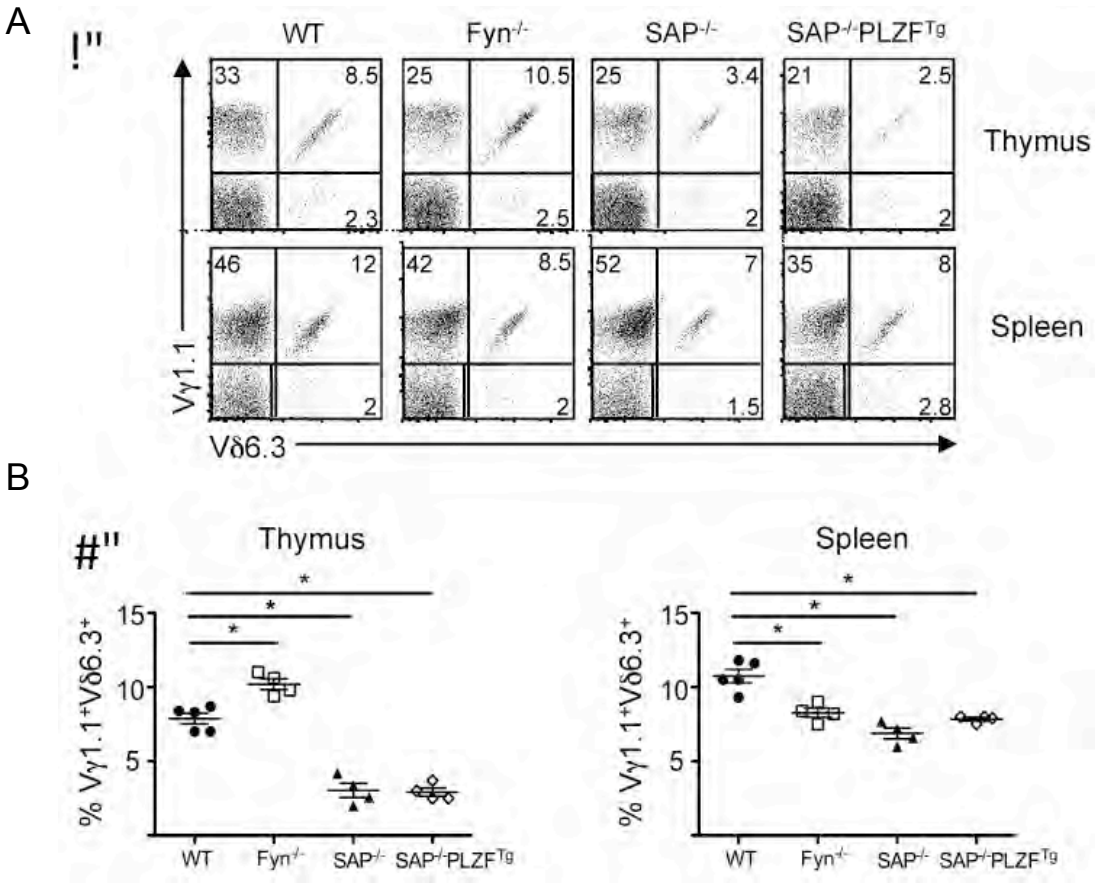

Figure 14. $V\gamma1.1^{+}V\delta6.3^{+}$ in Fyn- and SAP-deficient mice and SAP-deficient PLZF-transgenic mice. (A) FACS analysis of indicated γδ subsets thymocytes (top) and splenocytes (bottom) in indicated mouse strains. (B) Percent frequency of $V\gamma1.1^{+}V\delta6.3^{+}$ among thymocytes and splenocytes from wild-type (n = 5), Fyn-deficient (n = 4), SAP-deficient (n = 4), and SAP-deficient (n = 4) mice. Each symbol represents an individual mouse. *P = 0.05.

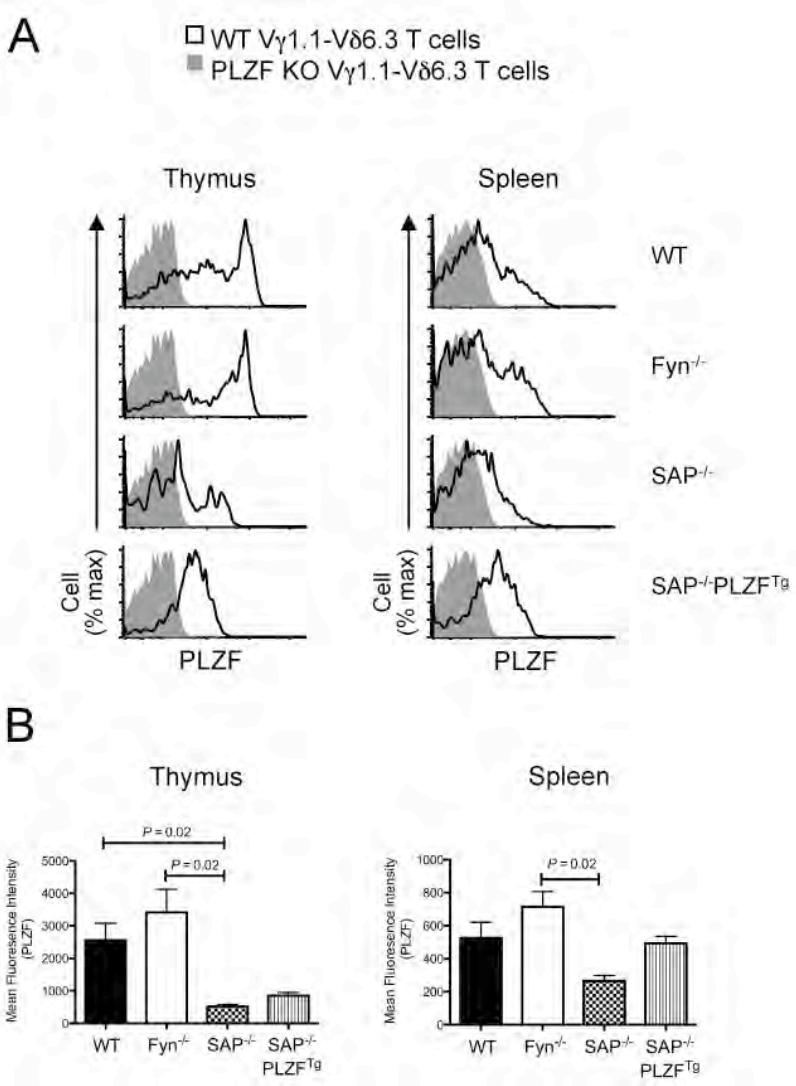

Figure 15. PLZF expression in $V\gamma1.1^+V\delta6.3^+$ in Fyn- and SAP-deficient PLZF-transgenic mice. (A) PLZF expression in $V\gamma1.1^+V\delta6.3^+$ thymocytes (left) and splenocytes (right) in indicated mouse strains (B) Mean fluorescence intensity (W/m^2) of PLZF levels in $V\gamma1.1^+V\delta6.3^+$ thymocytes: wild-type 2259±1049; Fyn KO =3464±1407; SAP KO 517±127; SAP KO-PLZF Tg=856±171; $V\gamma1.1^+V\delta6.3^+$: wild-type=527±187; Fyn KO 715±186; SAP KO=316±69; SAP KO-PLZF Tg=494±72. Error bars represent the SD. Data are representative of at least three experiments.

(CD244), or SLAMF6 (LY108) in PLZF-negative, -positive, or –deficient Vγ1Vδ6.3 T cells (data not shown). Together, these data show that unlike for NKT cells, Fyn is largely dispensable, but SAP plays an important role in the development of Vγ1Vδ6.3 T cells.

Signaling requirements for the development of PLZF-expressing γδ T cells

Data suggest that developing NKT cells require strong TCR-mediated signals for proper development[238]. Strength of signaling models have been proposed for directing thymocytes into the CD8αα and regulatory T cell lineages[239]. All of these T cell subsets are believed to express high-avidity, self-reactive TCRs that result in strong TCR signaling during development. It was also shown that strong TCR-mediated signals are necessary for commitment of thymocytes to the γδ T cell lineage, whereas weaker signals favor the αβ T cell lineage[165,166]. The restricted TCR profile of the PLZF-expressing γδ T cells suggested that the specificity of the TCR may play a pivotal role in directing the cells into this lineage. Therefore, the strength of TCR signal might affect Vγ1Vδ6.3 T cell development. To test this possibility, recently characterized SLP-76 (Src homology 2-domain-containing leukocyte phosphoprotein of 76 kDa) mutant mice, which have tyrosine to phenylalanine mutations 'knocked-in' at key sites of phosphorylation, were analyzed[240].

A mutation of the tyrosine at position 145 (Y145) within the n-terminal acidic domain of SLP-76 disrupts the interactions with IL-2 inducible T cell kinase (ITK) in Jurkat cells[241]. Consistent with the cell line studies, SLP76:Y145F mutant mice were found to phenocopy results from ITK-deficient mice[240]. In contrast, (Y112:128F) within the N-terminal domain of SLP-76 disrupts functional interactions with Vav1, a ρ-family GFP exchange factor, and Nck, an adaptor protein[242-244]. Thus, defects in proximal TCR

signaling in Y145F and Y112:128F mutant mice differentially affect some aspects of signaling.

In sharp contrast to the development of NKT cells, the Y145F and Y112:128F mutations resulted in an increased percentage of γδ T cells in the thymus (Figure 16A). The increase in absolute numbers of γδ T cells in the thymus of the mutant mice was not statistically significant; however, it was attributable to a decrease in total thymic cellularity (Figure 16B). The increased percentage of γδ T cells in the thymus as due to a 2- to 5-fold increase in the frequency and absolute numbers of Vγ1Vδ6.3 T cells in Y112:128F and Y145F mice, respectively (Figure 16A-C). There also was a significant increase in the percentage and absolute numbers of Vγ1Vδ6.3 T cell population in the spleen and lymph nodes of the two mutant mice. Furthermore, the frequency of PLZF-expressing γδ T cells was also increased, particularly in the spleen where nearly all of the Vγ1Vδ6.3 T cells in the mutant mice expressed high levels of the transcription factor (Figure 16D). This increase is PLZF expression was specific to Vγ1Vδ6.3 T cells, as we did not find elevated levels of PLZF expression among non-Vγ1Vδ6.3 γδ T cell subsets.

We activated pooled Vγ1Vδ6.3 T cells from the spleen and lymph nodes of wild-type, Y112:128F and Y145F mice with PMA/ionomycin followed by intracellular cytokine staining. In both mutant mice, the Vγ1Vδ6.3 T cells, but not the other γδ T cell subsets had significant increases in proportion of cells that expressed IFN-γ and IL-4 simultaneously, or IL-4 alone (Figure 17). Furthermore, there was an increase in both the frequency and amount of TNF-α on a per cell basis produced by the mutant Vγ1Vδ6.3 T cells (Figure 17). These findings indicate that altered TCR signaling, particularly at the SLP76-ITK interface, dramatically affects the differentiation and function of Vγ1Vδ6.3 T cells.

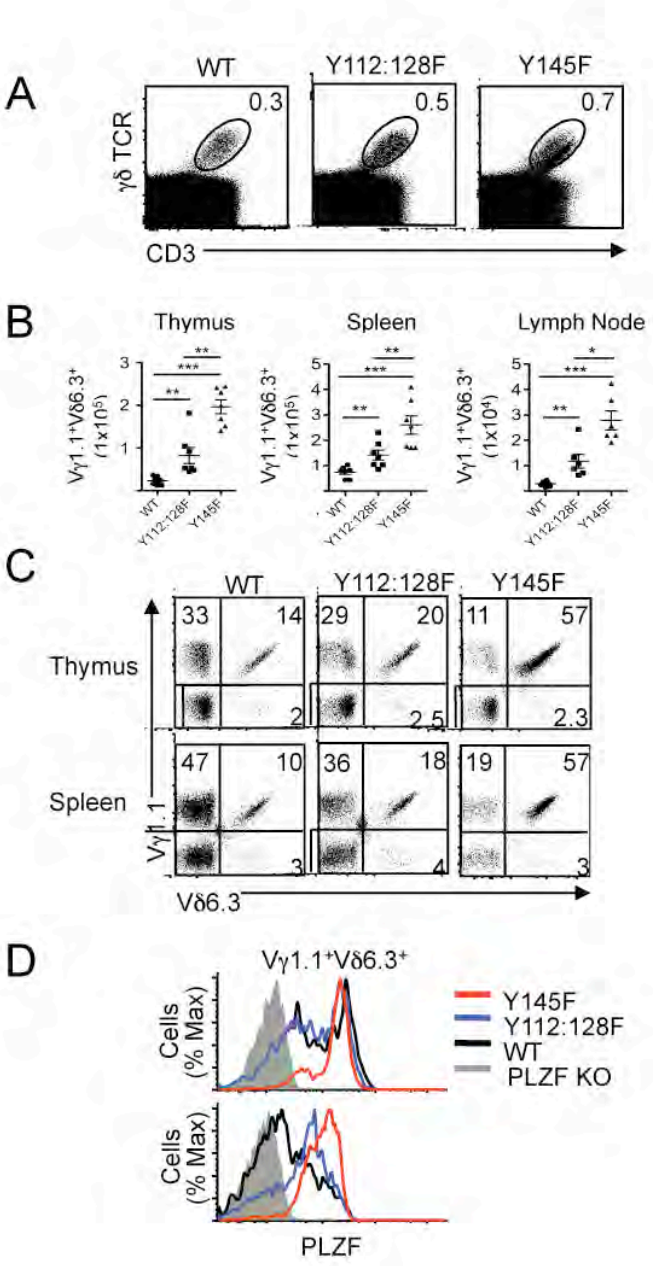

Figure 16. Reduced TCR signal strength enhances the development of Vγ1.1$^+$Vδ6.3$^+$ T cells. (A) Frequency of γδ T cells in the thymuses from wild-type, SLP-76 Y112:128F, and SLP-76 Y145F mutant mice. (B) The absolute numbers of indicated γδ subsets and PLZF expression analysis in Vγ1.1$^+$Vδ6.3$^+$ T cells in the thymus, spleen and lymph nodes of the indicated mouse strains (C) The frequency of γδ subsets and (D) PLZF expression analysis in Vγ1.1$^+$Vδ6.3$^+$ T cells in thymuses and spleen of indicated mouse strains.

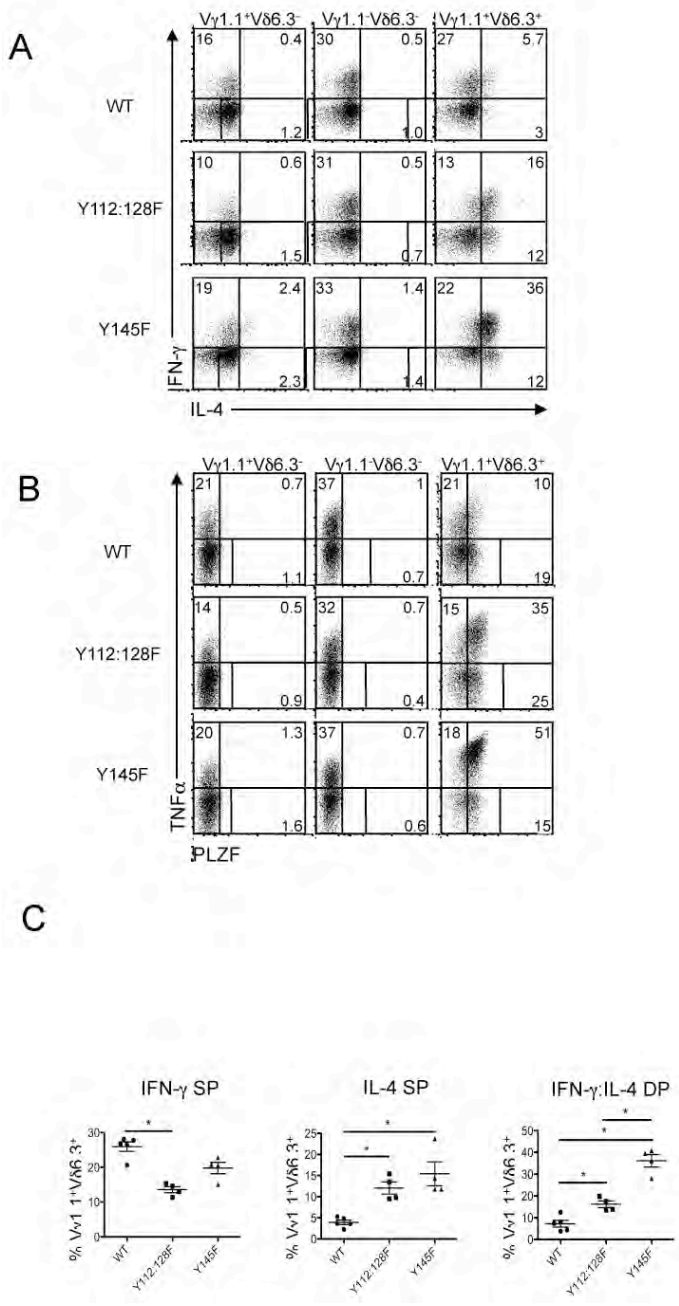

Figure 17. Reduced TCR signal strength enhances $V\gamma1.1^{+}V\delta6.3^{+}$ T cell function. Intracellular staining for IFN-γ and IL-4 (A) or TNF-α and PLZF (B) in indicated γδ subsets. (C) The frequency of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells that are SPs for IFN-γ (left), and IL-4 (middle) or DP (right) for IFN-γ and IL-4 from indicated mice. Each symbol represents *P = 0.05.

Id3 controls the development of Vγ1Vδ6.3 T cells

Strong TCR mediated signals are required to direct developing thymocytes into the γδ T cell lineage[165,166]. TCR signal strength, as measured by ERK1/2 phosphorylation, leads to increased transcription of Egr genes. Reduced Egr expression results in increased αβ T cell development, whereas increased Egr expression leads to enhanced γδ T cell development. Egr1 directly regulates transcription of Id3 (inhibitor of differentiation gene 3)[44]. In the absence of Id3, increased Egr expression no longer enhances the development of γδ T cells. Therefore, MAPK, ERK, Egr and Id3 appear to be in a linear pathway that regulates αβ- versus γδ T cell lineage decision[165,166]. To determine if Id3 was potentially a downstream gene target of altered strength of signaling in Vγ1Vδ6.3 T cells, Id3-deficient mice were examined.

Thymus cellularity in Id3-deficient mice is not altered, suggesting that early T cell development events are not under the control of Id3. Positive selection of αβ T cells, on the other hand, is significantly reduced in the absence of Id3[40]. In sharp contrast, we found that the percentage of γδ T cells in Id3-deficient mice was markedly increased the other hand, is significantly reduced in the absence of Id3 (Figure 18). Nearly identical to what was found in the TCR signaling mutant mice (Figures 17, 18), the frequency and absolute number of Vγ1Vδ6.3 T cells was increased (Figure 18A, B). Similar to the SLP76 mutants, the percentage of Vγ1Vδ6.3 T cells expressing high levels of PLZF was also greatly increased (Figure 18C). The phenotype of Vγ1Vδ6.3 T cells in Id3-deficient mice closely resembled what was observed in both SLP76 mutant mice, particularly with increases in the proportion of $NK1.1^+$ and $CD4^+$ cells, as well as upregulation of CD44, downregulation of CD62L.

Similarly to the SLP76 mutants, there was a significant increase in the frequency of Vγ1Vδ6.3 T cells that simultaneously produced both IFN-γ and IL4 (Figure 19A). There

was a two-fold increase in IL-4 single producers, and a five-fold increase in Vγ1Vδ6.3 T cells that expressed TNF-α (Figure 19). Overall these data show that Id3 deficiency leads to a Vγ1Vδ6.3 T cell phenotype that is very similar to what is found in mice with disrupted TCR signaling.

Discussion

In the absence of PLZF, NKT cells do not acquire innate T cell effector functions and do not express activation markers typically expressed by innate-like T cells[187,188]. Here we have shown that in addition to NKT cells, PLZF is also highly expressed by ~70% of thymic and ~40% of peripheral of Vγ1Vδ6.3 T cells (Figure 9). The presence of Vγ1Vδ6.3 T cells in PLZF-deficient mice and the significant reduction of only the PLZF-expressing Vγ1Vδ6.3 T cells in SAP-deficient mice strongly suggested that PLZF-positive and PLZF-negative Vγ1Vδ6.3 T cells represent two distinct lineages. Interestingly, the Vγ1Vδ6.3 iIELs also did not express PLZF, although it is possible that these cells developed extrathymically.

PLZF-positive and PLZF-negative Vγ1Vδ6.3 T cells were phenotypically similar (Figure 11A). Compared with the other γδ T cell subsets, both populations expressed high levels of CD44 and about half expressed NK1.1. Low expression of CD62L, however, clearly distinguished PLZF-positive from PLZF-negative Vγ1Vδ6.3 T cells. CD44 expression was also diminished in PLZF-deficient Vγ1Vδ6.3 T cells. In contrast to NKT cells, the absence of PLZF does not alter NK1.1 expression in Vγ1Vδ6.3 T cells. We did not observe any noteworthy changes in the phenotype of non-Vγ1Vδ6.3 γδ T cell subsets from PLZF-deficient mice.

Some of the activated PLZF-positive Vδ6.3 T cells simultaneously secreted IFN-γ and IL-4, while PLZF-negative Vδ6.3 T cells only secreted IFN-γ (Figure 12).

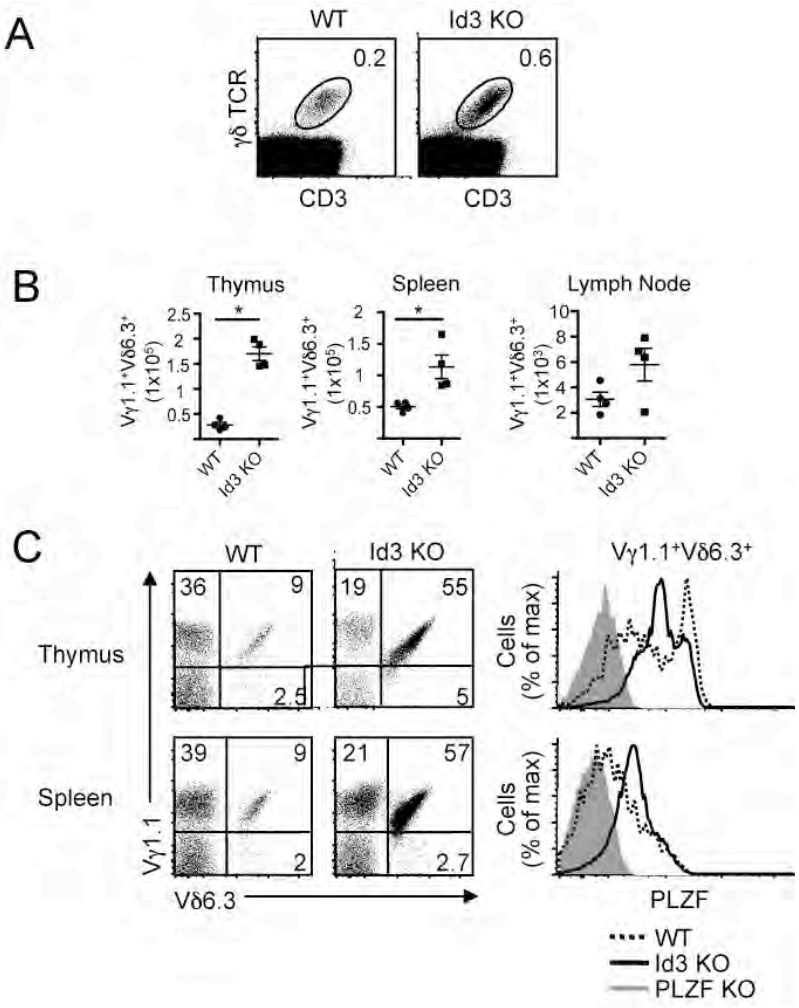

Figure 18. Id3 controls the development of PLZF-expressing $V\gamma1.1^{+}V\delta6.3^{+}$ T cells. (A) The frequency of γδ T cells in the thymuses of wild-type and Id3-deficient mice. (B) The absolute numbers of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells in the thymus, spleen, and lymph nodes of wild-type and Id3 KO mice. **P*=0.0005. (C) The frequency of γδ T cell subsets in WT and Id3 KO mice (left) and PLZF expression analysis of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells thymocytes and splenocytes of WT and Id3 KO mice (right).

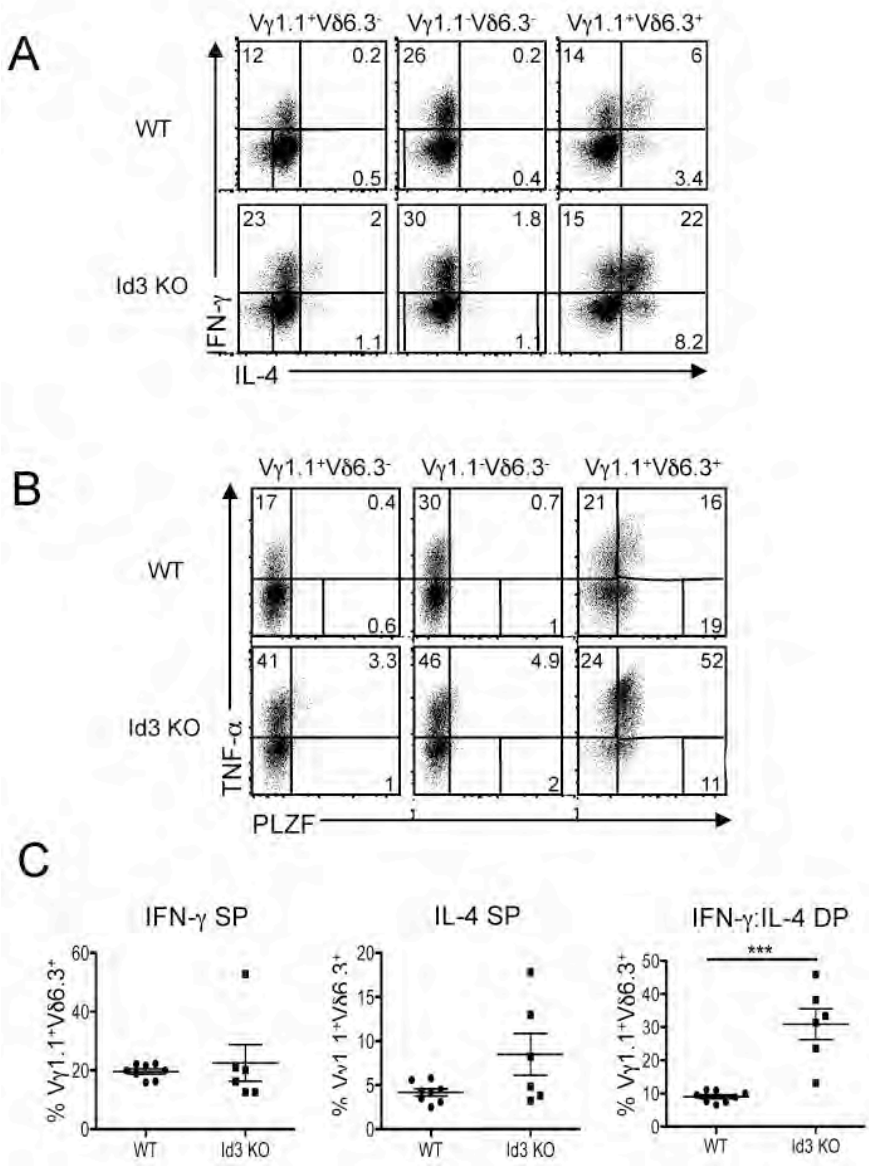

Figure 19. Gain of function phenotype in Id3-deficient $V\gamma1.1^{+}V\delta6.3^{+}$ T cell function. (A) Intracellular staining for IFN-γ and IL-4 (A) or TNF-α and PLZF (B) in indicated γδ subsets from pooled splenocytes and lymphocytes in wild-type and wild-type and Ide KO mice. (C)The frequency of $V\gamma1.1^{+}V\delta6.3^{+}$ T cells that are SPs for IFN-γ (left) and IL-4 (middle) or DPs for IFN-γ and IL-4 (right) in wild-type and Id3 KO mice. Data representative of at least four experiments. Error bars represent the SD.

Furthermore, Vδ6.3 T cells from PLZF-deficient mice produced less IFN-γ and no IL-4, reflecting functional data observed for PLZF-deficient NKT cells. In addition, PLZF-positive Vδ6.3 T cells secreted large amounts of MIP1α and RANTES, chemokines important for recruiting macrophages and other cells to sites of infection (Figure 12B). This finding appears to be consistent with the immunoregulatory role of Vγ1Vδ6.3 T cells during microbial infections. Taken together, these data demonstrate that PLZF defines the functional potential of Vγ1Vδ6.3 T cells and distinguishes these cells from other γδ T cells.

We found that a substantial percentage of γδ T cells expressed ThPOK. High ThPOK expression was almost exclusively found in the PLZF-expressing Vγ1Vδ6.3 T cells, although the majority of these cells did not express CD4 (Figure 13). Nonetheless, ThPOK is clearly required for CD4 expression in Vγ1Vδ6.3 T cells since in the absence of this transcription factor, the CD4-expressing cells appear to switch to CD8 expression (Fgiure 14C). It was recently shown that ThPOK expression is required to maintain CD4 T cell effector functions. In ThPOK-deficient mice, there was ~50% decrease in PLZF expression in Vγ1Vδ6.3 T cells (Figure 13B). It is interesting to speculate that ThPOK is required for some aspects of Vγ1Vδ6.3 T cell function, but it's function is modified by PLZF.

PLZF-expressing Vγ1Vδ6.3 T cells and NKT cells appear to have many features in common. However, the factors that control the development of NKT cells and PLZF-expressing Vγ1Vδ6.3 T cells appear to be different. The loss of Fyn nearly ablates NKT cell development, but has no impact Vγ1Vδ6.3 T cells (Figure 15). Even the loss of SAP, which is essential for NKT cells, only partially affects Vγ1Vδ6.3 T cell development (Figure 15). In SAP-deficient mice, however, there was a substantial decrease in the frequency and level of PLZF expression in Vγ1Vδ6.3 T cells.

Strong TCR-mediated signals are critical for the development of several T cell lineages, including gd T cells and NKT cells. Therefore, we examined the development of Vγ1Vδ6.3 T cells in mice carrying mutations of key tyrosines in SLP76. The SLP76-Y145F mutation phenocopies mice deficient for expression of ITK. Remarkably, the frequency and absolute numbers of Vγ1Vδ6.3 T cells expressing high levels of PLZF were dramatically increased these mice (Figure 16). The percentage of cells that produced IFN-γ and IL-4 simultaneously also significantly increased, as did the percentage of cells that produced only IL-4 (Figure 17). The SLP76-Y112:128F mutation also resulted in a significant increase of cells with these characteristics, but the change was not as great as with the Y145F mutation. Both mutations also resulted in a higher percentage of $CD4^+$ and $NK1.1^+$ PLZF-expressing cells.

Interestingly, the loss of ITK promotes the development of innate-like CD8 T cells. Therefore, reduced TCR signal strength requirements may be a common feature for some innate-like T cells. Indeed, ITK mice were also recently shown to have increased numbers of Vδ6.3 T cell. However, both of the SLP76 mutant mice and, also ITK deficient mice have substantially reduced numbers of NKT cells. The Y145F and Y112:128F mutations differentially alter TCR-induced calcium flux and MAPK/ERK phosphorylation and both mutations also result in reduced NFAT activity. Indeed, the reduced NFAT could be involved in the NKT cell development defect since NFAT controls early growth response 2 (Egr2), which is necessary for NKT cell development.

The downstream affects of the Y112:128F mutation of SLP76 are not entirely clear since this mutation likely disrupts the function of the guanine exchange factor Vav1 as well as the adapter protein, Nck. Nck plays a role in actin cytoskeletal reorganization following TCR activation and also directly interacts with CD3. Interestingly, Nck was also shown to interact with SAP. Therefore, it is possible that altered Nck activity in

Y112:128F mutant mice might reduce SAP activity, thereby counterbalancing the effects of altered TCR signaling in these mice. This might explain why the Vγ1Vδ6.3 T cell phenotype in the SLP76-Y112:128 mutant mice was less dramatic compared with Y145F mutant mice. In support of this, SLP76-Y145F, SAP-deficient mice do not have increased numbers of PLZF-expressing Vγ1Vδ6.3 T cells (not depicted). Therefore, the increased frequency of Vγ1Vδ6.3 T cells that is a consequence of altered TCR signaling does not supersede the requirement for SAP-mediated signaling.

Commitment to the γδ lineage is a consequence of strong TCR signaling that results in increased MAPK/ERK activity that leads to the induction or Erg1[166]. Transcription of Id3, a molecular target of Egr1, inhibits E protein activity, which is thought to set signaling thresholds that influence αβ/γδ-lineage choice. We found that Id3-deficient mice had increased numbers of Vγ1Vδ6.3 T cells and that nearly all of these cells express high levels of PLZF (Figure 18). Remarkably, the phenotype of Vγ1Vδ6.3 T cells in the Id3-deficient mice was nearly identical to what was found in the SLP76 mutant mice. Id3-deficient mice had an increased frequency of TNF-α single producers and IFN-γ and IL-4 double producer Vγ1Vδ6.3 T cells (Figure 19). These data, in some ways, appear to contradict previously established models of TCR signaling in γδ T cell development. For example, it has been shown that for γδ T cell development between fetal stages E15-E18, the lack of Id3 impedes the development of γδ T cells. However, neither Vγ1 nor Vδ6.3 TCRs are actively being rearranged during fetal development. Furthermore, several studies have shown that specific γδ T cell populations rely on certain molecules for their development, but those same molecules are seemingly dispensable for other γδ T cell populations.

It remains to be addressed whether PLZF-positive and-negative Vγ1Vδ6.3 T cells have different specificities due to variability in the TCR CDR3 regions. This might lead to

different strengths of signaling during selection in the thymus. For example, a recent study showed that in the absence of MHC class I-like molecules T10/T22 in β2m-KO mice, γδ T cells that normally recognize these ligands developed, but had a profound change in their effector functions[145]. Two studies relevant to this question were recently published[178,245]. Kreslavsky et al. showed that T cells in mice carrying transgenes encoding a Vγ1Vδ6.3 TCR were strongly, but not completely, skewed toward the PLZF-expressing lineage[178]. They also showed that PLZF is induced in γδ thymocytes after culturing on OP9-DL1 cells for 5 d in the presence of anti-γδ TCR Ab[178]. Together with data showing that CD5 levels were higher on the Vγ1Vδ6.3 thymocytes, it was proposed that strong TCR-mediated signals are involved in inducing PLZF expression[178]. Contrary to this conclusion, our data clearly showed that, *in vivo*, reduced TCR signal strength results in a dramatic increase in the PLZF-expressing γδ T cell lineage. Furthermore, only ~70% of the Vγ1Vδ6.3 TCR transgenic thymocytes and ~60% of WT Vγ1Vδ6.3 T cells expressed PLZF[178]. Therefore, despite expressing the identical TCR, PLZF was not induced in all cells. In contrast, once the TCR signal strength was reduced by the Y145F mutation to SLP-76, nearly all of the Vγ1Vδ6.3 T cells expressed high levels of PLZF (Figure 16). Together, these data seem to make a simple TCR avidity-based model for inducing PLZF expression tenuous.

Similar to our studies, Lauritsen et al.[245] and Ueda-Hayakawa et al.[246] recently showed that Vγ1 T cells are increased in the absence of Id3. Ueda-Hayakawa et al.[246] suggested that, in the absence of Id3, there is an increased window of opportunity for thymocytes to rearrange TCRγ-chains. In contrast, Lauritsen et al.[245] argued that the alteration in γδ T cells is due to the failure to negatively select thymocytes expressing the Vγ1 TCR. Neither of these explanations seems to fully account for our observations.

In the study by Ueda-Hayakawa et al.[246] it was proposed that double-negative cells that had failed to rearrange a functional TCRβ-chain were able to undergo further TCR rearrangement at the TCRγ-locus in the absence of Id3. Therefore, it was proposed that Id3 restricts the 'window' of TCR gene rearrangement in the double negative thymocytes. However, it is not clear how the same reasoning can be used to explain the nearly identical increase in Vγ1 T cells that we found in the SLP-76 mutant mice (Figure 16), because the signaling mediated by SLP-76 at the double-negative stage is expected to be dependent on pre-TCR expression. It was shown that the complete loss of SLP-76 disrupts TCRβ allelic exclusion. Therefore, it remains possible that disrupted allelic exclusion allows for the additional TCRγ-chain rearrangements.

Decreased negative selection due to reduced TCR signals, as proposed by Lauritsen et al.[245], at first seems to be more consistent with our data. However, a clear role for negative selection of developing γδ T cells remains to be defined. Furthermore, the idea that NK1.1^{+} γδ T cells are self-reactive is largely based upon comparisons with NKT cells. WT NKT cells have a constitutively activated phenotype, which was believed to a consequence of their TCR specificity for a ubiquitously expressed self-Ag. However, this concept has fallen from favor, now that it has been shown that the activated NKT cell phenotype is a consequence of the expression of PLZF[187]. Furthermore, it is difficult to understand why most Vγ1Vδ6.3 TCR-expressing thymocytes are negatively selected, whereas other expressing the same TCR mature. Finally, reduced negative selection does not explain the increase in PLZF-expressing Vγ1Vδ6.3 T cells from ~60% to nearly 100% in the Id3-deficient and SLP-76 mutant mice.

We propose that the increase in Vγ1Vδ6.3 T cells in SLP-76 mutant and Id3-deficient mice is a consequence of cells being directed into the PLZF-expressing lineage. PLZF-expressing NKT cells are known to go through an enormous proliferative

burst early in development. Therefore, it is likely that the PLZF-expressing γδ T cells also undergo through a similar expansion, although this has not been directly tested. Furthermore, NKT cells rapidly accumulate in mice, and it is possible Vγ1Vδ6.3 T cells also have this feature, perhaps as the result of both cell types during the postnatal period.

Overall, our work demonstrates the presence of two functionally distinct subsets of Vγ1Vδ6.3 T cells based on PLZF expression. Our data suggests a model in which reduced TCR signaling strength leads to a decrease in Id3 transcription, likely to reduced Egr activity. The lack of Id3 then directs cells into the PLZF-expressing Vγ1Vδ6.3 T cell lineage rather than the non-PLZF expressing γδ T cell lineage. The similarity of the phenotypes observed in the SLP-76:Y145F mice and the Id3-deficient mice is consistent with a linear relationship, analogous to what was reported in γδ T cells. This model is supported by recent data showing that conditional deletion of E2A in Id3-deficient T cells prevents the increase of Vγ1 γδ T cells[246]. Our work provides new insight into γδ T cell differentiation and function, while highlighting the role of BTB-ZF proteins in these enigmatic cells.

RESULTS II

Aberrant RORγt-activity restricts Id3-deficient γδ NKT cells from developing into the dominant T lymphocyte[c]

Introduction

The presence of two distinct T lymphocytes indicates that higher vertebrates evolved two unique modes of T cell-mediated immunity. αβ- and γδ T cells have many overlapping functions, however, the mechanism by which they recognize antigen is clearly different. The factors that control αβ- versus γδ lineage commitment are uncertain. The general model outlining αβ/γδ bifurcation argues that cell fate to either lineage depends on quantitative signaling generated by TCR:ligand interactions. This 'strength of signal' model posits that progenitor T cells are neither pre-committed to one lineage or the other, nor are they instructed to adopt a particular fate that is dependent upon the TCR type they express. The model is largely built upon data suggesting that γδ T cells require 'strong' TCR signals while αβ T cells require 'weak' TCR signaling for development. The E protein inhibitor Id3, a direct target of TCR signaling pathways, plays a pivotal role in the strength of signal model. Id3 activity is directly proportional to the magnitude of TCR signals generated by the cell. In summary, the signal strength model suggests that developing T cells have a certain degree of 'plasticity' that enables them to adopt the opposite lineage fate based on the relative strength of TCR signaling and Id3 activity.

Recently, we and others, showed a dramatic and selective expansion of Vγ1Vδ6.3 (γδ NKT) T cells in both TCR signaling mutant and Id3-deficient mice[179,245-248]. This expansion was not at the expense of other γδ sublineages. These findings would appear to contradict the signal strength model, which predicted an increase in the αβ lineage at the expense of the γδ lineage in these mice. Importantly, these results clearly

[c] *For this section, Vγ1.1$^+$Vδ6.3$^+$ T cells will be referred to as Vγ1Vδ6.3 T cells, or γδ NKT cells.*

demonstrated that different γδ sublineages interpret TCR signaling differently. Several explanations for the expanded γδ NKT cell population have been proposed. One model supports the view that γδ NKT cells require strong signals for development and, therefore, the absence of Id3 permits survival of cells that would normally undergo negative selection as a result of exceptionally strong TCR signaling. However, an increasing body of evidence indicates that γδ T cells do not undergo ligand-mediated selection. An alternative view is that in the absence of Id3, progenitors have an extended 'window' of time to rearrange TCR genes, as a consequence of diminished inhibition of E2A, whose role in TCRγ and -β chain recombination is well-documented[246]. This explanation seems unlikely, since this extended window of TCR rearrangement would have to be specific to γδ NKT cells. We suggest that these observations reveal the existence of two distinct populations of γδ T cells: Id3-independent and PLZF/Id3-dependent γδ T cells. As a result, a single model of development for both lineages is unsatisfactory.

E proteins have a clear role in regulating several aspects of T cell development[39,204,249]. E2A expression is rapidly induced at DN2 where it controls transcription of a T cell-specific gene module through regulation of Notch expression, which is required for T lineage commitment[205,249]. Notably, E2A promotes TCR rearrangement by directly regulating chromatin accessibility in a dose-dependent manner[39]. HEB has explicit roles at the β-selection checkpoint, and together with E2A, functions in DP cells to inhibit early transition into the CD8 SP T cell lineage[41,46]. Overall, it is clear that the level of E proteins can have important consequences during T cell development. It is noteworthy, that the expansion of *Id3*$^{-/-}$ γδ NKT cells is restored to near wild-type levels when cells are also deficient in E2A. These data undoubtedly implicate E2A in the expansion of γδ NKT cells in Id3-deficient mice[246,248].

Previous experiments showed the spontaneous acquisition of a Th17 phenotype in *Id3*$^{-/-}$ γδ NKT cells that was controlled by the transcription factor RORγt. To further clarify mechanisms controlling the expansion of *Id3*$^{-/-}$ γδ NKT cells, we asked if other targets of the Egr-Id3-E2A pathway might be aberrantly expressed during development. Indeed, the expression of RORγt was detected by flow cytometry in *Id3*$^{-/-}$ γδ thymocytes. The generation of *RORc*$^{-/-}$*Id3*$^{-/-}$ mice revealed a striking increase of γδ T cells at the expense of αβ T cells. Nearly all (~99%) of these γδ T cells expressed Vγ1Vδ6.3 TCRs and PLZF, indicating they belong to the γδ NKT lineage. This dramatic phenotype appears to be T cell-specific, as these mice had normal frequencies of bone marrow progenitor populations, NK and B cells, and myeloid cells. In addition, the overall thymic architecture appeared normal. However, analysis of thymic progenitors revealed major alterations in DN2 – DN4 stages of development. Our findings uncover a fundamental regulatory pathway that favors the development of γδ NKT cells, while restricting the maturation of progenitor T cells into the αβ lineage.

Results

Development of Th17 γδ NKT cells in Id3$^{-/-}$ *mice*

Functional analysis of γδ NKT cells from *Id3*$^{-/-}$ mice revealed the spontaneous acquisition of a Th17 effector program (Figure 20A, B). The differentiation of Th17 cells is controlled by the orphan nuclear receptor transcription factor RORγt. Since γδ NKT cells do not normally produce IL-17, we assessed the expression of RORγt in WT and *Id3*$^{-/-}$ γδ NKT cells by intracellular staining and flow cytometry. We detected RORγt in ~25% of *Id3*$^{-/-}$ γδ NKT cells from the thymus, but little or none was found in cells from the spleen (Figure 20 C,D). Consistent with previous reports, we did not detect the presence of RORγt in WT γδ NKT cells.

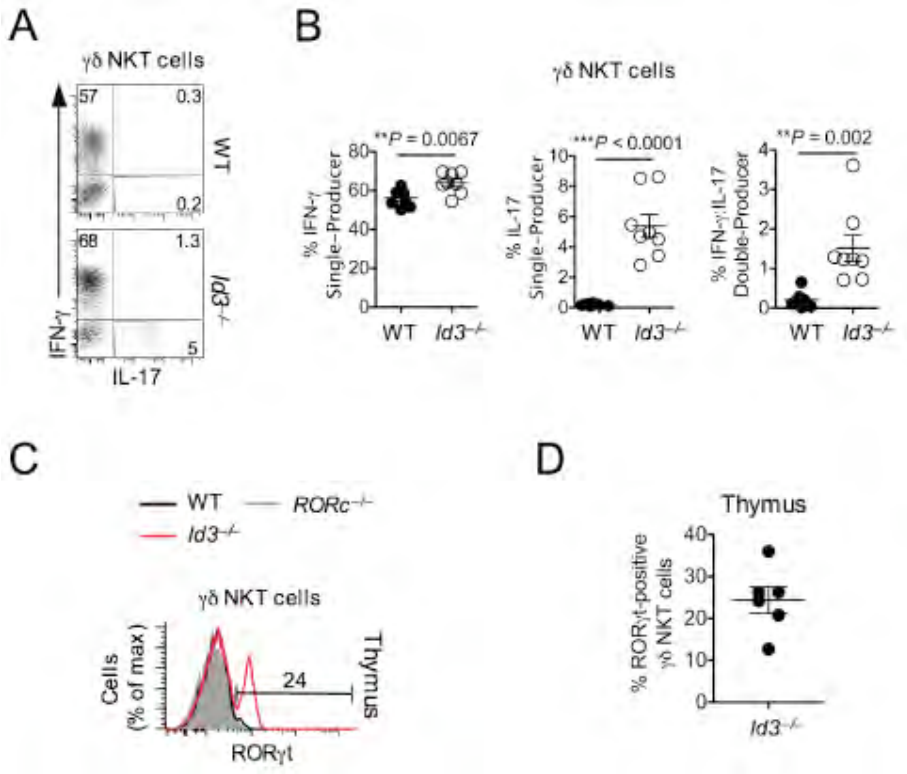

Figure 20. Spontaneous development of Th17 γδ NKT cells in Id3-deficient mice. (A,B) Id3-deficient γδ NKT cells produce a significant amount of IL-17. (C,D) ~25% of Id3-deficient γδ NKT cells express high levels of RORγt in the thymus. Data are representative of at least six independent experiments.

RORγt controls differentiation of PLZF-expressing γδ NKT cells

While αβ T cell development was severely affected in the absence of RORγt, there was no defect in frequencies γδ sublineages in either the thymus or spleen (Figure 21A,B). Unexpectedly, however, we observed a marked reduction in the frequency (~50%) of γδ NKT in the thymus and periphery that express PLZF in *RORc*$^{-/-}$ mice (Figure 22A). There was also a decrease of PLZF on a per cell basis, as the majority of *RORc*$^{-/-}$ γδ NKT cells expressed low levels of PLZF relative to wild-type cells (Figure 22A). PLZF-deficient γδ NKT cells fail to acquire all the salient features typically associated with innate T cells. Paradoxically, *RORc*$^{-/-}$ γδ NKT thymocytes exhibited a more robust innate-like phenotype compared to wild-type controls. Nearly all *ROR*$^{-/-}$ γδ NKT thymocytes expressed high levels of CD44 and NK1.1 while CD62L and CD24 were virtually absent (Figure 22B). However, the phenotype was remarkably similar between WT and *RORc*$^{-/-}$ γδ NKT cells in the spleen (Figure 22B).

The mechanism by which PLZF programs the innate effector functions of γδ NKT cells is unclear, however, the level of PLZF expression in these cells indeed impacts overall cytokine production, particularly IL-4. To evaluate whether the lack of RORγt had any impact on the cytokine secretion among various γδ T cell populations, we activated wild-type and *RORc*$^{-/-}$ splenocytes and stained for IFN-γ and IL-4, the signature cytokines of γδ NKT cells. *RORc*$^{-/-}$ γδ NKT cells produced far less IL-4 (~60%) compared with wild-type γδ NKT cells (Figure 22C). In particular, there were considerably fewer cells producing IFN-γ and IL-4 simultaneously (Figure 22C). This data is consistent with previous publications demonstrating that PLZF is critical for IL-4 production in γδ NKT cells. The loss of RORγt did not affect the IFN-γ secretion by γδ NKT cells, or in other γδ sublineages (Figure 22C).

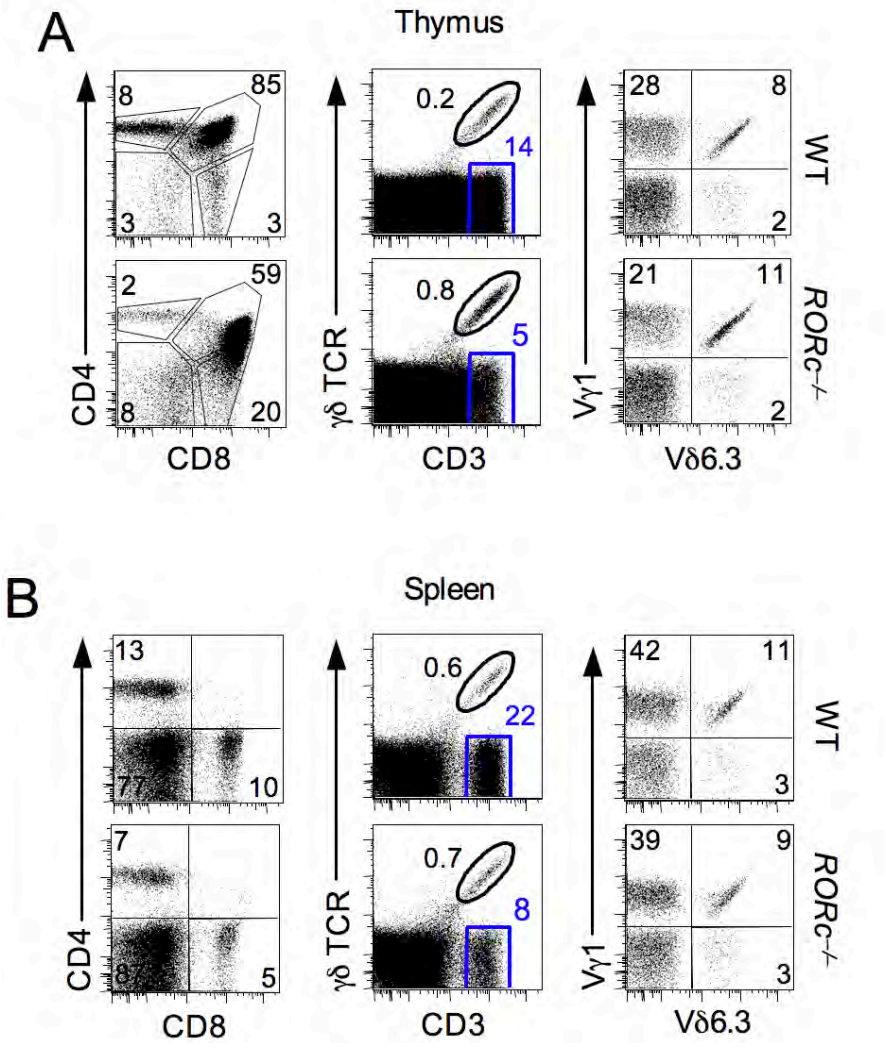

Figure 21. Overall frequency of γδ sublineages are similar between wild-type and RORγt-deficient mice. (A) thymus (B) spleen. Data are representative of at least six independent experiments.

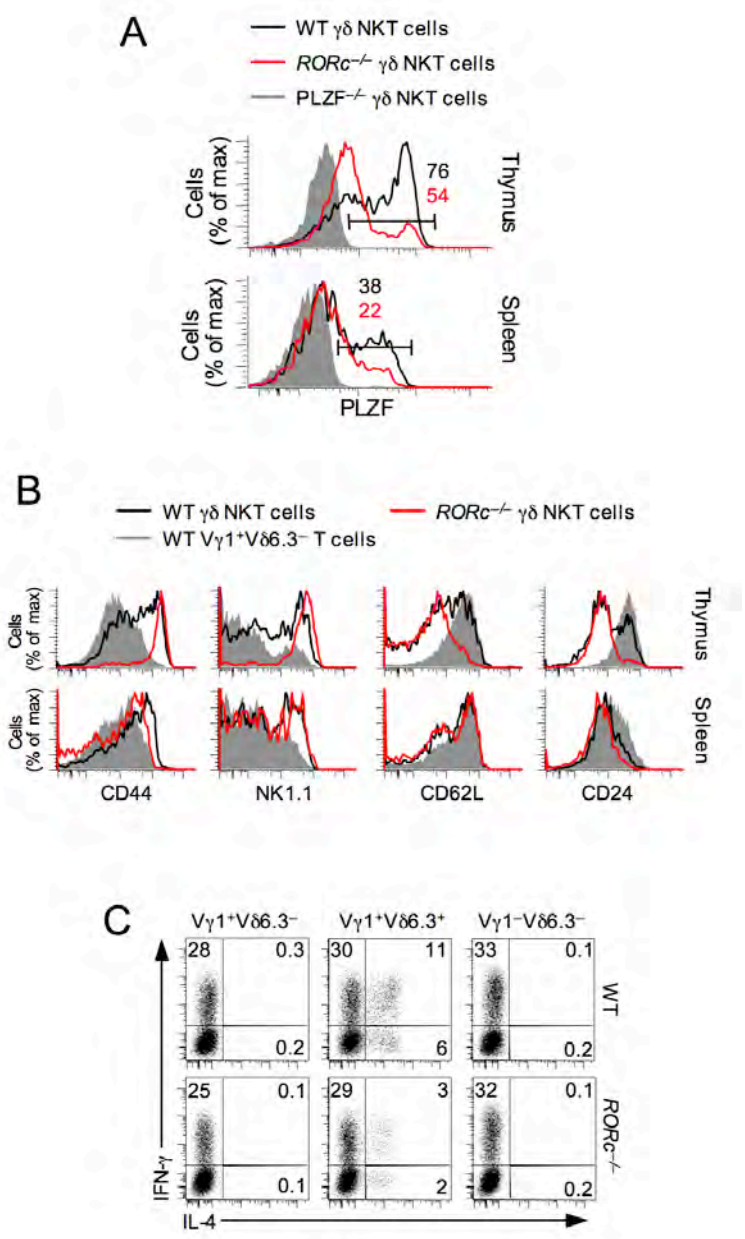

Figure 22. Altered development of γδ NKT cells in RORγt-deficient mice. (A) The frequency of PLZF expression is significantly lower in the thymus and spleen in RORγt-deficient mice (B) CD44, NK1.1, CD62L, and CD24 expression on RORγt-deficient γδ NKT cells resemble a more 'innate-like' phenotype compared to wild-type γδ NKT cells. (C) IL-4 production is substantially reduced in RORγt-deficient γδ NKT cells compared to wild-type controls. Data are representative of at least six independent experiments.

The altered development of PLZF-expressing γδ NKT cells in *RORc*$^{-/-}$ mice was peculiar since these cells did not appear to express RORγt in wild-type mice (Figure 20C). The failure to detect RORγt in γδ NKT thymocytes suggested that these cells might develop from a RORγt-expressing progenitor that is required to imprint the PLZF effector program, but is not required to maintain it. To assess whether γδ NKT cells arose from a RORγt^{+} progenitor, we chose a fate-mapping approach where transgenic mice expressing cre-recombinase under the control of the RORγt promoter were bred to *ROSA26*^{YFP} mice. In this reporter system, cells that expressed RORγt at any point would become permanently marked by YFP expression. Remarkably, we did not find YFP-positive γδ NKT cells in the thymus or the spleen (Figure 23). The bulk of YFP expression was easily detectable among Vγ1^{-}Vδ6.3^{-} (Vγ4) T cells and single-positive CD4 and CD8 αβ T cells (Figure 23). These data indicated that wild-type γδ NKT cells do not develop from RORγt-expressing γδ progenitor cells.

γδ T cells are the dominant T lymphocyte RORc$^{-/-}$*Id3*$^{-/-}$ *mice*

A significant and selective expansion of γδ NKT cells occurs in mice lacking Id3 (cite). The expansion of γδ NKT cells is dependent on E2A, as the number of γδ NKT cells is restored back to wild-type numbers in *Id3*$^{-/-}$*E2A*$^{f/f}$*-Lck*^{Cre} mice[246]. It is noteworthy that E2A regulates RORγt expression in developing thymocytes and during the differentiation of Th17 CD4 T cells. Therefore, de-repression of E2A in *Id3*$^{-/-}$ thymocytes could induce the spontaneous expression of RORγt in developing γδ NKT cells. To assess the role of RORγt in *Id3*$^{-/-}$ γδ NKT thymocytes, we generated *RORc*$^{-/-}$*Id3*$^{-/-}$ *mice.* Analysis of *RORc*$^{-/-}$*Id3*$^{-/-}$ (DKO) thymocytes populations revealed a striking expansion of γδ T cells that resulted in over 50% of thymocytes expressing the γδ TCR (Figure 24A).

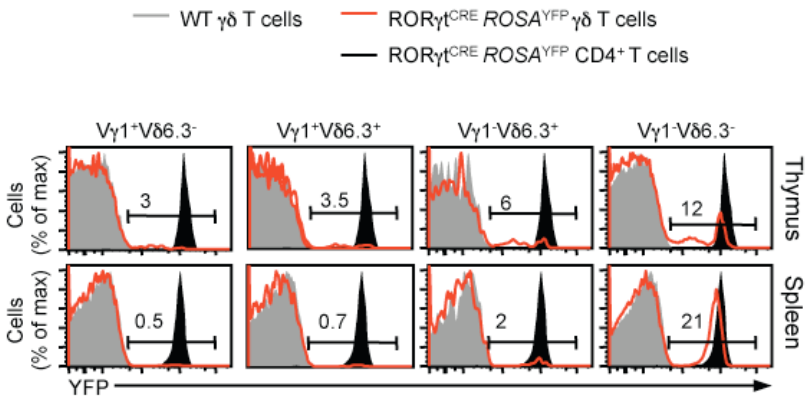

Figure 23. Fate-mapping analysis reveals that RORγt expression is absent in γδ NKT cells. The bulk of RORγt expression is found mostly among Vγ4 T cells. Data are representative of four independent experiments.

To evaluate the frequency of each γδ sublineage, we stained DKO thymocytes with antibodies against Vγ1 and Vδ6.3 TCRs. Remarkably, nearly all (~99%) γδ T cells expressed Vγ1Vδ6.3 TCRs, demonstrating that these cells were of the γδ NKT lineage (Figure 24A). The other γδ sublineages could be detected in DKO mice, albeit at substantially reduced levels (Figure 24). The expansion of the γδ lineage appeared to be at the expense of αβ lineage, as only between 1-3% of thymocytes were αβ T cells (Figure 24A). The absolute number of γδ NKT cells was considerably higher (~1000 fold) in DKO mice, while the absolute number of αβ T cells was significantly lower. Analysis of the αβ lineage the thymus of DKO mice revealed an even more profound reduction in the frequency of DP and SP αβ T cells than observed in *RORc*$^{-/-}$ mice (Figure 24A). To test whether the reduced frequency of DP and SP thymocytes was due to an increase in cells undergoing apoptosis, we cultured bulk thymocytes for 24 hours at 37°C in complete media. Annexin V analysis revealed a similar frequency of cell death among wild-type and *Id3*$^{-/-}$ thymocytes and, as expected, increased death in the absence of RORγt. Importantly, the cell death was not substantially increased in the DKO thymus as compared to the *RORc*$^{-/-}$ thymus (Figure 24B).

The drastic alteration in T cell populations observed in DKO mice prompted an assessment of the overall thymic structure by immunofluorescence. Comparative analysis of medulla-cortex architecture by staining revealed no gross abnormalities in organization of thymic epithelial cells in DKO mice (Figure 25). We did observe, however, that thymuses from *RORc*$^{-/-}$ mice consistently had smaller cortex regions than did WT, *Id3*$^{-/-}$, or DKO mice (Figure 25). The organization of thymic cortico-medullary epithelial cells appeared normal in DKO mice, therefore it is unlikely that a structural thymic defect is responsible for this phenotype. The apparent dysregulation in the proportion of γδ to αβ T cells in the thymus might begin with alterations within the

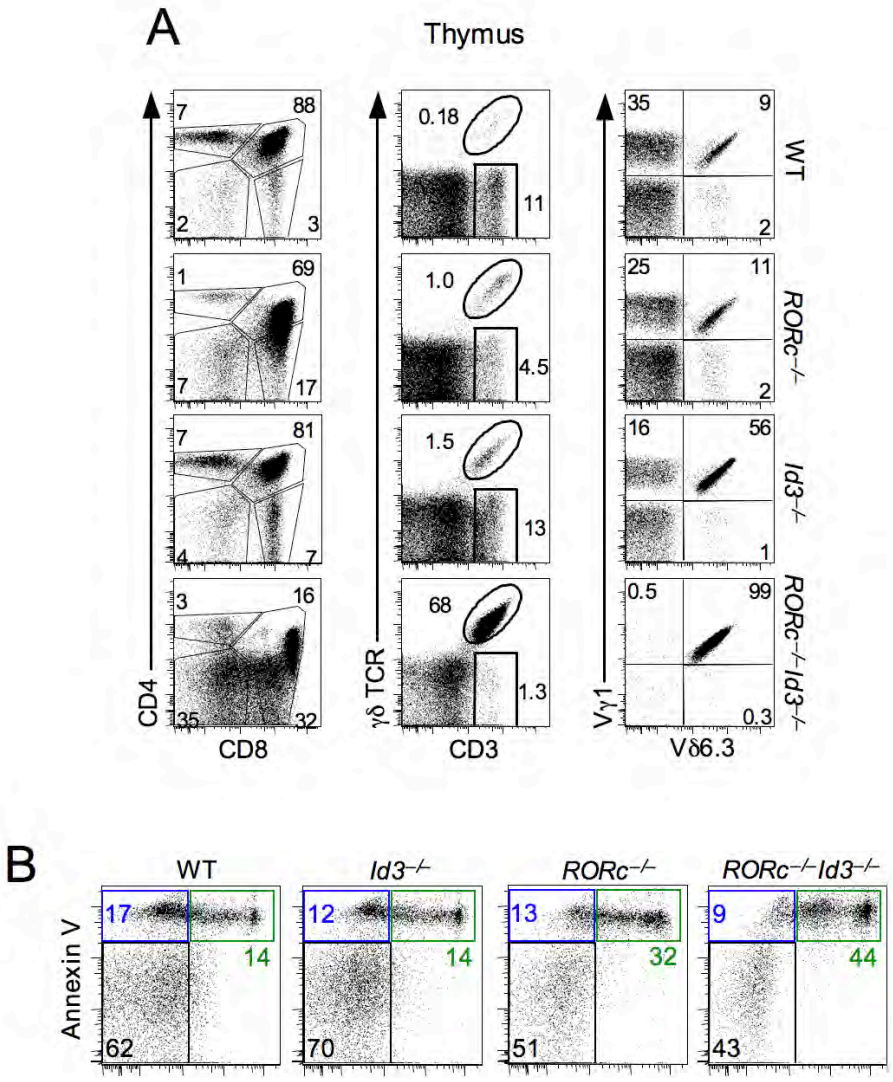

Figure 24. DKO thymocytes have a striking increase in γδ T cells at the expense of αβ T cells. (A) Between 50-70 of thymocytes express Vγ1Vδ6.3 TCRs (B) The high level apoptosis that occurs in RORγt-deficient thymoctes is not substantially increased in DKO thymusus Data are representative of at least 14 experiments (A) and four experiments (B), respectively.

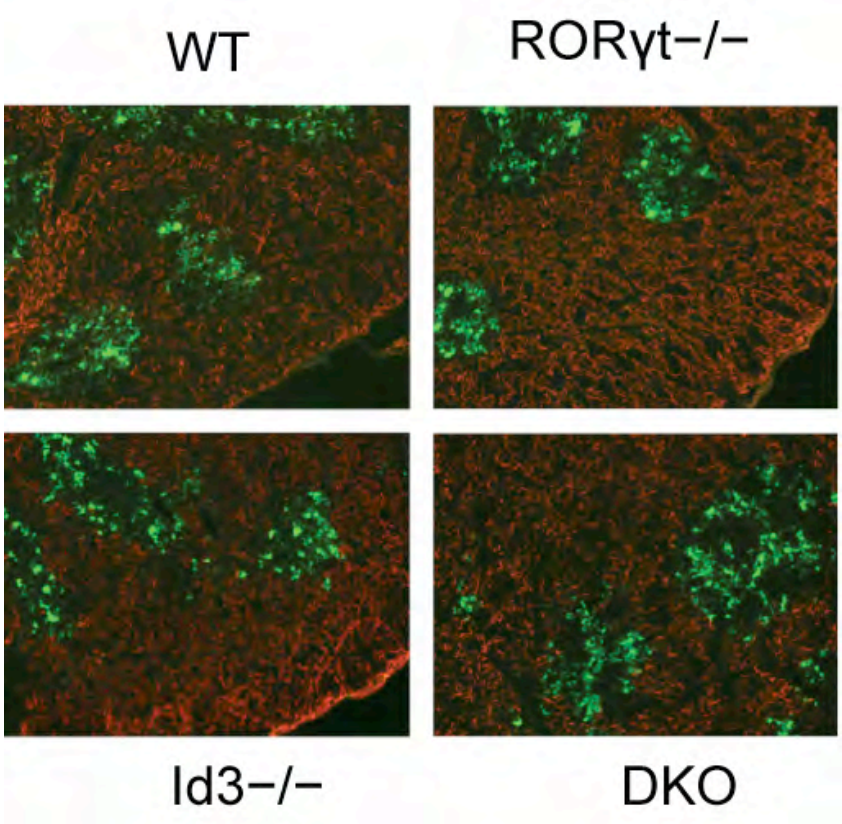

Figure 25. Thymic structure appears normal in DKO mice. Thymic sections were made from wild-type, RORγt-KO, Id3-KO, and DKO mice. Thumuses were stained with anti-DEC205 (cortex, red) and UAE (medulla, green) to evaluate the overall organization of cortico-medullary structures. Data representative of three independent experiments.

hematopoietic bone marrow (BM) progenitors compartment, particularly LSK cells which are the principle progenitor that give rise to T cells. However, there were no significant alterations in the frequency or absolute numbers of the various BM progenitor populations in the single- or double-gene deficient mice (Figure 26).

Since RORγt-deficient mice lack lymph nodes and peyer's patches, it is thought that B and T cells accumulate in the spleen. Nonetheless, the absolute numbers of DKO γδ T cells remained far above normal and were significantly higher compared wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ mice (Figure 27A). There was a decrease in the ratio between the γδ- and αβ T cell lineages, which might reflect homeostatic proliferation of αβ T cells in the periphery. Alternatively, γδ NKT cells normally accumulate in the liver and therefore, are more prominent in the hepatic lymphocyte population compared to the spleen. Indeed, we found that the ratio of γδ- to αβ T cells in the liver was 1:10 and DKO at 10:1, respectively (Figure 27B). γδ NKT cells are a minor population of the IEL compartment, as the majority of γδ T cells in the s-IEL are DETCs while Vγ7 T cells predominate in the i-IEL. Intriguingly, we found normal frequencies of DETCs and Vγ7 T cells in the s-IEL and i-IEL compartment of DKO mice, respectively (Figure 28). Previous reports showed altered DETC development in the absence of Id3, however, we found normal percentages of these cells in both *Id3*$^{-/-}$ and *RORc*$^{-/-}$ mice. The normal frequency of γδ NKT cells in the IEL compartment indicates that these cells traffic normally and importantly, that development of DKO DETCs during early ontogeny is likely normal. The frequency of B and NK cells were similar between *RORc*$^{-/-}$ and DKO mice (Figure 29). Overall these data indicate that the lack of RORγt and Id3 is specific to the T cell compartment, as all other cell populations appear normal.

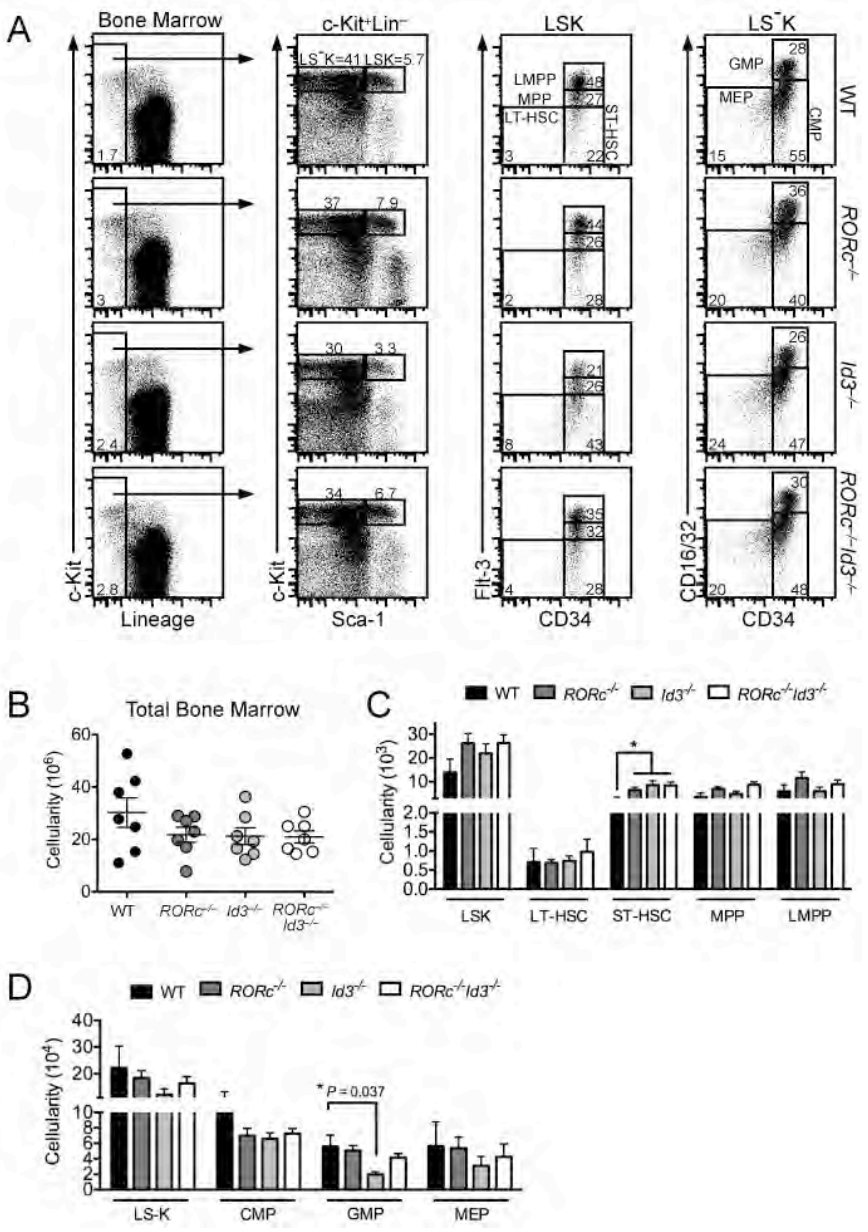

Figure 26. Stem cell populations in the bone marrow appear normal in DKO mice. (A) Analysis of LS$^-$K and LSK populations for various myeloid and lymphoid progenitor cells and short- and long-term hematopoietic stem cells. (B) Variability in the total cellualrity was apparent in widl-type mice but overall numbers were similar between indicated mouse strains (C, D) Absolute numbers for various LSK subpopulations (C) and LS$^-$K subpopulations. Data are representative of at least four independent experiments.

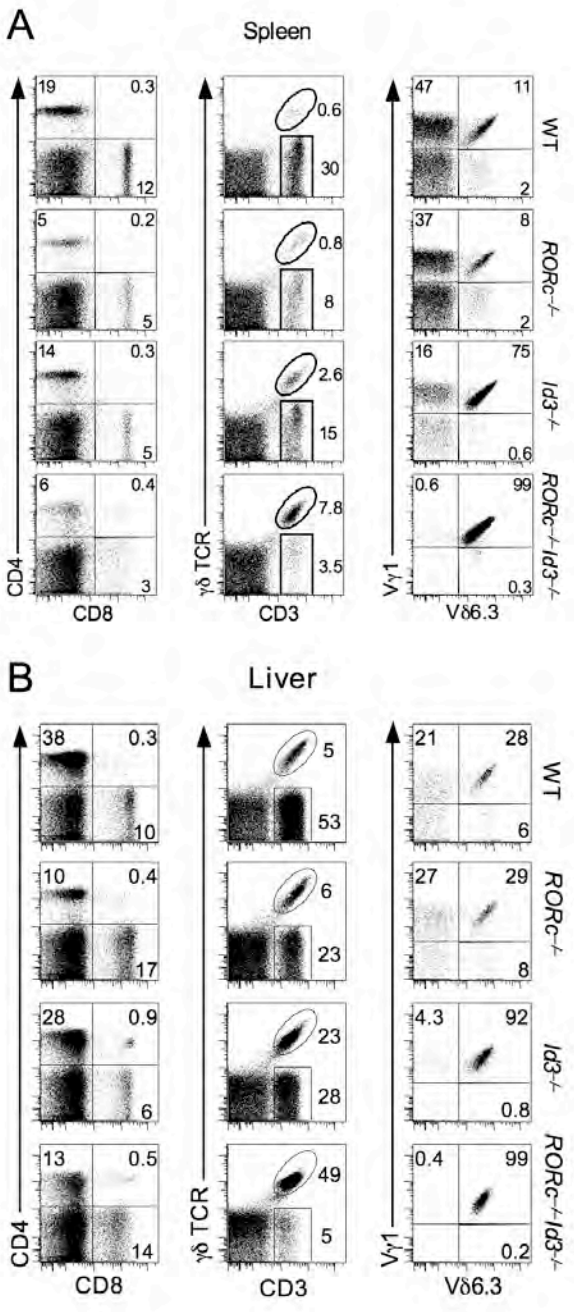

Figure 27. The dramatically expanded γδ NKT cells are also apparent in the periphery. (A) γδ NKT cells far out number αβ T cells in the DKO spleen, but the ratio narrows. (B) γδ NKT cells are highly enriched in the liver, as the ratio of γδ NKT cells to αβ T cells is similar to the ratio found in the thymus. Data are representative of at least 14 experiments (A) and 8 experiments (B), respectively.

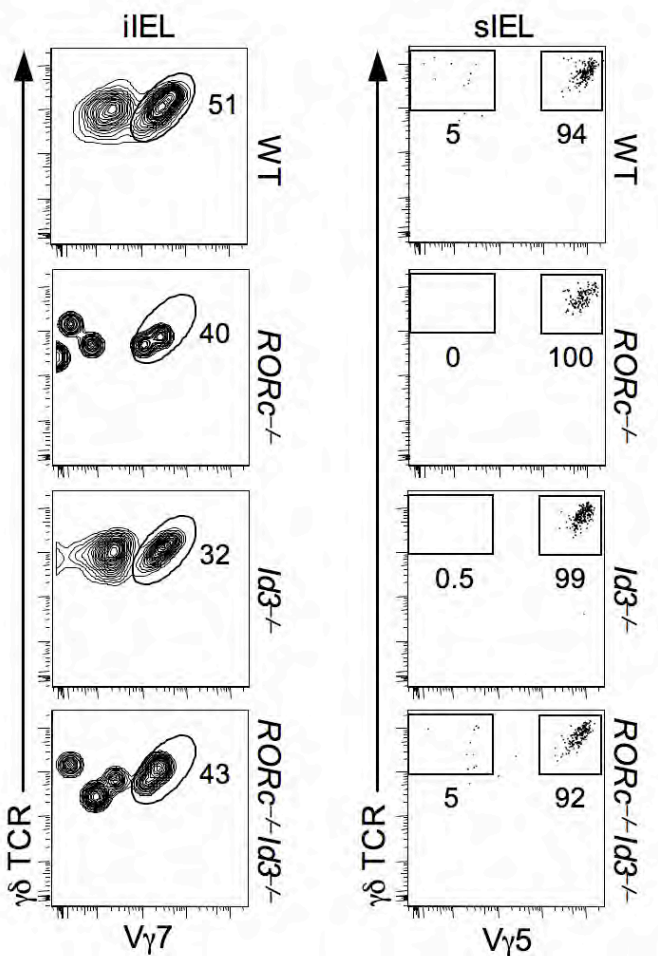

Figure 28. The IEL compartment is normal in DKO mice. A normal frequency of Vγ7 T cells are found in the i-IEL of indicated mouse strains (left) and DETCs are also found in at a normal frequency in the s-IEL of all mouse strains indicated (right). Data are representative of three independent experiments.

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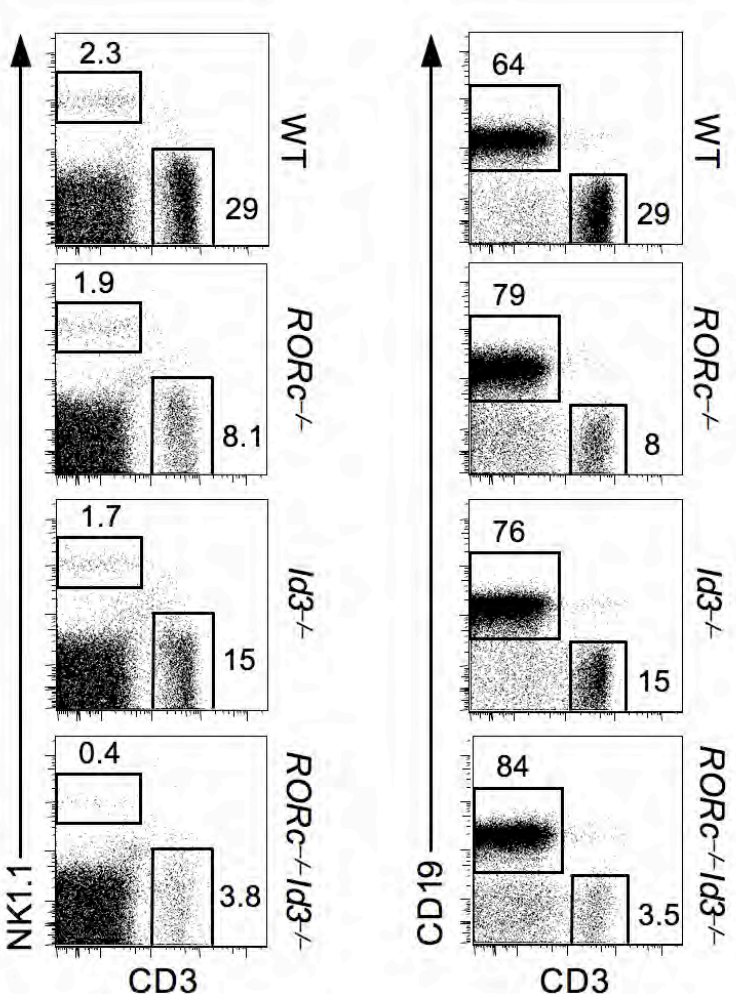

Figure 29. NK and B cells appear normal in DKO mice. The frequency of NK cells are similar among indicated mouse strains, except DKO where the frequency is ~60% less on average than wild-type and single-gene deficient mice (left). $CD19^{+}$ B cells are also similar in the spleens of indicated mouse strains. Data are representative of at least eight independent experiments.

The expansion of γδ NKT cells

To assess whether the dramatic expansion of DKO γδ NKT cells might reflect an increase in proliferation, we pulsed mice with BrdU and assessed proliferation 18 hours later. Surprisingly, we found that the frequency of cells that had proliferated in the thymus and spleen was similar in γδ T cells in wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO mice. (Figure 30). However, when we specifically evaluated γδ NKT cells we found that *Id3*$^{-/-}$ γδ NKT thymocytes proliferated far less compared to γδ NKT thymocytes from WT, *RORc*$^{-/-}$, and DKO mice (Figure 30). In the spleen, we found a small, but significant reduction in the proliferation of bulk *RORc*$^{-/-}$ γδ T cells compared to wild-type, *Id3*$^{-/-}$, and DKO mice (Figure 30). Surprisingly, the proliferation rate of γδ NKT cells was reduced slightly reduced in DKO mice (Figure 30). Therefore, excessive proliferation is not responsible for the expanded population of γδ NKT cells in *RORc*$^{-/-}$*Id3*$^{-/-}$ mice. Our observation that there is a slight difference in proliferation between bulk *Id3*$^{-/-}$ and wild-type γδ T cells is likely due an unexpected but considerable decrease specifically in the proliferation of *Id3*$^{-/-}$ γδ NKT cells.

The cytokine production from Id3$^{-/-}$ *and RORc*$^{-/-}$*Id3*$^{-/-}$ *of γδ NKT cells are comparable*

We and others, previously reported that nearly all *Id3*$^{-/-}$ γδ NKT cells express high levels of PLZF and produced copious amounts of IL-4[179,248]. We found a similar pattern of PLZF expression between *Id3*$^{-/-}$- and DKO γδ NKT cells in all tissues examined (Figure 31A). We activated γδ NKT cells from the spleens of wild-type, *ROR*$^{-/-}$, *Id3*$^{-/-}$, and DKO mice with PMA/ionomycin, followed by intracellular staining for IL-4 and IFN-γ. The percentage of cells producing cytokines was comparable between *Id3*$^{-/-}$ and DKO γδ NKT cells, with a slight but consistent skewing toward IFN-γ over IL-4 in DKO γδ NKT

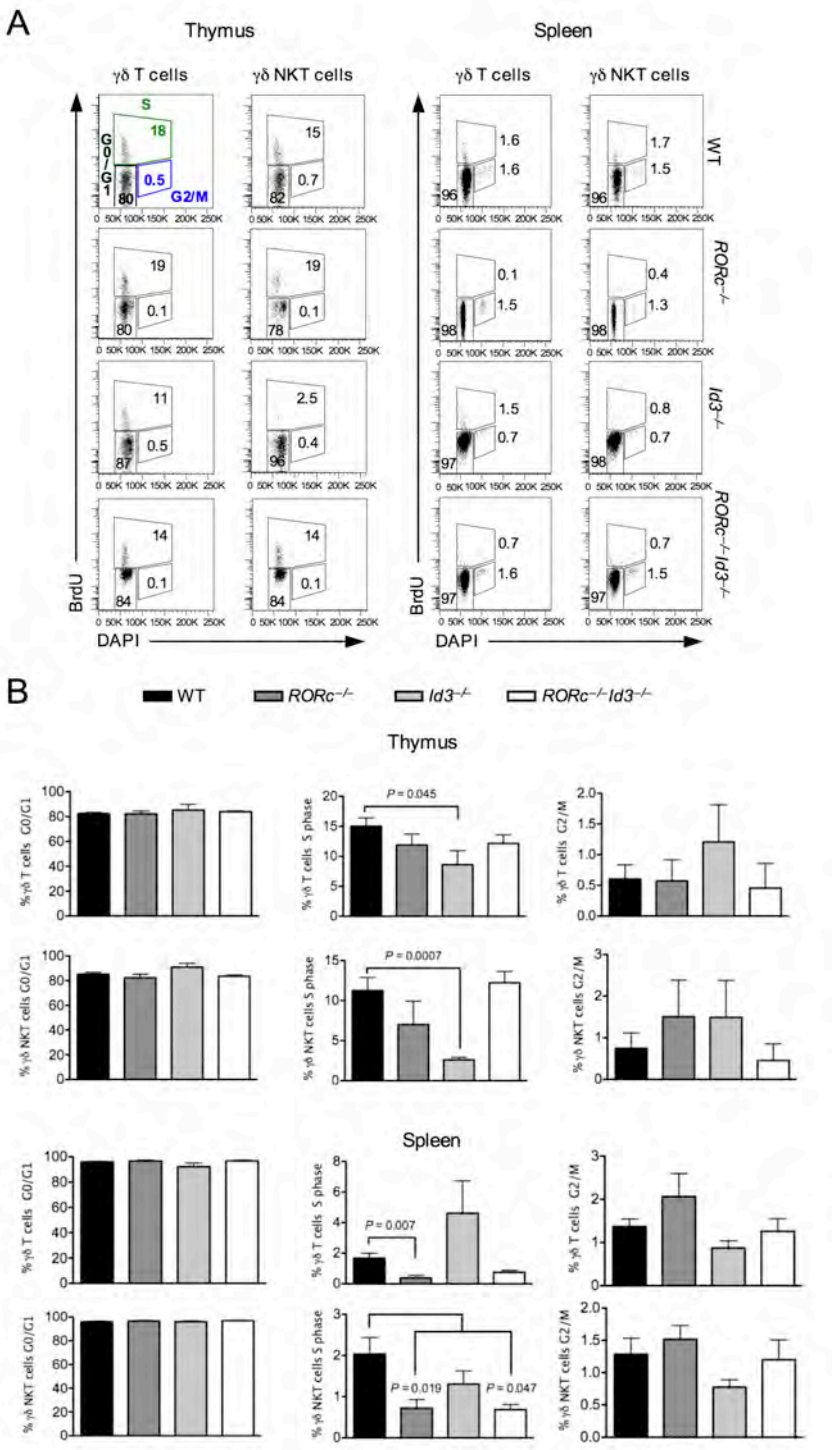

Figure 30. Proliferation rates of γδ T cells in the thymus and spleen. (A) Animals were injected IP with 1mg of BrDU, thymuses and spleens were harvested 18 hours later and analyzed for the presence of BrDU and DAPI (B) Statistical analysis of BrDU experiments. Data representative of 4 independent experiments.

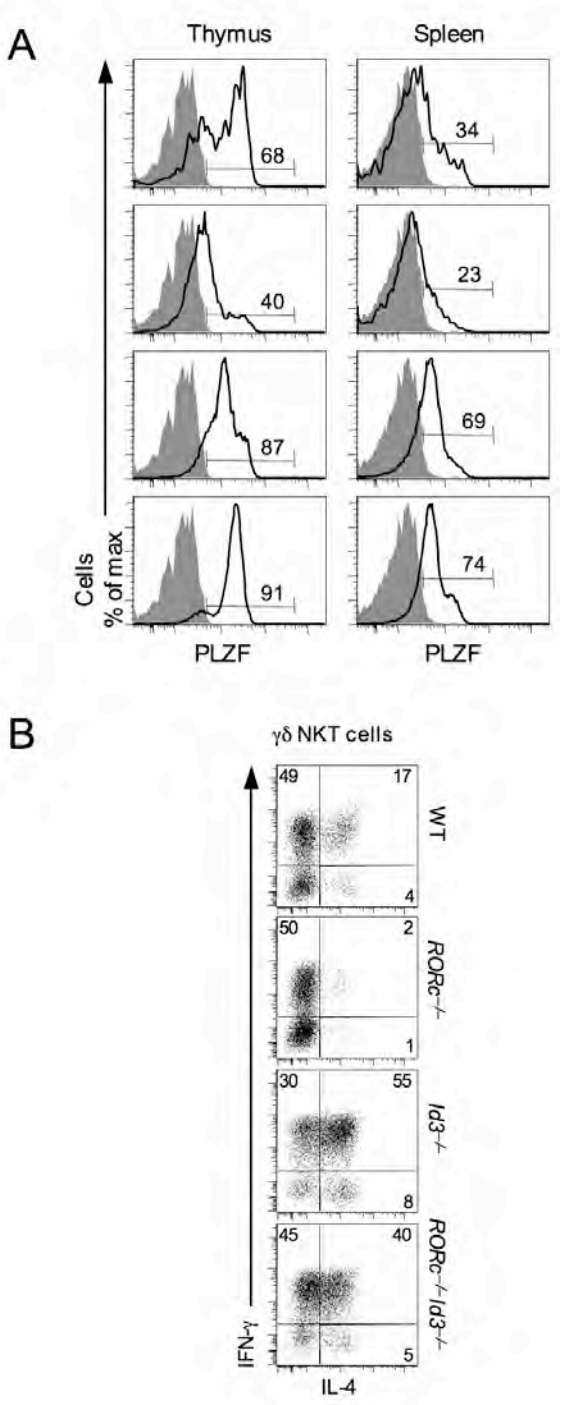

Figure 31. PLZF expression and cytokine production in γδ NKT cells. A) PLZF expression analysis in γδ NKT thymocytes and splenocytes in indicated mouse strains (blue) and PLZF-deficient mice (red). (B) Intracellular cytokine secretion analysis for IFN-γ and IL-4 in γδ NKT cells in indicated mouse strains.

cells (Figure 31B). Consistent with our previous results, we observed a significant loss of IL-4 production in activated *RORc*$^{-/-}$ γδ NKT cells (Figure 31B). These data indicate that the DKO γδ NKT cells behave more like *Id3*$^{-/-}$ rather than *RORc*$^{-/-}$ γδ NKT cells, demonstrating that the loss of RORγt has little impact on the function of *Id3*$^{-/-}$ γδ NKT cells.

The single- and double gene-deficient mice have varying defects in β-selection

Both γδ- and αβ T cells emerge from a common progenitor. Therefore, we wanted to evaluate how the absence of RORγt and Id3 affected γδ- versus αβ lineage commitment. There were significantly fewer cells at every DN stage in *RORc*$^{-/-}$*Id3*$^{-/-}$ and DKO mice due to the overall reduced cellularity. Thus, we compared the frequency of DN 1 – 4 in our single- and double-deficient mice. We observed fewer DN1, and DN2 in *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO thymocytes compared to wild-type controls. Closer analysis of c-Kit^{+}CD44^{+} ETPs revealed a significant reduction in the frequency DN1 cells in *Id3*$^{-/-}$ (Figure 32A). However, the ratio of DN2a/b cells appeared similar in all mice (Figure 32B).

The majority of γδ T cells are proposed to develop from the DN3a stage, while αβ lineage cells make it beyond the β-selection checkpoint traverse onward through DN3b to DN4. The frequency of the bulk DN3 population was similar between all mice (Figure 32B). By sub-setting DN3a and DN3b, however, we found a significant reduction in the frequency of DN3b cells in *Id3*$^{-/-}$ and to wild-type mice (Figure 32C). We also observed a slight reduction of in *RORc*$^{-/-}$ DN3b cells compared to wild-type controls, however these cells were significantly more abundant compared to DN3b cells from both *Id3*$^{-/-}$ and DKO mice (Figure 32C). Interestingly, DN3 cells from DKO consistently expressed higher levels of CD44 between experiments. These analyses of DN3b cells indicated

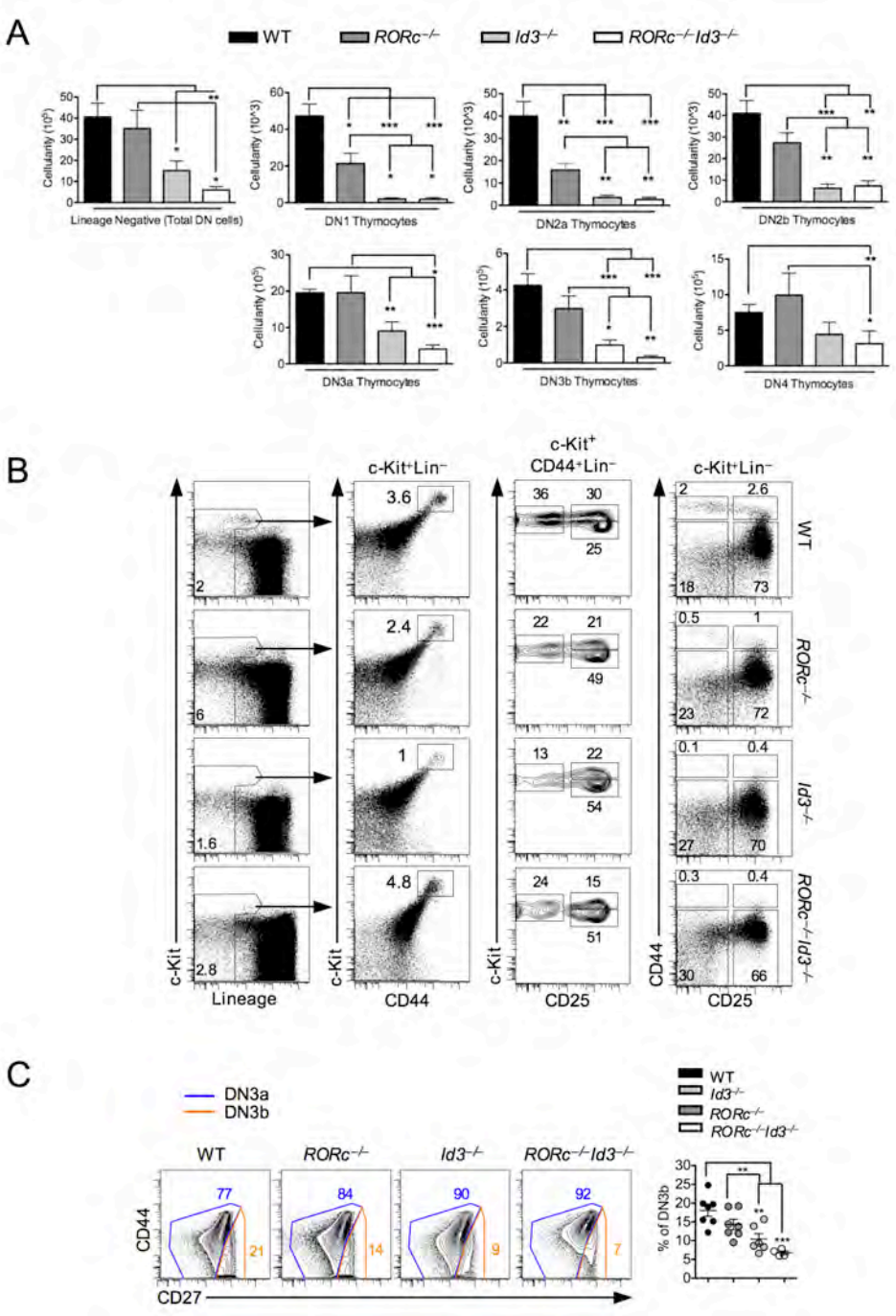

Figure 32. Developmental block in b-selection in single- and double-gene KO mice. (A) Progenitor T cell populations are drastically reduced in RORγt-KO and DKO mice. (B)The frequency of DN2 cells are similar between mouse strains indicated. DN3 cells appear to slightly different in DKO mice. (C) Reveals a difference in the frequency of β-selected DN3b cells in RORγt- and Id3-deficient mice with the most significant reduction found among all indicate mouse strains.

DN3b cells indicated defects in β-selection of varying degrees from high to low in DKO, *Id3*$^{-/-}$, and *RORc*$^{-/-}$ mice, respectfully.

Altered IL-7R expression on RORc$^{-/-}$*Id3*$^{-/-}$ *DN thymocytes*

IL-7 signaling is required for γδ T cell development[34-38,85]. IL-7 receptor expression peaks at DN2 and controls TCR-γ recombination through activation of STAT5, which regulates accessibility of TCR-γ elements by interacting with Jγ germline promoters and TCR-γ enhancers through chromatin remodeling[34-38,85]. Thus, IL7/STAT5 and E2A are critical factors that induce alterations to chromatin structure at the TCR-γ locus, leading to germline transcription and V(D)J recombination. Since IL-7R^{hi} DN2 cells preferentially give rise to γδ T cells[85], we wanted to determine the level of surface IL-7R expression on DN1 – 4 thymocytes in wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO mice. In agreement with previous findings, IL-7R expression is barely detectable on DN1 cells although we found a significant reduction of IL-7R on DN1 cells from *RORc*$^{-/-}$ mice (Figure 33). Peak IL-7R expression occurred at DN2b in all mouse strains, however, IL-7R expression was significantly lower on DKO thymocytes compared to wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ (Figure 33). In wild-type mice, IL-7R expression progressively decreases from DN2b to DP stage where it is re-expressed on CD4 and CD8 SP T cells (Figure 33)[86]. Interestingly, in DKO mice, IL-7R expression levels did not decrease between the DN2b to DN3 transition, rather the level of IL-7R expression was maintained at a constant level from DN2b – DN4 (Figure 33). As a result, IL-7R receptor expression was significantly higher on DN3a and DN4 DKO thymocytes compared to wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ cells (Figure 33). Although not statistically significant, IL-7R expression levels were also higher on DKO DN3b thymocytes compared to wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ thymocytes.

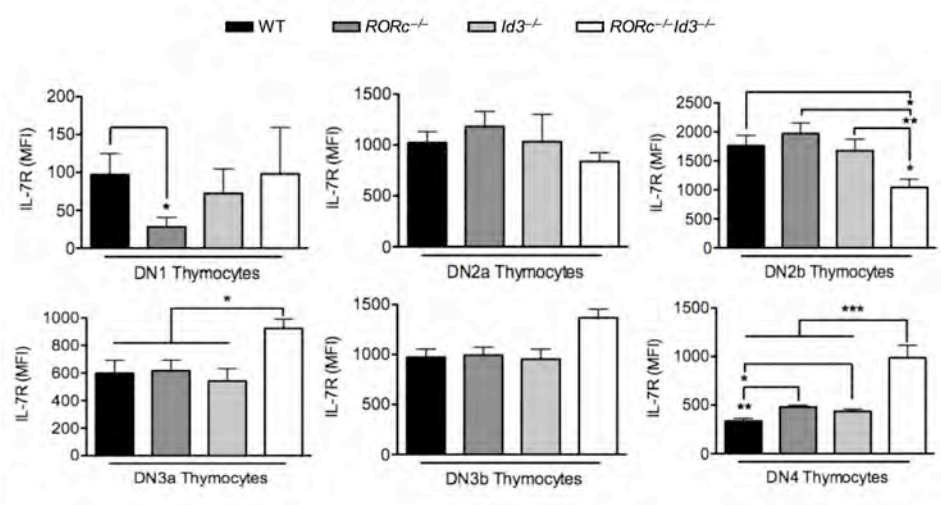

Figure 33. IL-7 receptor expression is altered during thymic development in DKO mice. IL-7R expression is significantly lower in DKO DN2b thymocytes compared tow wild-type, *RORc*$^{-/-}$, and *Id3*$^{-/-}$ DN2b thymocytes. IL-7R levels on DKO DN3a cells are maintained at significantly higher levels compared to DN3a wild-type, *RORc*$^{-/-}$, and *Id3*$^{-/-}$ *cells.* IL-7R levels on DN3b and DN4 DKO thymocytes appear to increase compared to DN3 and remain significantly higher compared to wild-type, *RORc*$^{-/-}$, and *Id3*$^{-/-}$ thymocytes.

Interestingly, IL-7R levels were significantly higher on *RORc*$^{-/-}$ and *Id3*$^{-/-}$ DN4 thymocytes compared to wild-type indicating a premature re-expression of IL-7R on these cells (Figure 33). With the exception of DN4, we find a similar pattern of IL-7R expression on DN cells between wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ mice. In contrast, our data clearly indicate a significant alteration in the pattern of IL-7R expression on DN2b – DN4 thymocytes in DKO mice.

Gene expression profile is drastically altered in Id3$^{-/-}$ *and RORc*$^{-/-}$*Id3*$^{-/-}$ *γδ NKT cells.*

To compare the gene expression profiles of γδ NKT cells from single- and double-gene deficient mice, we isolated RNA from FACS sorted γδ NKT thymocytes from WT, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO mice and performed microarray analysis. Genes with a two-fold or more change in expression level between indicated mutant strains and wild-type were clustered into a Venn diagram (Figure 34). We found drastic changes in the gene expression patterns of γδ NKT cells from both *Id3*$^{-/-}$ (1400) and DKO (1572) mice compared to wild-type (Figure 34). Interestingly, only 691 genes were changed similarly in *Id3*$^{-/-}$ and DKO γδ NKT cells. In contrast, only 33 genes were differentially expressed in *RORc*$^{-/-}$ γδ NKT cells. These data indicate a profound change in the genetic programming of γδ NKT cells in the absence of Id3. γδ NKT cells do not normally express RORγt, and predictably, only a handful of genes are altered in the absence of RORγt. An overlapping but distinct gene program is observed in DKO γδ NKT cells, indicating that spontaneous RORγt activity in *Id3*$^{-/-}$ γδ NKT cells controls a unique subset of genes, and only in the absence of Id3, does RORγt appear to substantially impact the development of γδ NKT cells.

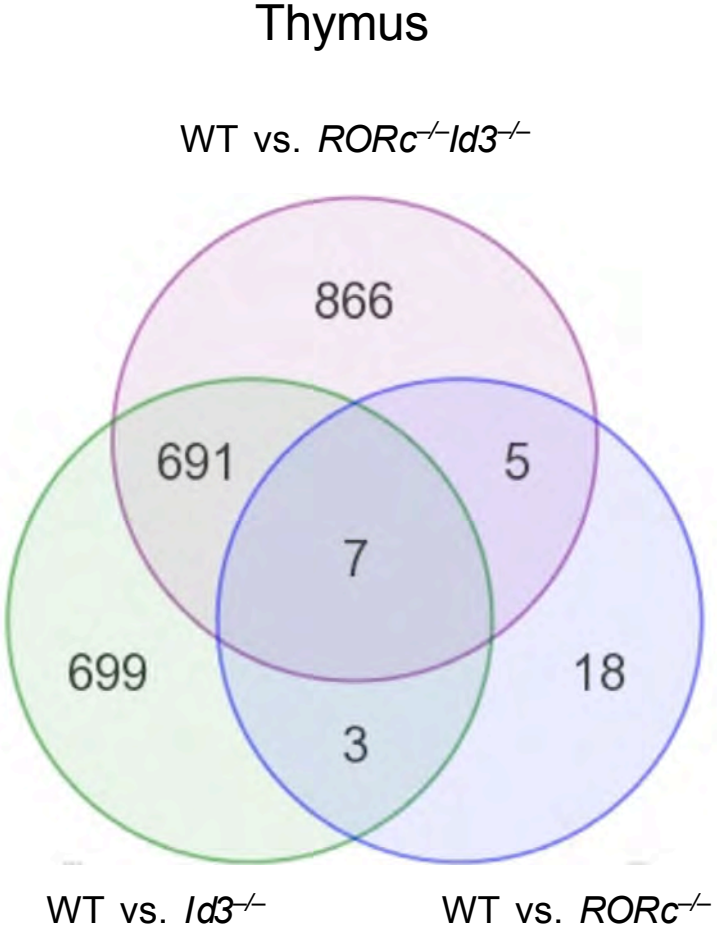

Figure 34. Sweeping changes in gene-expression profile of *Id3*$^{-/-}$ and DKO γδ NKT thymocytes. Labeled RNA was hybridized to Affemetrix gene chips. Data analysis was performed using Partek software with the data limits set at a <0.05 false-discovery rate and a 2-fold or more change in the absolute gene expression level. Data are representative of 3 independent FACS experiments.

Discussion

Although γδ NKT cells do not appear to express RORγt at any time during their development, we show that the differentiation and function of these cells are clearly impacted in *RORc*$^{-/-}$ mice. In the absence of RORγt, γδ NKT cells exhibit defects in PLZF expression and IL-4 production. Interestingly, we detected the presence of IL-17 producing γδ NKT cells in Id3-deficient mice. Intracellular staining analysis showed spontaneous expression of RORγt in *Id3*$^{-/-}$ γδ NKT thymocytes. Therefore, we sought to elucidate the role of RORγt in Id3-deficient mice by generating mice deficient in both genes. Analysis of lymphocyte populations in DKO mice revealed a remarkable expansion of the γδ NKT cell population at the expense of the αβ lineage. This expansion was not the result of increased cell death of αβ T cells or enhanced proliferation of γδ NKT cells. There was no major difference in the pattern or expression levels of PLZF between Id3-deficient and DKO γδ NKT cells. Furthermore, cytokine production was nearly identical between DKO- and *Id3*$^{-/-}$ γδ NKT cells. These data clearly indicate that the loss of RORγt in Id3-deficient mice does not significantly alter PLZF expression or cytokine secretion, but does significantly impact αβ– versus γδ lineage commitment.

We wondered whether the thymuses in *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO mice where altered in any obvious way. Staining of thymic tissue sections revealed no obvious defects in the cortex or medulla in any mutant mouse strain. The absence of any apparent abnormalities in the overall thymic structure in the mutant mice suggested an intrinsic defect in DKO progenitor T cells that might begin within the HSC compartment in the bone marrow. However, analysis of the overall HSC compartment, including LSKs which give rise to T cells, showed normal frequencies and absolute numbers in *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO bone marrow. We also found normal frequencies of NK and B cells in the spleen of

DKO mice. Altogether, these data indicate that the lymphoid compartment is intact in DKO mice.

It seemed that the absence of RORγt and Id3 mostly affected the T cell compartment, particularly the development of γδ T cells. While the γδ NKT cell lineage made up the bulk of the γδ lineage in DKO mice, the other γδ sublineages were present. The ordered appearance of specific γδ T cell populations, each expressing distinct TCRs, during mouse ontogeny provided an opportunity to evaluate whether the development of other γδ T cell populations was disrupted in DKO mice. In mice, DETC development is exclusive to the fetal thymus, while mature DETCs are found only in the skin epithelium. Notably, analysis of the skin IEL compartment revealed the presence of DETCs at normal frequencies in the *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO mice. We also found normal frequencies of Vγ7 T cells in the i-IEL compartment in *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO mice. These findings were surprising for a few reasons. First, the normal frequencies of DETCs and Vγ7 T cells clearly demonstrated that development of these cell populations is not substantially affected in DKO mice. Secondly, the relative absence of γδ NKT cells in the IEL compartment indicates that these cells traffic normally. Thirdly, and importantly, these data suggest that the development of DKO DETCs during early ontogeny is normal, indicating that the ordered recombination of distinct Vγ genes is intact. Altogether, these findings indicate that the defect in T cell development in the absence of RORγt and Id3 is γδ NKT cell-specific.

A massive increase in the proliferative capacity of γδ NKT cells could explain the drastic increase in the absolute numbers of these cells in DKO mice. Notably, however, we found that the proliferation rates of γδ NKT thymocytes were similar between WT, *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO mice. This surprising result ruled out the possibility that the

dramatic expansion of DKO γδ NKT cells was simply due to an increase in proliferation, and suggested that the phenotype in these mice was more complex.

Since the HSC compartment appeared normal in DKO bone marrow, we focused on the DN stages of development in the thymus to determine if progenitor cells were altered in the absence of RORγt and Id3. Remarkably, we did not find an obvious defect in the frequency of DN1 – 4 cells in the thymuses of *RORc*$^{-/-}$, *Id3*$^{-/-}$, or DKO mice. This result was surprising because of the substantial disparity between the αβ- and γδ T cell populations in the thymus. αβ- versus γδ lineage commitment is thought to occur at DN3. Therefore, we assessed the frequency of DN3a and DN3b cells in WT, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO thymocytes. Interestingly, we found a significant and increasing defect in the frequency of DN3b cells in *RORc*$^{-/-}$, *Id3*$^{-/-}$, DKO thymocytes, respectively. Notably, we observed an additive defect in αβ lineage commitment in DKO mice. These results demonstrate a partial block in DN3 cells committing to the αβ lineage in each of the mutant mouse strains, with the most severe block occurring in DKO thymocytes. Therefore, these data suggest a novel role for RORγt and Id3 in the regulation of the β-selection checkpoint.

Our data demonstrate a drastic and specific increase in γδ NKT cells in *RORc*$^{-/-}$ *Id3*$^{-/-}$ mice. Remarkably, ~70% of thymocytes belong to the γδ NKT lineage in the absence of RORγt and Id3. Thus, there is ~100 fold increase in the absolute numbers in γδ NKT cells in DKO versus *Id3*$^{-/-}$ mice. Initially, we hypothesized that the spontaneous RORγt expression in *Id3*$^{-/-}$ γδ NKT cells promoted the expansion of these cells in these Id3-deficient mice. However, based on the enormous expansion DKO γδ NKT cells it was clear that RORγt instead *restricted* the development of *Id3*$^{-/-}$ γδ NKT cells. Gene expression analysis revealed that 871 genes were changed in DKO versus *Id3*$^{-/-}$ γδ NKT thymocytes indicating that RORγt controls a large, distinct gene set. Careful investigation

should reveal novel gene targets of RORγt, potentially exposing transcriptional pathways critical for the expansion of γδ NKT cells in DKO mice. Our data clearly indicate a novel role for RORγt in controlling *Id3*$^{-/-}$ γδ NKT cell development.

Lauritsen and coworkers argue that γδ NKT cells that receive an exceedingly strong TCR signal are normally deleted by negative selection, while *Id3*$^{-/-}$ γδ NKT cells are able to escape negative selection resulting in a larger γδ NKT cell compartment. However, this conclusion was based on experiments using γδ TCR Tg mice that expressed a Vγ4 TCR. It is clear, however, that γδ TCR expressing cells are not one lineage, but rather are made up of several γδ lineages each with distinct developmental requirements. Notably, there are no obvious defects in the development of Vγ4 T cells in Id3-deficient mice, indicating these cells do not require Id3 for development. Based on these findings, we propose that there are two distinct γδ lineages: Id3-independent and PLZF/Id3-dependent γδ T cells. Therefore, the conclusions made by Lauritsen and coworkers regarding the role of Id3 during γδ NKT cell selection by using *Id3*$^{-/-}$KN6 mice almost certainly does not reflect the biology that occurs in *Id3*$^{-/-}$ γδ NKT cells. Until the discovery of γδ NKT cell ligands occurs, the role of Id3 and TCR signaling in γδ NKT cell selection will remain tenuous.

Notably, while the spontaneous expression of RORγt in *Id3*$^{-/-}$ γδ NKT cells is clearly restricting the expansion of these cells, currently there is no known role for RORγt in the selection of T lymphocytes. Based on Lauritsen and coworker's negative selection model, the dramatic expansion of γδ NKT cells in DKO mice would be a direct result of ~100 fold increase in γδ NKT cells escaping negative selection compared to Id3 deficient mice. However, it is difficult to imagine a scenario where ~60-70% of thymic progenitors are directed into the γδ NKT lineage, the bulk of which undergo negative selection, to ultimately give rise to ~0.0002% (50,000 cells) thymocytes in wild-type mice. Under

these circumstances, nearly two-thirds of the total DN3 population would contain rearranged Vγ1Vδ6.3 TCR transcripts, which is not the case[250].

In both RORγt- and Id3-deficient mice, we observed a reduction in the frequency of β-selected DN3 thymocytes. Our data clearly indicate a defect in αβ lineage commitment prior to the DP stage where both RORγt and Id3 have previously been shown to have distinct roles in development of αβ T cells. Based on data from RORγt reporter mice, it was concluded that RORγt is expressed only between late DN4 and DP stage of T cell development[211,214]. However, a recent study showed RORγt expression as early as DN3 in wild-type mice[251]. Although there is some evidence that DP thymocytes trans-condition γδ T cell development, it has not been formally demonstrated that DP cells also trans-condition DN3 cells to promote β-selection. Our data supports a model where RORγt has a modest, but important role in β-selection.

Id3, on the other hand, has been shown to have an important role in αβ- versus γδ lineage commitment. The strength of signal model argues that weaker TCR signals would promote the development of the αβ lineage. However, others and we recently showed that γδ NKT cells are expanded nearly 10-fold in the absence of Id3 without any major decrease in the frequency of αβ T cells[179,245,246,248]. Notably, we see a significant decrease in β-selected DN3b cells in Id3-deficient mice. Therefore, our data indicate that the absence of Id3 results in a partial block in progenitors committing to the αβ lineage and more cells committing to the γδ NKT cell lineage. Interestingly, there is an even more significant block in β-selection in DKO mice. The combined loss of RORγt and Id3 results in an additive defect, with a two-thirds decrease in the frequency of DN3b cells compared to wild-type mice. Collectively, our data demonstrate varying defects in progenitor thymocytes committing to the αβ lineage in *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO mice.

It is possible that the dramatic expansion of DKO γδ NKT cells could be due to a redirection of progenitor cells away from the αβ lineage and into the γδ NKT lineage. However, unlike the strength of signal model, this redirection would occur prior to surface TCR signaling, during TCR gene transcription and recombination. E2A has a critical role in regulating TCR gene transcription during T cell development[39,42]. Ueda-Hayakawa and coworkers argue that *Id3*$^{-/-}$ DN progenitors that failed to produce a functional pre-TCR have an extended 'window' to rearrange TCR-γ due to increased E2A activity that results in these cells being directed into the γδ lineage[246]. Interestingly, the expansion of *Id3*$^{-/-}$ γδ NKT cells is dependent on E2A[205,246]. Strong evidence indicates that TCR-δ and TCR-γ genes undergo recombination prior to TCR-β, most likely due to high levels of IL-7 signaling at DN2[79,85,161,250,252,253]. While TCR-β chain rearrangement is also initiated at DN2, V-DJβ rearrangement does not occur until DN3[246]. Thus, it is generally accepted that functional γδ TCRs are produced at DN2 - 3, prior to pre-TCR surface expression[87,254]. It is also thought that pre-TCR signaling induces Id3-mediated repression of E2A activity on target genes, one of which is RORγt[206]. Thus, it is plausible that the levels of E2A activity directly correlate with the levels of RORγt expression. It is noteworthy that RORγt is thought to regulate RAG2 expression[206]. Under these circumstances, pre-TCR signaling would normally block RORγt expression at DN3 indirectly. However, in the absence of Id3, high levels of E2A would be maintained at DN3 potentially redirecting some cells into the γδ NKT cell lineage through enhanced Vγ1Vδ6.3 TCR recombination. We hypothesize that elevated levels of RORγt in Id3-deficient DN3 cells prevents further Vγ1Vδ6.3 TCR recombination, perhaps by maintaining high levels of recombination at the TCRβ gene locus through control of RAG2 expression. In the absence of both RORγt and Id3, we suggest that the dramatic expansion of γδ NKT cells is a direct result of a significant loss of gene

regulation at the TCRβ gene locus along with enhanced Vγ1Vδ6.3 TCR recombination, resulting in an overall loss of DN3 progenitors that successfully produce surface pre-TCRs required to pass the β-selection checkpoint. Interestingly, we did not observe a major decrease in the frequency of DN4 cells in DKO mice. It is possible that those cells are late-stage 'redirected' γδ NKT cells, or an expanded population of the few β-selected cells traversing toward the DP stage of development.

It is important to note that IL-7 signaling is required for chromatin remodeling and gene transcription of the TCR-γ locus[34-36]. Recently, it was shown that STAT5, a direct target of IL-7 signaling, is required for TCR-γ gene transcription[37,38]. IL-7 signaling peaks at DN2, the stage at which TCR-γ transcription is the most robust. While it is clear that IL-7 signaling is required for the development of γδ T cells, it has not yet been investigated whether the level of IL-7/STAT5 controls distinct Vγ gene units. In other words, it is possible that progenitors that experience high levels of IL-7/STAT5 might recombine one Vγ cluster over the other (see Figure 35). In wild-type mice, IL-7R expression progressively decreases from DN2b to DP stage where it is re-expressed on CD4 and CD8 SP T cells. We observed a relatively normal pattern of IL-7R expression on DN thymocytes from *RORc*$^{-/-}$ and *Id3*$^{-/-}$ mice. Notably, we found that the pattern of IL-7R expression on DN2b – DN4 thymocytes was significantly altered in DKO mice, with IL-7R expression levels remaining relatively constant between DN2a – DN4 (Figure 33). It is clear that IL-7R^{hi} DN2 cells preferentially produce γδ T cells while IL-7R^{lo} produce αβ T cells[85]. Thus, a particular threshold of IL-7 signaling is required to induce chromatin remodeling at the TCR-γ locus for germline transcription and recombination. However, it is unknown whether a particular level of IL-7 signaling is required to access distinct Vγ gene clusters, or if reaching a specific threshold opens the entire TCR-γ locus, resulting in stochastic gene transcription and recombination. Clearly, the ordered

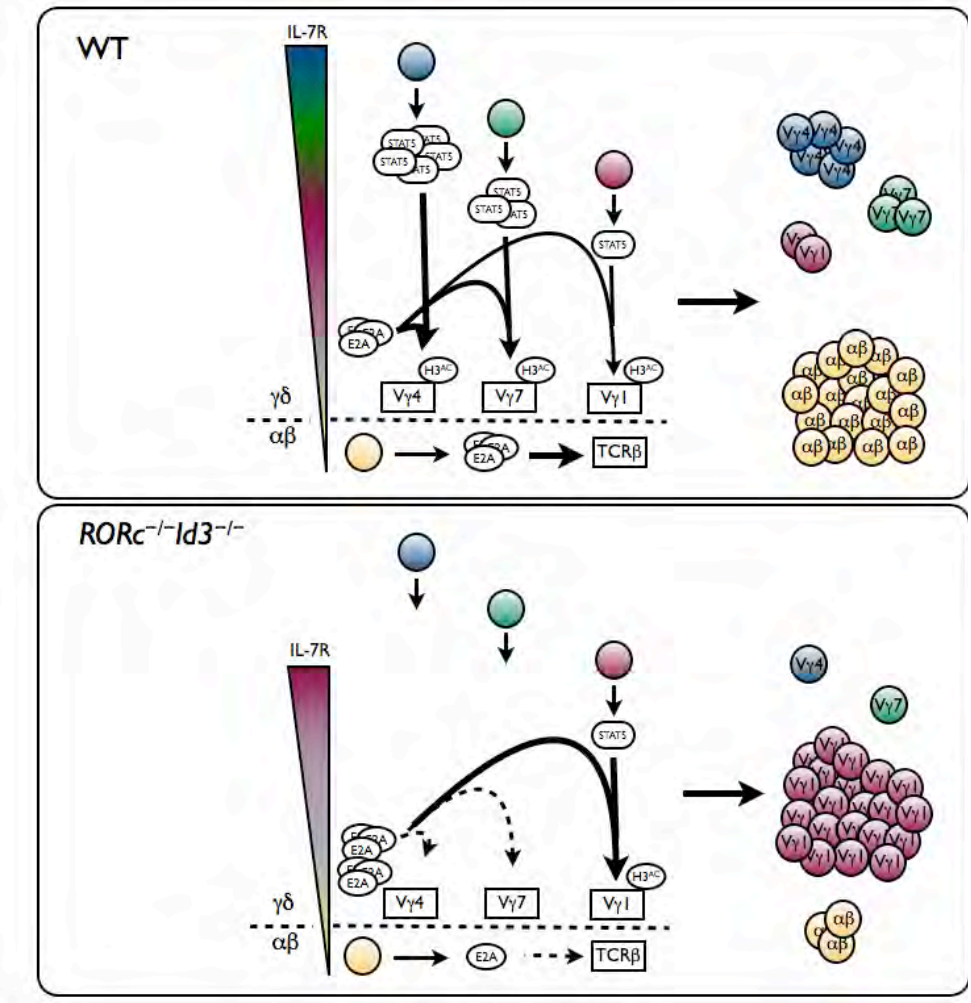

Figure 35. Model of enhanced γδ NKT cell development in DKO mice. (A) We hypothesize that there is a spectrum of IL-7R/STAT5 signaling that controls distinct Vγ chain recombination. E2A regulation of the TCR-γ locus occurs simultaneously with IL-7R/STAT5 signaling at DN2, maintaining TCR gene recombination throughout DN3. (B) In the absence of RORγt and Id3, low IL-7R results in low IL-7-mediated STAT5 transcription of TCR-γ (dotted arrows). However, low levels of IL-7/STAT5 results in a focused recombination of Vγ1 (large arrow). E2A regulation is unhindered in DKO thymocytes, which exacerbates and/or maintains focused gene recombination at the Vγ1 gene locus (large arrow). The enhanced recombination of Vγ1 results in reduced recombination of other Vγ TCR genes and TCRβ (dotted arrows).

development of specific γδ T cell subsets that express distinct VγVδ TCRs is a direct result of fastidious gene regulation during fetal and adult thymopoiesis[35,42]. Thus, it is difficult to imagine that TCR-γ regulation is random after reaching a particular IL-7 signaling threshold.

We favor a model where constant levels of IL-7R expression in DKO DN thymocytes might result in a quantitative difference in STAT5 signaling, perhaps producing an enhanced focus in germline transcription and recombination at the Vγ1 gene cluster over other TCR-γ clusters. The comparative level of IL-7R expression among DKO DN2a – DN4 thymocytes might maintain active Vγ1 gene regulation throughout the DN stages of T cell development. Furthermore, studies have demonstrated that high levels of IL-7 signaling enhance the development of DN precursors and γδ T cells but prevent development of αβ T cells[255]. Therefore, the relatively high levels of IL-7R on DKO DN3 thymocytes might maintain Vγ1 gene regulation instead of TCR-β (V-DJβ) recombination, preventing DN3 progenitors from generating pre-TCRs required for β-selection. Since high levels of Id3 at DN3 are thought to inhibit E2A activity, it is possible that in the absence of Id3, abnormally high levels of E2A in DKO DN3 thymocytes exacerbate the enhanced IL7/STAT5-mediated regulation at the Vγ1 locus. Thus, concurrent with altered STAT5 regulation of TCR-γ genes, it is appealing to consider that the unhindered activity of E2A in DKO DN thymocytes might redirect chromatin remodeling complexes to γδ NKT lineage-specific genes.

Overall, we propose that aberrant RORγt activity in Id3-deficient thymocytes operates to restrict γδ NKT lineage commitment. In the absence of RORγt and Id3, we observe a dramatic and selective expansion of γδ NKT cells at the expense of αβ T cells. Importantly, we observed a significant block in DN3 thymocytes passing the β-

checkpoint. We favor a model where a constant and moderate levels of IL-7R expression from DN2a – DN4 favor optimal conditions that result in enhanced STAT5-induced transcription and recombination of the Vγ1 gene cluster in DKO thymocytes. High levels of E2A might cooperate with IL7/STAT5 at DN3 or earlier to enhance and/or maintain focused Vγ1 gene regulation at the expense of TCR-β gene regulation. Our findings uncover a fundamental regulatory pathway that favors the development of γδ NKT cells, while restricting the maturation of pro-T cells into the conventional T cell lineage.

DISCUSSION[d]

PLZF: the innate T cell determinant

The BTB/POZ-ZF [Broad complex, Tramtrack, Bric à brac (BTB) or poxvirus and zinc finger (POZ)-zinc finger] protein family of transcription factors has been found to control a wide variety of biological processes[215]. These BTB-zinc finger (BTB-ZF) family members are defined by the presence of an N-terminal protein-protein interaction domain (BTB/POZ) and C-terminal zinc finger domains. Several members of this gene family have been shown to have transcriptional repressor activity. Notably, several BTB-ZF genes have been shown to play critical roles in the development or function of some cells of the hematopoietic system[256].

Naïve, conventional T cells must go through activation-induced differentiation, followed by secondary activation by the same antigen, prior to producing effector cytokines. Therefore, it is days, if not weeks, before full participation of conventional T cells in an immune response. In sharp contrast, NKT cells produce vast amounts of a multitude of effector cytokines minutes after activation. Furthermore, NKT cells can simultaneously produce both IL-4 and IFN-γ; a feature essentially never found among conventional T cells[25]. NKT cells are distinct from naïve T cells in that they constitutively express the activation markers CD44 and CD69. Indeed, acquisition of these markers is a sign of functional maturity in the thymus. In addition to enhanced effector functions, NKT cells are also distinguished by their uneven tissue distribution; mature cells accumulate in the thymus and liver, but are relatively scarce in the lymph nodes. Recent data has shown that the BTB-ZF transcription factor PLZF (Promyelocytic leukemia zinc finger) controls the development of all of these effector functions and characteristics. In

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the absence of PLZF, NKT cells still develop, but phenotypically and functionally resemble naïve CD4 T cells[187,188].

Ectopic expression of PLZF in conventional T cells results in the spontaneous acquisition of memory/effector phenotypes and functions[224,225]. T cells in mice carrying a T cell-specific PLZF transgene were, like NKT cells, found to be nearly all be $CD44^{hi}$ and $CD62L^{lo}$ [224,225]. These cells also were found to produce large amounts or several cytokines upon primary upon activation. Therefore, PLZF expression appears to be necessary, sufficient and cell intrinsic for many of the salient features that characterize innate T cell function and phenotype.

Recently, several labs reported that in addition to NKT cells, a subset of mouse γδ T cells expresses high levels of PLZF[178,179,247,248]. These $V\gamma1^{+}V\delta6.3^{+}$ T cells (γδ NKT cells) were also shown to have the innate T cell capacity to co-express IFN-γ and IL-4[178,179]. In unpublished work, we have also found that PLZF is expressed at high levels of non-invariant, CD1d-restricted T cells as well as in non-CD1d restricted innate T cells. Finally, human NKT cells also express PLZF and, importantly, are dependent upon PLZF for their development[187]. Therefore, rather than use a conglomeration of multiple markers, it might be more accurate to simply define the innate T cell lineage as "PLZF-expressing T cells".

Innate CD8 T cells

In addition to its cell intrinsic functions, PLZF has recently also been shown to affect the functions of T cells *in trans*. Several genetic mutations that influence TCR signaling, such as Itk or SLP76, or targets of TCR signaling such as Id3, have been reported to cause the development of an innate-like CD8 T cell population (discussed further below). The impact of these mutations on the development of these innate CD8 T

cells has been reported to be cell intrinsic[257]. For example, if Itk deficient bone marrow is used to reconstitute wild type mice, innate CD8 T cells develop. Two recent studies, however, convincingly demonstrated that acquisition of the innate T cell characteristics was not intrinsic[258,259]. This became apparent when wild type mice were reconstituted with a 50:50 mix of bone marrow carrying the mutation of interest along with wild type bone marrow. Unexpectedly, the wild type CD8 T cells still acquired the innate T cell phenotype. The culprit was found to be an expanded population of PLZF-expressing T cells that developed from the mutant bone marrow. The PLZF-expressing cells release low levels of IL-4 in the thymus, which is captured by the IL-4R, which is expressed by some CD8 T cells. These CD8 T cells upregulate eomesodermin (eomes) expression, take on a memory phenotype and produce large amounts of IFN-γ following primary activation. Interestingly, increased Eomes expression is not only a characteristic of the phenotype, but actually appears to be required[260].

An increased frequency of PLZF expressing T cells explains another odd phenotype that has been reported. Although Itk deficient T cells have a reduced capacity to generate Th2 responses, the mice have increased germinal center activity and increased serum IgE[247]. Leslie Berg and colleagues, however, found that eliminating the PLZF expressing $\gamma\delta$ T cells from the Itk deficient mice by crossing to TCRδ-/- mice reduced the both the serum IgE levels and the frequency of PNA^+ B cells back to near wild type mouse levels[247].

PLZF has also been reported to influence interferon-mediated innate immunity in mice. In particular, a recent study suggested that NK cell function in PLZF-deficient mice is altered[261]. Sensitive assays conducted in our laboratory have failed to detect PLZF in the vast majority of NK cells. Therefore, it is possible that the interferon-mediated response is also a “trans” effect of PLZF expressing cells.

TCR signaling in innate T cell development

Data from several labs have shown that genetic mutations or deletions in proximal TCR signaling molecules (e.g., $LAT^{-/-}$, $Itk^{-/-}$, $SLP76^{Y145F}$), or lack of Id3, resulted in a dramatic and selective expansion of PLZF-expressing T cells[179,247,248,262,263]. Surprisingly, the frequency of invariant NKT cells in these mice was actually reduced (E.S.A. unpublished data)[240]. Rather the expansion occurred in a rather obscure population of so-called γδ NKT cells. These γδ T cells, which are functionally distinct from the majority of γδ T cells, all express a TCR composed of a Vγ1.1 and Vδ6.3 gene segments. Importantly, we have now shown that expansion of the γδ NKT cells is actually dependent upon PLZF (unpublished).

The expansion of $PLZF^{+}$ of γδ NKT cells in TCR signaling mutant mice and Id3-deficient mice is clear; however, the explanation for this phenotype widely varies. The controversy is largely related to *in vitro and in vivo* studies that support a model whereby αβ/γδ lineage commitment is a result of the relative signaling strength through the pre-TCR (weak) or γδ TCR, which 'secures' the fate of precursor T cells by initiating a particular lineage-restricted gene expression program[165,166]. The finding that the changes in the frequency of γδ versus αβ T cells was actually a consequence of the expansion of PLZF-expressing NKT γδ T cells has necessitated some revisions of the strength of signal model for γδ T cell development. Lauritsen and coworkers, for example, have suggested that the increase in γδ NKT cells in the absence of Id3 is due to failed negative selection[245]. These T cells, they suggest, receive exceedingly strong TCR signals during selection and, therefore, would normally be deleted. This hypothesis is largely based on experiments using KN6 γδ TCR transgenic mice, which can bind to the MHC Ib-like molecules. However, a clear role for either positive or negative selection in γδ T cell development is tenuous. Rather, there is support from studies that show γδ

TCR:ligand interactions have profound effects on the differentiation into particular Th effector lineages. Also we, and others, did not observe obvious changes in the absolute numbers of other γδ T cell subsets in Id3-deficient mice[179,248]. Therefore, if strength of TCR mediated signals is critical, different γδ T cell subsets must differ in their requirements for TCR signaling and Id3.

Interestingly, even within Vγ1Vδ6.3 γδ NKT cell subpopulation, not all cells express PLZF. For example, in the spleen of wild type mice, PLZF is expressed in as low as ~10% to as high as ~60% of the γδ NKT cells[178,179]. There also is a complete absence of PLZF-expressing γδ T cells among iIELs, even among the Vγ1Vδ6.3 TCR population[178,179]. Furthermore, sequencing efforts strongly suggest that, similar to the invariant nature of the NKT TCR, these TCRs are identical to each other, even within the potentially variable CDR3 segments[248]. Therefore, two types of PLZF-expressing T cells (αβ- and γδ NKT), both of which are thought to require strong signaling for development, respond differently to alterations in the strength of TCR signals. Indeed, even among $V\gamma1^{+}V\delta6.3/6.4^{+}$ TCR transgenic thymocytes, PLZF only appears to be induced in ~70% of the cells[178]. We believe that these observations are inconsistent with expected results based a simple strength of TCR signaling model. In summary, we argue that these data suggest that signals generated from the TCR alone cannot fully induce PLZF expression and, therefore, other signals must be necessary for the development of PLZF-expressing innate T cells.

It has also been proposed by Udea-Hayakawa et al that the increase in γδ T cells that occurs in the absence of Id3 is a consequence of changes in the dynamics of TCR rearrangements[246]. There hypothesis is that, in the absence of Id3, double negative thymocytes that fail to successfully rearrange a functional TCRβ chain have an extended 'window' of time that permits further rearrangements at the TCRγ loci. This idea,

however, would only appear to be correct if Id3 plays a specific role at the Vγ1 and Vδ6.3 loci during this atypical 'window' of TCR rearrangement. There is no data to support this and, as such, it is difficult to reconcile how only one type of γδ TCR would be rearranged under this scenario.

It should also be noted that PLZF expression is not entirely restricted to γδ NKT cells, as nearly 20% of Vγ1 SP and Vδ6.3 SP (most likely $V\gamma7^{+}V\delta6.3^{+}$) express PLZF[179]. Comparison of gene expression profiles among PLZF-expressing T cells should aid in both identifying common signaling pathways that regulate PLZF activity, and identify transcriptional targets of PLZF necessary to define the robust effector phenotype of innate T cells. It is interesting to speculate that TCR:ligand interactions might be required for some aspects of γδ T cell differentiation, rather than positive or negative selection. Since PLZF expression is found among various γδ T cell subsets, it is possible that there is a 'PLZF-like' precursor that gains or loses particular traits that favor the development of PLZF-expressing γδ T cells. The role of soluble factors, such as cytokines and chemokines, on the development of PLZF-expressing T cells has also not been addressed. It is clear that lymphocytes can affect the development of other T cell populations *in trans.* Although DP thymocytes do not appear to have a role on the development of PLZF-expressing γδ T cells[179], it is possible that factors yet to be identified might influence the differentiation of these cells.

If not TCR signals, then what does control innate T cell development?

αβ NKT cells are selected on CD1d expressed on DP thymocytes[110]. Recently, data showed that αβ NKT cells also require homotypic interactions with SLAM family receptors on DP cells[193]. The requirement for SLAM mediated signaling was made evident by previous work that showed that the adaptor molecule SAP was essential for

NKT cell development[191]. Previous studies also had shown that in the absence of the Src kinase Fyn, which is know to bind SAP, also results in a severe block in αβ NKT cell development[190,192]. FACS analysis and qRT-PCR analysis of early stage αβ NKT cells from SAP- and Fyn-deficient mice, however, showed that PLZF expression could occur independently of these molecules[188,225]. Furthermore, in the absence of SAP and Fyn, γδ NKT cells develop and express PLZF, although PLZF levels are reduced in the absence of SAP[179]. As mentioned earlier, ectopic PLZF expression in conventional T cell results in a gain of NKT cell-like functionality. Importantly, the ability of PLZF to endow cells with these properties is also independent of these two signaling molecules[225]. Altogether, although these data suggest some differential requirements for SAP and Fyn in αβ- and γδ NKT cells for development, they eliminate the possibility that this pathway is essential for PLZF expression.

ThPOK, another BTB-ZF transcription factor, has clearly been shown to be necessary and sufficient for the differentiation of αβ CD4 T cells[201]. Recent studies have, however, also defined a role for ThPOK in innate T cell development[179,264-266]. Surprisingly, ThPOK expression was found in all αβ NKT cells. Utilizing ThPOK reporter mice, we showed that the transcription factor had a heterogeneous expression pattern in γδ T cell populations[179]. PLZF-expressing γδ NKT cells, however, like αβ NKT cells were found to express high levels of ThPOK; levels that were equivalent to what was found in conventional CD4 T cells. Interestingly, a large percentage of αβ NKT cells and nearly all of the γδ NKT cells do not express CD4, clearly indicating that ThPOK does not carry out the same function in innate T cells as it does in conventional T cells.

In the absence of functional ThPOK defects in the differentiation and function of both γδ and αβ NKT cells were apparent. First, in the absence of ThPOK both cell types now had a substantial percentage of cells that expressed CD8[179,264]. More interestingly,

both cell types also lost the capacity to produce IL-4 upon primary activation[179,264]. This loss of function, however, might be a consequence of reduced PLZF expression, at least in the γδ NKT cells[179].

It is interesting that there is a dramatic increase $CD4^+$ γδ NKT cells in TCR signaling mutant-and Id3-deficient mice[179,248]. While ThPOK expression is apparent in CD4-negative αβ-and γδ NKT cells, the increased frequency of $CD4^+$ γδ NKT cells in these mice is suggestive of an increase in ThPOK activity. Park and coworkers, however, reported a decrease in ThPOK mRNA in γδ T cells from Id3-deficient mice. It is tempting to speculate that PLZF and ThPOK cooperate to control some aspects of NKT cell development[265]. While it is clear that ThPOK has an important role in innate T cell development, future experiments should define how ThPOK impacts the development of these unique cell types.

The alternative pathway for T cell development: thymocyte-thymocyte selected T cells express PLZF

It is clear from the previous two sections that the induction of PLZF in innate T cells requires new transcriptional networks that are intertwined with potentially novel signaling pathways. What is also clear is that there is, at this time, no clear resolution to the question of how PLZF is induced. Clues to problem should come from the interesting selection pathway required for NKT cells that requires interactions with CD1d expressed by double positive DP thymocytes[110]. The interaction with DP thymocytes is obligatory; forced expression of CD1d on thymic epithelial cells does not support NKT cell development[111,112]. In contrast, the development of CD4 T cells was long believed to require unique interactions with thymic epithelial cells. Again, these interactions appeared to be obligatory since MHC class II expressing non-thymic derived epithelial cells were shown to be unable to support T cell development. This concept was, at least

partially, refuted by studies showing that if MHC class II expression on thymocytes was sufficient to support the development of CD4 T cells[113-115]. Therefore, in mice and humans, there is an alternative pathway for the selection of CD4 T cells.

The phenotype of T-CD4 T cells (CD4 T cells selected via the alternative pathway) immediately suggested that these cells were somehow distinct from E-CD4 T cells (CD4 T cells selected via thymic epithelial cells). In particular, the T-CD4 T cells tended to have an activated phenotype ($CD44^{hi}$), lower TCR levels and the capacity to produce cytokines upon primary activation[113,114]. The discovery that a subset of T-CD4 T cells were induced to express high levels of PLZF offered a least a partial explanation for the phenotype and function of these cells. More importantly, these cells provided a second clear example of the need for the alternative pathway to induce PLZF expression. Intriguingly, however, only some (~30%) of the T-CD4 T cells are induced to express PLZF. We expect that the differences between the signals received by these two cell populations will be very informative.

But what the PLZF-expressing $\gamma\delta$ NKT cells? Do these cells also require interactions with DP thymocytes to induce PLZF expression? In Figure 5, we suggest several possibilities. First, PLZF expressing $\gamma\delta$ T cells, like $\alpha\beta$ NKT cells and T-CD4 T cells, might require a DP stage of development, while non-PLZF expressing $\gamma\delta$ T cells develop directly from double negative thymocytes. Although our published work shows that $\gamma\delta$ NKT cells develop and express PLZF in TCRβ-deficient mice, TCRβ-deficient mice do still have DP thymocytes; the DP cells simple express the $\gamma\delta$ TCR[179]. These data would suggest that if $\gamma\delta$ NKT cells require a DP stage of development, it likely is somewhat distinct from what is required for $\alpha\beta$ NKT cell development. A second idea is that both PLZF-expressing and non-expressing $\gamma\delta$ T cells arise from the DN stage, but the precursor cells are in some way distinct. Intriguingly, PLZF expression can be

detected at fairly high levels in a subset of DN2a thymocytes. It is possible that expression of PLZF in a precursor cell type “sets the stage” for later development into a PLZF-expressing lineage. Since at this time we cannot discriminate between DN and DP pathways, we must also consider that both are in play and, therefore, signaling required for γδ NKT cells development can occur at either stage. Overall, we believe that comparison of the developmental requirements for each of these lymphocyte subsets will be of great help in uncovering the signals that induce PLZF expression.

Overall, it is has become clear that the BTB-ZF family of transcriptional regulators is necessary for fundamental steps during immune system development and function. Our findings show that PLZF has a specific, non-redundant and essential function for the development of a complete immune system. PLZF is an extraordinarily powerful transcription factor. Our preliminary data shows that the phenotype and function of any T lymphocyte that expresses or is forced to express PLZF is dramatically altered. These findings suggest that PLZF might be useful therapeutically to enhance T cell responses, for example, against tumors. On the other hand, aberrant expression of PLZF in nominally self-reactive T cells may lead to autoimmunity. There is a growing appreciation that numerically minor subsets of T cells can potently modulate immune responses. In particular, the fast and robust response of innate T cells has led to a series of clinical trials designed both to enhance immunity against cancer and to dampen immunity against self. Our discovery that PLZF controls the function of these T cell responses should provide new insight into how to control and manipulate this lineage of cells.

An unusual suspect: RORγt in altered γδ T cell development

We were interested in outlining an underlying mechanism for the expansion of PLZF-expressing γδ NKT cells in *Id3*$^{-/-}$ mice. Spontaneous IL-17 production and RORγt expression in *Id3*$^{-/-}$ γδ NKT cells indicated that RORγt might promote the increase in γδ NKT cells. Remarkably, we found an extreme expansion (~1000 fold) of γδ NKT cells in DKO mice. The excessive numbers of these cells did not appear to be due to proliferation, nor due to an increase in apoptosis of αβ T cells. This surprising finding prompted an analysis of the DN compartment to identify any obvious defects that might contribute to the phenotype observed in DKO mice. Since IL-7R expression is required for γδ T cell development, we sought to assess the levels of IL-7R expression on DN1 – 4 in wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$, and DKO thymocytes. While the overall pattern of IL-7R expression was similar among wild-type, *RORc*$^{-/-}$, *Id3*$^{-/-}$ thymocytes, we observed a moderate but constant level of IL-7R expression throughout DN2a – DN4 in DKO thymocytes. Since IL-7 signaling induces STAT5 activity, we propose that a certain threshold of IL7/STAT5 might enhance Vγ1 gene regulation over other TCR-γ gene clusters. We also argue that increased E2A activity the absence of Id3 in DKO thymocytes might exacerbate IL7/STAT5-mediated Vγ1 TCR recombination. We observed a statistically significant decrease in the frequency of DN3b cells in DKO thymuses. Therefore, it appears that DKO progenitors are failing to commit to the αβ lineage. Our data support a model where DKO DN progenitors are skewed toward the γδ NKT cell lineage as a result of altered IL-7/STAT5/E2A regulation that increases Vγ1 recombination at the expense of TCRβ recombination. Alterations in the transcriptional network in *RORc*$^{-/-}$*Id3*$^{-/-}$ DN thymocytes could target chromatin remodeling complexes to γδ NKT lineage-specific genes. We hypothesize that skewing toward the γδ NKT cell lineage could occur early in thymic development and throughout all four DN stages.

Specifically, 'priming' of DN cells might favor the development of γδ NKT cells over αβ T cells in DKO mice, and cells that initially commit to the αβ lineage may be redirected into the γδ NKT lineage. Perhaps, alterations in key transcriptional pathways could program progenitor cells toward the γδ NKT lineage. Transcription factors often regulate a large number of genes either directly or indirectly. Gene regulatory networks are controlled at 'nodes' by transcription factors, and disruption of even one 'node' can drastically alter global gene expression patterns in gene-deficient cells. Global gene expression analysis of γδ NKT cells clearly demonstrates that a large cohort of genes was altered in Id3-deficient mice. An even larger cohort of genes was altered in the DKO γδ NKT cells, but surprisingly, this gene set is distinct, as only 691 genes changed similarly between Id3 and DKO γδ NKT thymocytes. Notably, only 33 genes were altered in *RORc*$^{-/-}$ γδ NKT cells. Thus, it is clear that RORγt activity only occurred in the absence of Id3. Based on global gene expression patterns, it is apparent that the 'identity' of DKO γδ NKT cells was dramatically different from both wild-type and Id3-deficient γδ NKT cells.

PLZF expression was altered in *RORc*$^{-/-}$ γδ NKT cells, which is likely the cause of reduced IL-4 production. However, both intracellular staining and fate-mapping experiments failed to detect RORγt in γδ NKT cells. RORγt is highly expressed in DP thymocytes, but can be detected as early as DN3. While it has been proposed that DP thymocytes *trans-condition* the acquisition of the γδ effector program, we previously demonstrated that PLZF-expression is unaffected in the absence of DP thymocytes. This confounding result indicates that either another RORγt-expressing thymocyte population *trans-conditions* the development of PLZF-expressing γδ NKT cells, or that the RORγt fate-mapping mice do not faithfully recapitulate RORγt expression in all cells. Future experiments should illuminate this intriguing phenotype.

While it is clear that there is a profound defect in the T cell compartment of *RORc*$^{-/-}$*Id3*$^{-/-}$ mice, it is unclear what mechanism controls this dramatic phenotype. We argue that we have uncovered a fundamental regulatory pathway that favors the development of γδ NKT cells, while restricting the maturation of pro-T cells into the conventional T cell lineage. Phylogenetic analysis of the constant regions of Igs and TCR genes indicated that γδ T cells are the most ancient lymphocyte[117]. While γδ T cells can become activated against viral antigens, they are thought to control microbial infections and regulate tissue homeostasis. Some have proposed that the immune system evolved a more sophisticated method of detecting rapidly evolving viral antigens through the development of αβ T lymphocytes. While several genes have been identified to control various aspects of αβ T cell development, it is noteworthy that few of these significantly impact γδ T cell development. With the exception of Sox13, no transcription factors that regulate γδ lineage commitment have been identified. Thus, it seems that γδ T cells express a 'skeleton' gene expression program that is buried under several layers of transcriptional regulation that has evolved to promote the development of the αβ lineage. We propose that at least two of these layers are controlled by Id3 and RORγt. Why is the expansion limited to Vγ1Vδ6.3 TCR expressing cells in DKO mice? Others and we have shown the presence of Id3-dependent and -independent γδ T cell lineages. Clearly, the absence of Id3 specifically impacts the development of γδ NKT cells, which is greatly exacerbated when RORγt is also absent.

It is tempting to speculate that other transcriptional networks might restrict the development of other γδ sublineages in a similar manner as RORγt and Id3 do in the context of γδ NKT cells. The drastic expansion of γδ NKT cells in *RORc*$^{-/-}$*Id3*$^{-/-}$ mice provides a new framework to study the development of this atypical T cell population. Future studies of *RORc*$^{-/-}$*Id3*$^{-/-}$ γδ NKT cells might reveal new insight into the on-going

challenge of understanding γδ T cell development and function, while potentially exposing opportunities for future therapeutic application.

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